Inhibitory Effect of 4-Acylaminophenol Derivatives (T-0799 and T-0757), Novel Lipoxygenase Inhibitors, on Arachidonic Acid-Induced Infiltration of Polymorphonuclear Leukocytes in Rat Skin

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ABSTRACT—Effects of 4-acylaminophenol derivatives, novel 5-lipoxygenase inhibitors, on the neutrophil infiltration in arachidonic acid (AA) (10 mg/site)-induced skin inflammation in rats were examined. Myeloperoxidase (MPO) activity in the skin lesion, used as an indicator of neutrophil infiltration, was significantly increased after intradermal injection of AA. Dual inhibitors of cyclooxygenase and 5-lipoxygenase, phenidone (100 mg/kg × 2, i.p. and p.o.) and BW-755C (50 mg/kg × 2, p.o.), and 5-lipoxygenase inhibitors, AA-861 (100 mg/kg × 2, i.p.) and the 4-acylaminophenol derivatives T-0799 and T-0757 (10-100 mg/kg × 2, p.o.), inhibited the increase in MPO activity 5 hr after AA-injection, but the cyclooxygenase inhibitor indomethacin (5 mg/kg, i.p.) showed no effect. These results suggest that products of lipoxygenase, but not of cyclooxygenase, are involved in the MPO activity increase (i.e., neutrophil infiltration), and that this model is useful for in vivo evaluation of 5-lipoxygenase inhibitors. It is suggested that the 4-acylaminophenol derivatives may be useful as orally active drugs for treatment of some leukotriene-mediated diseases.

Keywords: Inflammation, Neutrophil infiltration, Myeloperoxidase, Lipoxygenase inhibitor, 4-Acylaminophenol derivative

The acute inflammatory reaction is implicated as a host defense mechanism at the site of the microcirculation that comprises exudative and cellular responses. These responses are manifested by increased vascular permeability and leukocyte infiltration. The exudative response is relatively easy to study because of the paw (1) or ear (2) edema formation technique. However, simple and quantitative methods for evaluation of the leukocyte infiltration are scarce. It has been reported that various cytotoxic inflammatory mediators are produced and released by leukocytes infiltrating into the inflamed tissue, causing the later phases of inflammation. Therefore, regulation of the leukocyte infiltration is important in the treatment of inflammatory diseases, and can be an attractive target for the development of new anti-inflammatory agents.

Many reports have shown that leukocyte infiltration is induced by various chemotactic mediators such as complement fragment (C5a), leukotriene B_4 (LTB_4) or platelet activating factor (PAF). It has also been reported that anti-inflammatory steroids inhibit the leukocyte infiltration by inhibiting production of chemotactic mediators (3) and leukocyte motility (4). On the other hand, arachidonic acid metabolites, leukotrienes (LTs) in particular, are likely to be implicated in various inflammatory diseases such as asthma (5), rheumatoid arthritis (6), hepatitis (7), glomerular nephritis (8, 9) and psoriasis (10). Therefore, much effort has been focused on the development of inhibitors of 5-lipoxygenase, the rate limiting enzyme of LT synthesis, trying to regulate the production of the biologically active LTs in various disease states (11).

In the present study, we used AA-induced skin inflammation to evaluate the cellular response caused by AA-metabolite(s) and measured myeloperoxidase (MPO) activity at the inflammation site as an indicator of neutrophil infiltration (12, 13). With this model, we evaluated the efficacies of novel lipoxygenase inhibitors, 4-acylaminophenol derivatives (14), and compared them to those of other known inhibitors.

MATERIALS AND METHODS

Animals

Seven-week-old male Wistar rats (Charles River Japan, Atsugi) were used.

