

Effect of AA-861, a 5-Lipoxygenase Inhibitor, on Leukotriene Synthesis in Human Polymorphonuclear Leukocytes and on Cyclooxygenase and 12-Lipoxygenase Activities in Human Platelets

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AA-861, a selective inhibitor of 5-lipoxygenase of arachidonic acid, was tested for ability to inhibit leukotriene C_4 and leukotriene B_4 synthesis in human polymorphonuclear leukocytes after calcium ionophore stimulation. AA-861 dose-dependently inhibited leukotriene B_4 and leukotriene C_4 generation in human polymorphonuclear leukocytes; the concentration required to inhibit generation by 50% (IC_{50}) was 3×10^{-7} M for leukotriene B_4 and 1×10^{-8} M for leukotriene C_4 . BW-755C inhibited the generation of leukotriene C_4 with an IC_{50} of about 10^{-5} M, indicating that AA-861 is about 1000 times more potent than BW-755C. AA-861 did not affect the activity of either cyclooxygenase or 12-lipoxygenase at a concentration up to 10^{-5} M in human platelets. AA-861 did not inhibit histamine release from human basophils. These results indicate that AA-861 selectively inhibits 5-lipoxygenase but not cyclooxygenase or 12-lipoxygenase in human specimens.

Key words: leukotriene B_4 ; leukotriene C_4 ; lipoxygenase.

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Leukotrienes, formerly known as slow-reacting substances of anaphylaxis, are much more potent constrictors of human airway smooth muscle than histamine. Leukotrienes are recognized to be among the most important pathological mediators in bronchial asthma (4, 11, 12, 25). Since 5-lipoxygenase is an initial enzyme in the biosynthesis of leukotrienes from arachidonic acid, a selective inhibitor of 5-lipoxygenase would reduce formation of leukotrienes (2). Development of selective inhibitors of 5-lipoxygenase is being very actively investigated because such a drug should be useful as therapeutic agent for bronchial asthma (3, 9, 10, 16, 22, 26).

AA-861 (2-(12-hydroxydodeca-5,10-dinyl)-3, 5, 6-trimethyl-1, 4-benzoquinone), a benzo-

quinone derivative, is a selective inhibitor of 5-lipoxygenase in animal enzyme preparations and in *in vivo* studies it was effective in animal models of allergic asthma (1, 27). The objects of the studies reported here were to examine whether AA-861 prevents the generation of leukotrienes in human polymorphonuclear leukocytes and whether AA-861 influences the activity of cyclooxygenase and 12-lipoxygenase of arachidonic acid in human platelets.

MATERIAL AND METHODS

Material

The following reagents were purchased from commercial companies: calcium ionophore

A23187 (Calbiochem), Lymphoprep (Nye-gaard), a radioimmunoassay kit for leukotriene C_4 (New England Nuclear), a radioimmunoassay kit for leukotriene B_4 (Amersham). The leukotriene C_4 antiserum is reported to have about 60% cross-reactivity with leukotriene D_4 and 8.6% cross-reactivity with leukotriene E_4 . In this report, leukotrienes measured by the radioimmunoassay kit for leukotriene C_4 were expressed as leukotriene C_4 in spite of the kit showing cross-reactivity for leukotriene C_4 , leukotriene D_4 and leukotriene E_4 , because separation of the supernatant by high speed liquid chromatography demonstrated that only about 3% of leukotriene C_4 was converted into leukotriene D_4 during incubation. The cross-reactivity of the anti-leukotriene B_4 antiserum with leukotriene C_4 , leukotriene D_4 and the other prostanooids was less than 0.03%, and diastereoisomers of 5, 12-dihydroxy-6, 8, 10-trans-14-cis-eicosatetraenoic acid showed 3.3% cross-reactivity. None of the drugs used in this study interfered with these radioimmunoassays.

AA-861 was provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). It was dissolved in dimethyl sulfoxide before being used. The final concentration of dimethyl sulfoxide added to the cells was 0.5% and its concentration had no effect on calcium ionophore-induced leukotriene generation. BW-755C and dimethylcarbamazine were supplied by the Wellcome Research Laboratories (Beckenham, England) and Tanaba Pharmaceutical Co., respectively. Tyrode buffer was composed of NaCl 137 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.1 mM, $NaHCO_3$ 11.9 mM, NaH_2PO_4 0.4 mM, and D-glucose 5.6 mM.

