Selective inhibition of 5-lipoxygenase by natural compounds isolated from Chinese plants, *Artemisia rubripes* Nakai

Yasuko Koshihara, Tomohiro Neichi, Sei-itsu Murota, Ai-na Lao*, Yasuo Fujimoto* and Takashi Tatsuno*

Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Sakaecho, Itabashi-ku, Tokyo 173 and **The Institute of Physical and Chemical Research, Wako-shi, Saitama 351, Japan*

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Three of four natural compounds, which are caffeic acid, eupatilin and 4'-demethyleupatilin, isolated from Chinese plant, *Artemisia rubripes* Nakai selectively inhibited 5-lipoxygenase of cultured mastocytoma cells. Half-inhibition doses (ID_{50}) for caffeic acid, eupatilin and 4'-demethyleupatilin were 3.7, 14 and 18 $\times 10^{-6}$ M, respectively. The inhibition by caffeic acid was non-competitive types. Prostaglandin synthase activities were little inhibited by eupatilin and 4'-demethyleupatilin, but rather stimulated by affeic acid. The formation of leukotriene C_4 and D_4 by mast tumor cells was almost completely suppressed by these compounds at 10^{-4} M.

5-Lipoxygenase *Inhibitor leukotriene* *Caffeic acid* *Prostaglandin synthease* *Lipoxygenase*

1. INTRODUCTION

Leukotrienes which comprise slow reacting substance of analylaxis (SRS-A) have various physiological and pharmacological activities, and are presumed as mediators for allergic and anaphylactic reaction and inflammation. At the initial step in the course of leukotriene biosynthesis, 5-lipoxygenase catalyzes oxygenation of arachidonic acid, and produces 5-hydroperoxyeicosatetraenoic acid (5-HPETE) through which all the leukotrienes are formed. The specific or selective inhibitor for 5-lipoxygenase should be useful not only as a tool for investigating the regulation mechanism of leukotriene biosynthesis but also as a drug for clinical use. We found some selective inhibitors for 5-lipoxygenase among sources of natural compounds isolated from a Chinese plant, *Artemisia rubripes* Nakai, which has been used for treatment of female diseases caused by blood hindrance. Four compounds isolated from the plant were investigated by a unique in vitro assay system established using a cloned mastocytoma P-815, 2-E-6 cells for assaying both lipoxygenase and prostaglandin synthetase activities [1,2]. Of 4 compounds tested, 3 inhibited 5-lipoxygenase activity without inhibiting prostaglandin synthetase activity at 5×10^{-5} M. Two of them belong to flavonoids, a group of natural compounds widely distributed in plants, which are well-known to have various pharmacological effects, such as inhibition of platelet aggregation and antiasthmatic effects. The other is caffeic acid which is commercially available. Here, we discuss the inhibitory effects of the natural compounds on 5-lipoxygenase and their inhibitory effects on SRS-A formation.

Abbreviations: 12-, 5- and Di-, 12-HETE, 5-HETE and 5,12-diHETE, respectively; D, E and F, prostaglandin D_2, E_2 and $F_{2\alpha}$, respectively

2. MATERIALS AND METHODS

The sources of the reagents used in this experiment were as follows: [1-^{14}C]arachidonic acid (spec. act. 55.5 Ci/mol), Radiochemical Centre

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(Amersham); foetal bovine serum (mycoplasma- and virus-free), Grand Island Biochemical (NY); sodium *n*-butyrate, Wako Chemical Co. (Osaka); authentic 5-hydroxyeicosatetraenoic acid (HETE), prostaglandin and leukotriene standards, Ono Pharmaceutical (Osaka); thin-layer silica gel plates (60 F_{254}; layer thickness 0.25 mm), Merck (Darmstadt).