Materials

Casein (Nacalai Tesque, Kyoto); *N*-(hydroxyethyl) piperazine-*N'*-2-ethanesulfonic acid (HEPES), *o*-dianisidine, hydrogen peroxide, arachidonic acid (AA), hexadecyltrimethylammonium bromide (HTAB) and indomethacin (all from Sigma, St. Louis, MO, USA); horse radish peroxidase (Boehringer Mannheim GmbH, Mannheim, Germany); and phenidone (Tokyo Kasei, Tokyo) were obtained commercially. AA-861, BW-755C and 4-acylaminophenol derivatives (shown in Fig. 1, T-0799: *N*-(3,5-dimethyl-4-hydroxyphenyl)-3-thenoic amide and T-0757: *N*-(3,5-dimethyl-4-hydroxyphenyl)-3,7-dimethyl-(2,*E*)-2,6-octadienoic amide) were synthesized in the Organic Chemistry Research Laboratory of Tanabe Seiyaku Co., Ltd. Other chemicals used were of the special grade.

Preparation of rat peritoneal neutrophils

Rats were injected intraperitoneally with a 2% sterile casein solution (100 ml/kg). Sixteen hours later, the rats were killed by bleeding from the carotid artery, and neutrophils were collected by washing the peritoneal cavity with 20 ml of 25 mM HEPES containing Hank's Balanced Salt Solution (HBSS). The neutrophils were centrifuged at 750 × *g* for 5 min at 4℃, and contaminating erythrocytes were removed by hypotonic lysis. The cell density was adjusted to 1×10^7 cells/ml. The purity of this neutrophil preparation was more than 90%.

Assay of MPO activity

MPO activity was measured according to the methods of Bradley et al. (12) and Lundberg and Arfors (13) with a slight modification. Ten milliliters of 0.5% HTAB containing 0.05 M potassium phosphate buffer (pH 6.0) was added to 1 ml of the above neutrophil suspension in HBSS. After vigorous shaking, the mixture was homogenized with a Polytron™, and the homogenate was centrifuged at 12,000 × *g* for 30 min at 15℃. Three milliliters of the supernatant was mixed with 3 ml of chloroform, vigorously shaken for 3 min and centrifuged at 1,500 × *g* for 5 min at 15℃, and the aqueous phase was used for the MPO assay. A 100-μl aliquot of the aqueous phase was preincubated at 37℃ for 3 min, mixed with 3 ml of 0.05 M potassium phosphate buffer (pH 6.0) containing 0.17 mg/ml of *o*-dianisidine and 0.0005% of hydrogen peroxide and then incubated for 15 min. The reaction was terminated by adding 2 ml of 0.1 N NaOH. The absorbance at 460 nm of the resultant reaction mixture was measured. MPO activity was expressed in units as the total activity of the homogenate using horseradish peroxidase as a standard. The activity of the standard horseradish peroxidase showed a good linearity to the absorbance below 2 units (background, 0.014 abs; 1.06 unit, 0.619 abs; 2.11 unit, 0.965 abs). The MPO activity was stable for 48 hr at room temperature, and the color was stable for 2 hr at least.

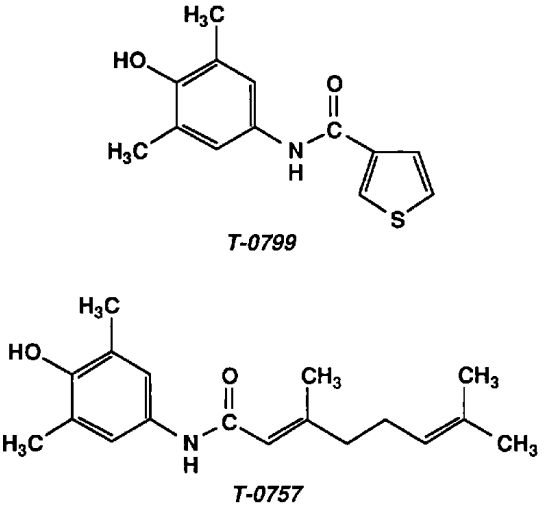

Fig. 1. Chemical structures of T-0799 and T-0757.