Effect of AA-861 on in vitro calcium ionophore-induced leukotrienes B_4 and C_4 generation in human polymorphonuclear leukocytes

Heparinized venous blood was drawn from asthmatic patients or normal healthy subjects. After dextran sedimentation for 30 min, the leukocyte-rich supernatant was collected and layered on Lymphoprep. After centrifugation for 30 min at $400 \times g$, the pellet was washed with tyrode buffer. The cells were then exposed

to 0.2% NaCl for 20 s to lyse erythrocytes. Following centrifugation, the polymorphonuclear leukocytes recovered in the pellet were resuspended in tyrode buffer to a concentration of approximately 1×10^6 cells/ml. The sample of cell suspension was preincubated for a few minutes at $37^\circ C$ with shaking in the presence or absence of various concentrations of the drugs. The cells were subsequently challenged with 2×10^{-6} M of calcium ionophore, which produces a submaximal stimulation of leukotriene C_4 synthesis (8). After incubation for 20 min, the reaction was terminated by centrifugation for 10 min at $400 \times g$ at $4^\circ C$ to remove the cells. In the case of leukotriene B_4 formation, the reaction was stopped at 10 min (17, 20). The concentration of leukotriene C_4 or leukotriene B_4 in the supernatant was quantitated by radioimmunoassay kits as reported previously (8).

Effect of AA-861 on cyclooxygenase and 12-lipoxygenase in human platelets

Platelets were obtained from citrated whole blood drawn from asthmatic patients or normal healthy subjects (23). The platelets were suspended in 25 mM Tris buffer (pH 7.6) containing 137 mM NaCl, 2.7 mM KCl, and 11 mM D-glucose, and an aliquot of the suspension (1×10^8 cells/0.5 ml) was incubated with ^{14}C -arachidonic acid (0.17 μCi , 6.2 μM) in the presence or absence of AA-861. After incubation for 20 min at $37^\circ C$, the reaction was terminated by acidification (pH 3.0) and the products were extracted with 2 ml of ethyl acetate three times. The extract was analyzed by silicagel thin layer chromatography using chloroform: methanol:acetic acid:water (95:5:0.5:0.5, v/v) as a developing solvent. The R_f values for thromboxane B_2 , 12-hydroxyheptadecatrienoic acid, and 12-hydroxyeicosatetraenoic acid were 0.07, 0.29, and 0.40, respectively. After the thin layer chromatography areas were identified by autoradiography, they were scraped off the plate and the radioactivity was determined with a liquid scintillation spectrometer.

Effect of AA-861 on 12-lipoxygenase in a cell-free system

Platelets were suspended in 50 mM phosphate buffer (pH 7.4) containing 0.1% gelatin and the suspension was homogenized using a Branson Sonifier cell disrupter 200 at a power setting of 4 for 4 s at 4°C. The homogenate was centrifuged at $100,000 \times g$ at 4°C for 30 min. The supernatant containing 12-lipoxygenase was used for the assay. The method was identical with that described for intact platelets.

Effect of AA-861 on histamine release from human basophils

Venous blood was collected from mite-sensitive asthmatic patients. The mixed leukocytes were collected by dextran sedimentation procedure as previously described (14). The leukocytes (5×10^6 cells) were transferred into a plastic tube. After incubation with AA-861 for a few minutes at 37°C, mite allergen (0.1 µg/ml) was added to the tube and the tube was incubated for 40 min to release histamine. The reaction was stopped by cooling on ice and the tubes were centrifuged at $400 \times g$ for 10 min at 4°C. Histamine in the supernatant was measured by an automated fluorometric method of Siraganian (21).