2.1. *Assay of 5-lipoxygenase and prostaglandin synthetase activities*

Both assays were done as in [3] except solvent system of thin-layer chromatography. In brief, a cloned mastocytoma P-815, 2-E-6 cells were treated with *n*-butyrate for 40 h to induce cyclooxygenase [4] and then were harvested by centrifugation at 200 × *g* for 5 min. The 10000 × *g* supernatant fraction as both enzyme sources was prepared from cell homogenate in 50 mM phosphate buffer (pH 7.4), 1 mM EDTA and 0.1% gelatin. Under the standard assay condition (1.0 ml) for lipoxygenase activity, the supernatant fraction (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°C for 5 min with shaking in an open tube. And for the assay of prostaglandin synthetase activity, $CaCl_2$ and indomethacin were omitted from above incubation mixture, and it was incubated at 37°C for 7 min. These conditions were specific and optimum for assaying each enzyme. Both reactions were terminated by adjusting the mixture to pH 3.0 with HCl. The synthesized HETEs and prostaglandins were extracted with 8 vol. 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 [5]. Labeled products separated on the plates were scanned, and radioactive zones were scraped off the plate for measurement of radioactivity by a liquid scintillation spectrometer. The activity of 5-lipoxygenase or prostaglandin synthetase activity of 10^7 cells were expressed as the sum radioactivities due to 5-HETE and 5,12-diHETE and the sum radioactivities due to prostaglandins synthesized, respectively. Inhibitors dissolved in ethyl alcohol of reagent grade (50 μl) or ethyl alcohol (50 μl) for control were transferred to each assay tube containing [^{14}C]arachidonic acid in toluene and to them one drop of mixture of propylene glycol/ethyl alcohol (1/3) was added. Organic solvents were then evaporated completely under N_2 gas stream before addition of enzyme to exclude any effect of these organic solvents.

2.2. *Preparation of inhibitors*

Dried whole plant (340 g) of *Artemisia rubripes* Nakai collected in China was extracted with ethyl alcohol and the alcohol solution was concentrated under reduced pressure to leave a darker oily material (31 g). On this oily material silica gel column chromatography was performed; it was separated into 7 fractions by successive elution with hexane/ethyl acetate (10/1), hexane/ethyl acetate (1/1), chloroform/methanol (3/1) and methanol. Finally, 10 compounds were isolated and purified from the fractions by repeated silica gel chromatography, and crystallized [6]. Four compounds among them were selected and tested for the activity on lipoxygenase. They were eupatilin, dammaradienyl acetate, caffeic acid and 4′-demethyleupatilin, and the yields were 220 mg, 80 mg and 160 mg from the starting material, respectively. Their structural formulae are shown in fig.1.

3. RESULTS AND DISCUSSION

Effects of 4 compounds isolated from Chinese plants, *Artemisia rubripes* Nakai, on both 5-lip-

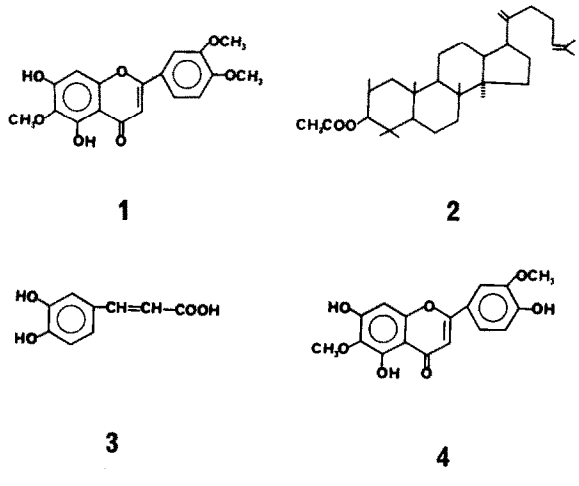

Fig.1. Chemical structure of 4 compounds isolated from a Chinese plant, *Artemisia rubripes* Nakai: (1) eupatilin; (2) dammaradienyl acetate; (3) caffeic acid; (4) 4′-demethyleupatilin.

oxygenase and prostaglandin synthetase activities were assayed by the method where a cloned mastocytoma P-815, 2-E-6 cell line was used [3]. The cells treated with *n*-butyrate for inducing cyclooxygenase had 5-lipoxygenase activity dominantly as compared with 12-lipoxygenase [1,2]. Therefore, the $10000 \times g$ supernatant fraction of the cells was prepared as the enzyme sources which could assay 5-lipoxygenase, 12-lipoxygenase and cyclooxygenase activity at the same time as described in section 2. The assay conditions were fixed at linearly increasing state of the enzyme activities. The radioactivities due to 5-HETE and 5,12-diHETE synthesized were designated as 5-lipoxygenase activity. However, the radioactivities due to prostaglandin D_2, E_2 and $F_{2\alpha}$, which were all of the prostaglandins synthesized by the cells, were designated as prostaglandin synthetase activity.