RESULTS

When incubated with calcium ionophore, human polymorphonuclear leukocytes synthesized both leukotriene C_4 and leukotriene B_4 by 5-lipoxygenation of arachidonic acid; unstimulated cells did not generate measurable amounts of leukotriene C_4 and leukotriene B_4 . Polymorphonuclear leukocytes stimulated with calcium ionophore released 9.0 ± 2.6 ng of leukotriene $C_4/10^6$ cells (mean $\pm$ SEM) and 14.0 ± 3.7 ng of leukotriene $B_4/10^6$ cells (mean $\pm$ SEM). Fig. 1 shows a dose-dependent inhibition of leukotriene C_4 generation by AA-861, BW-755C, and diethylcarbamazine. Complete inhibition was observed with 10^{-5} M of AA-861; the concentration required to inhibit the generation by 50% (IC_{50}) was about 10^{-8} M, when

graphically estimated. BW-755C and diethylcarbamazine inhibited leukotriene C_4 generation with an IC_{50} of about 10^{-5} M and over 10^{-3} M, respectively. AA-861 was also examined for its inhibitory effect on leukotriene B_4 synthesis in human polymorphonuclear leukocytes. AA-861 potentially inhibited leukotriene B_4 synthesis in a dose-dependent manner with an IC_{50} of 3×10^{-7} M (Fig. 2). AA-861 inhibited the generation of leukotriene C_4 and leukotriene B_4 in cell preparations of both normal subjects and asthmatic patients at a similar concentration range, and there was no difference in inhibition between these cell preparations.

To examine the influence of AA-861 on the activities of cyclooxygenase and 12-lipoxygenase, the production of ^{14}C -arachidonic acid

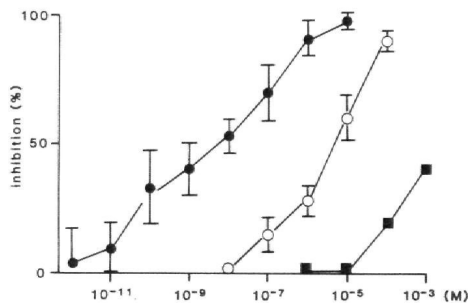


Fig. 1. Effect of AA-861 (●), BW-755C (○), and diethylcarbamazine (■) on calcium ionophore A23187 induced leukotriene C_4 release from human polymorphonuclear leukocytes. The results, except for a case of diethylcarbamazine ($n = 2$), are expressed as mean $\pm$ SEM of three experiments.

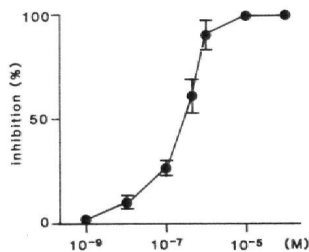


Fig. 2. Effect of AA-861 on calcium ionophore A23187 induced leukotriene B_4 release from human polymorphonuclear leukocytes. The results are expressed as mean $\pm$ SEM of four experiments.

metabolites in the presence or absence of AA-861 was examined in human washed platelets. The sum of the radioactivities due to 12-hydroxyheptadecatrienoic acid and thromboxane B_2 was designated as cyclooxygenase activity; 12-lipoxygenase activity was measured as the radioactivity of 12-hydroxyeicosatetraenoic acid formed during incubation of ^{14}C -arachidonic acid with platelets. The results are expressed as % inhibition and % stimulation with respect to the control. The % conversion of ^{14}C -arachidonic acid in the control was about 25 % for thromboxane B_2 , 20 % for 12-hydroxyheptadecatrienoic acid, and 25 % for 12-hydroxyeicosatetraenoic acid. AA-861 had no significant effect on the cyclooxygenase and 12-lipoxygenase products at a concentration up to 10^{-5} M. However, at the highest concentration of 10^{-4} M used in this experiment, AA-861 slightly stimulated 12-hydroxyheptadecatrienoic acid production by cyclooxygenase and suppressed 12-hydroxyeicosatetraenoic acid production by 12-lipoxygenase. On the other hand, when the $100,000 \times g$ supernatant of platelet homogenate was used in place of intact platelets, AA-861 did not affect the production of ^{14}C -12-hydroxyeicosatetraenoic acid at the same concentration range.

AA-861 did not show any inhibitory effect on allergen-induced histamine release from human basophils.