Fig.2 shows the inhibitory activities of the compounds on 5-lipoxygenase. The activities are expressed as percent inhibition to the control. The radioactivity due to 5-lipoxygenase metabolites in the control was 61400 dpm. Three out of 4 compounds tested had strong inhibitory activities, but dammaradienyl actate had as little activity as only 20% inhibition even at 5×10^{-4} M, where the other 3 compounds showed almost complete inhibition. Half-inhibition doses (ID_{50}) for caffeic acid, eupatilin and 4′-demethyleupatilin were 3.7, 14 and 18×10^{-6} M, respectively. For characterization of the mechanism of inhibition, 5-lipoxygenase was assayed at various concentrations of arachidonic acid in the presence of increasing concentrations of caffeic acid. A Lineweaver–Burke plot of the date showed that caffeic acid was a non-competitive inhibitor (not shown).

The effects of these 3 compounds on prostaglandin synthetase activities were next investigated (fig.3). The radioactivity due to prostaglandin synthetase activity for the control was 16000 dpm. Of the 4 compounds tested, 2 had a little stimulating activity at high concentration; caffeic acid especially stimulated the activity >60% at 10^{-4} M compared with the control. 4′-Demethyleupatilin had a slight inhibitory activity for prostaglandin synthetase at a high dose of 10^{-4} M. The ID_{50} for prostaglandin synthetase of this compound was 4.5×10^{-4} M, which was 25-times higher than that for lipoxygenase inhibition. These results were obtained in at least 3 different experiments on each figure.

The scanning profile of the thin layer chromatography for HETE and prostaglandin syntheses in the presence of 10^{-4} M eupatilin is shown in fig.4.

The effects of caffeic acid and eupatilin on SRS-A formation in mast tumor cells were investigated next. These assays were done as in [7]. Mast tumor cells (10^7 cells/ml) were incubated with each compound (1×10^{-4} M) in the presence of indomethacin, Ca^{2+}-ionophore A23187 and L-cysteine. The SRS-A activity in the incubation fluid was bioassayed for its contractile activity to guinea pig ileum in organ bath as in [8]. These compounds reduced the contraction to base line level. Since these compounds did not antagonize the contraction caused by leukotriene C and D, the reduction of the contractility can be attributed to the inhibition of SRS-A formation through 5-lipoxygenase inhibition (submitted).

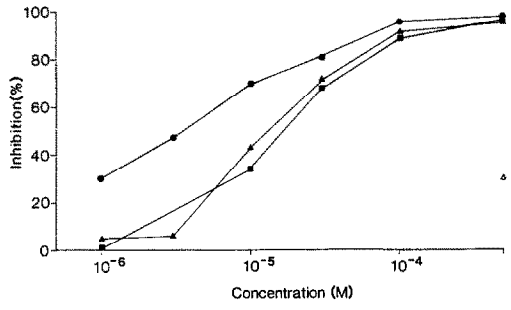

Fig.2. Inhibitory effects of natural compounds on 5-lipoxygenase activity. Details are described in section 2: (▲—▲) eupatilin; (△) dammaradienyl acetate; (●—●) caffeic acid; (■—■) 4′-demethyleupatilin.

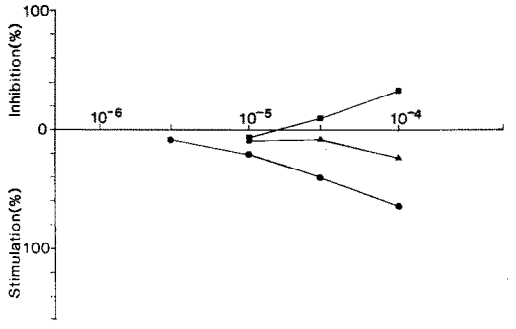

Fig.3. Effect of natural compounds on prostaglandin synthetase activity. Details are described in section 2. Symbols are as in fig.2.

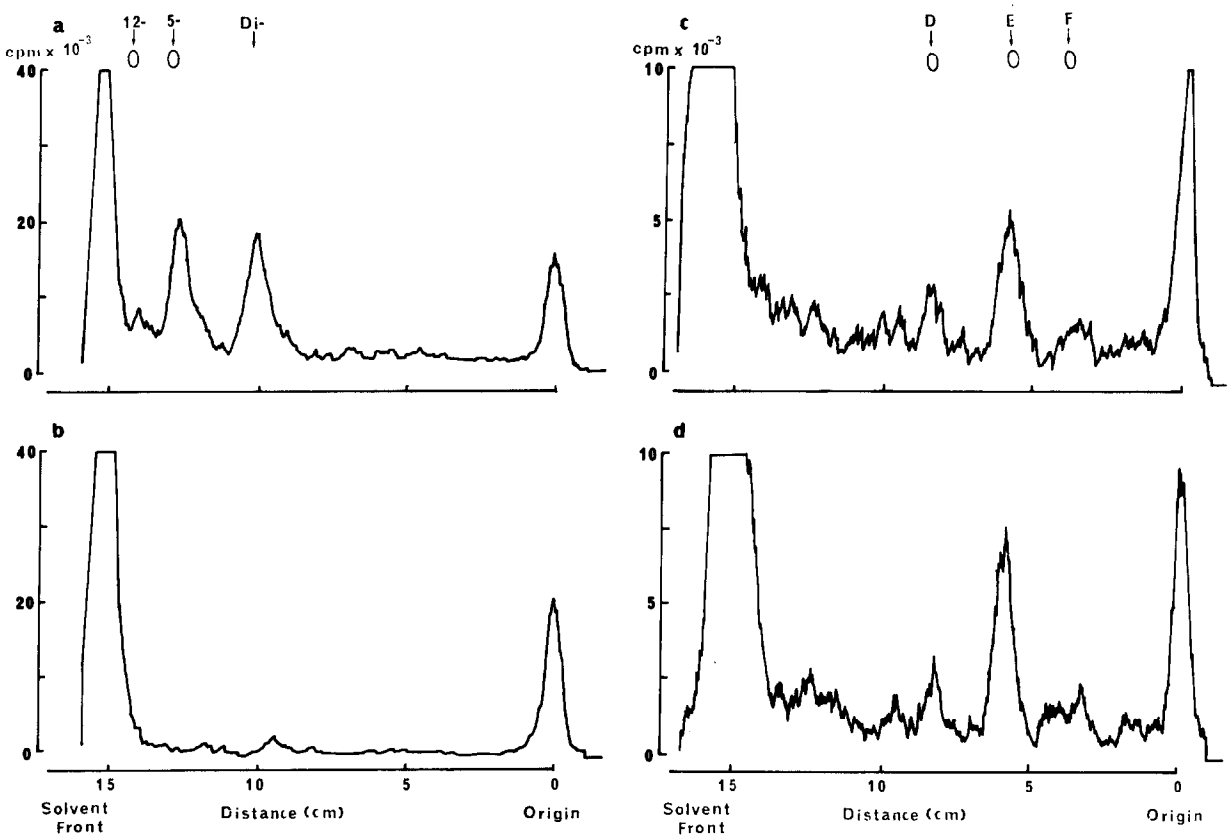

Fig.4. Radiochromatoscan showing HETE and prostaglandin syntheses with or without eupatilin (10^{-4} M). Details are described in section 2. The left 2 panels show HETE synthesis: (a) control; (b) eupatilin. The right 2 panels show prostaglandin synthesis: (c) control; (d) eupatilin.

Caffeic acid is one of the most selective inhibitors for 5-lipoxygenase reported. Eupatilin also has a good selective inhibitory activity for 5-lipoxygenase. Caffeic acid is commercially available and eupatilin can be obtained in high yield from original plants. Selective inhibitors as caffeic acid and eupatilin are very helpful and useful for examining physiological and pharmacological significance of leukotrienes. We are now investigating the effects of these drugs on the allergic reaction.

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