DISCUSSION

In addition to histamine, leukotrienes, 5-lipoxygenase metabolites of arachidonic acid, are considered to play a central role in the pathophysiology of allergic diseases. Inhibitors that selectively block the formation of products of the 5-lipoxygenase pathway without affecting other pathways of arachidonic acid would be desirable to investigate the physiological role of leukotrienes and to use as therapeutic agents in treating asthma. Some inhibitors, such as nordihydroguaiaretic acid, benoxaprofen, and 5, 8, 11-eicosatrienoic acid have been employed in biological and pharmacological studies, but they are not so specific for the 5-lipoxygenase pathway (7). AA-861 is a specific inhibitor of

5-lipoxygenase in animal enzyme preparations (26, 27). However, little information is available with regard to the potency and selectivity of 5-lipoxygenase in human blood cells. In this study, we examined the effects of AA-861 on the generation of leukotrienes in human polymorphonuclear leukocytes and on the enzymes of arachidonic acid in human platelets.

The inhibitory activity of AA-861 was compared to that of BW-755C, and diethylcarbamazine, another known leukotriene biosynthesis inhibitor. BW-755C is known to inhibit both cyclooxygenase and lipoxygenase of arachidonic acid (6). Diethylcarbamazine inhibits the enzymatic conversion of 5-hydroperoxyeicosatetraenoic acid to leukotriene A_4 and hereby inhibits leukotriene C_4 production (13). In the present study, when human polymorphonuclear leukocytes were treated with 10^{-5} - 10^{-11} M AA-861, a dose-dependent inhibition of leukotriene C_4 and leukotriene B_4 production was observed; the IC_{50} value for leukotriene C_4 production was estimated as 10^{-8} M. BW-755C and diethylcarbamazine also inhibited leukotriene C_4 production in a dose-dependent manner, but their IC_{50} values were 10^{-5} M and over 10^{-3} M, respectively. AA-861 was more than 1000 times as potent as BW-755C and diethylcarbamazine in inhibiting leukotriene C_4 production stimulated by calcium ionophore in this experiment. In a preliminary study using allergen challenge of passively sensitized human lung fragments, AA-861 suppressed leukotriene C_4 production with an IC_{50} value of 5×10^{-10} M (data not shown). These results suggest that AA-861 potentially inhibits leukotriene biosynthesis in human specimens.

The effects of AA-861 on 12-lipoxygenase and cyclooxygenase activities were determined by incubating the human platelets in the presence and absence of various concentrations of AA-861 before ^{14}C -arachidonic acid was added. AA-861 did not affect the activity of cyclooxygenase and 12-lipoxygenase at a concentration of 10^{-5} M, which completely inhibited the production of leukotriene C_4 and leukotriene B_4 . At the highest concentration used (10^{-4} M), AA-861 slightly stimulated the activity of cyclooxygenase, but suppressed the activity of 12-lipoxy-

genase by 60%. The mechanism of the increase in the cyclooxygenase product is not clear. These results suggest that, virtually, AA-861 does not affect the activity of cyclooxygenase or 12-lipoxygenase in human platelets.

Since lipoxygenase products of arachidonic acid metabolism, presumably 5-lipoxygenase products, have been reported to be necessary for allergen-induced histamine release (18, 19), it is possible that AA-861 may inhibit histamine release by allergen. However, the histamine release from rat mast cells was not affected by AA-861 (1). Furthermore, AA-861 failed to inhibit allergen-induced histamine release from human basophils of mite-sensitive asthmatic patients and from passively sensitized human lung tissues at a concentration up to 5×10^{-5} M. These findings are in accordance with the suggestion of other authors that 5-lipoxygenase products are not involved in the release of histamine from human lung tissues and human basophils (5, 15, 24).

The results presented here demonstrate that AA-861 inhibits the non-immunological generation of leukotrienes without affecting cyclooxygenase and 12-lipoxygenase in human platelets. AA-861 may be useful, not only as a tool for examining the physiological and pharmacological significance of leukotrienes, but also as a therapeutic agent for treating diseases in which leukotriene participation has been suggested.

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