Induction of rat skin inflammation

AA (100 mg/ml) was suspended in divalent cation-free phosphate-buffered saline (PBS(−)100 mg/ml) and sonicated in an ice bath. Rats, previously shaved on the dorsal skin, were injected intradermally with vehicle (PBS(−)) or an AA suspension (0.1 ml) of various concentrations under halothane anesthesia using a 1-ml syringe for tuberculin with a 27 gauge needle. After a designated time, the rats were killed by decapitation and bleeding, and the skin lesion was isolated from the dorsal skin with a punch (r=9 mm) and scissors with a scalpel. The skin lesion was cut into fragments, suspended in 10 ml of 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5% HTAB and homogenized by a Polytron™. The homogenate was centrifuged at 12,000 × *g* for 30 min at 15℃, and the supernatant was mixed with 3 ml of chloroform, shaken for 3 min and centrifuged at 1,500 × *g* for 5 min at 15℃. The aqueous phase was used for MPO assay.

Drug preparations and administration

Drugs were evenly suspended in distilled water or physiological saline with Tween 80™, for oral or intraperitoneal administration, respectively, and the control rats were administered a corresponding vehicle. Since pharmacokinetic data were not available, the drugs were given twice, 0.5 hr before and 2.5 hr after AA injection, except for indomethacin, which was administered once 0.5 hr before AA injection. The rats were fasted for 24 hr before oral administration.

Statistics

The data were statistically analyzed by Student's *t*-test. A P value of 0.05 or less was considered to indicate a significant difference.

RESULTS

Correlation between the neutrophil number and MPO activity

Correlation between the number of neutrophils and MPO activity was examined. The MPO activity was proportional to the number of neutrophils in the range of 1×10^5 to 1×10^7 cells as shown in Fig. 2 ($R=0.997$, $P<0.001$).

Time course of the skin inflammation

MPO activity of the inflamed skin homogenate 0, 1, 3, 5 and 7 hr after intradermal injection of 3 and 10 mg/site of AA was examined. As shown in Fig. 3, the MPO activity increased dose-dependently, and its increment was statistically significant 5 hr after AA injection at either dose. For evaluation of drug efficacy in the following experiments, we sacrificed the animals 5 hr after AA injection at the dose of 10 mg/site.

MPO activity in the skin lesion 5 hr after injection of AA at various doses

AA (0, 1, 3 and 10 mg/site) was injected intradermally in the dorsal skin of rats. Then, the MPO activity of the skin lesion was measured 5 hr after AA injection. As shown in Fig. 4, the MPO activity increased dose-dependently above the AA dose of 1 mg/site.

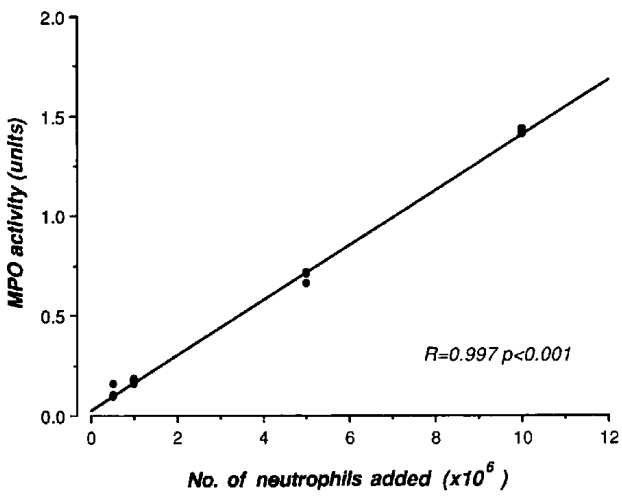

Fig. 2. Relationship between the number of neutrophils and MPO activity in vitro. The rat peritoneal neutrophils were lysed in HTBA solution. The MPO activity was assayed by the method of Bradley and Lundberg with a slight modification (see the text). Each point represents the individual data of a triplicate assay.

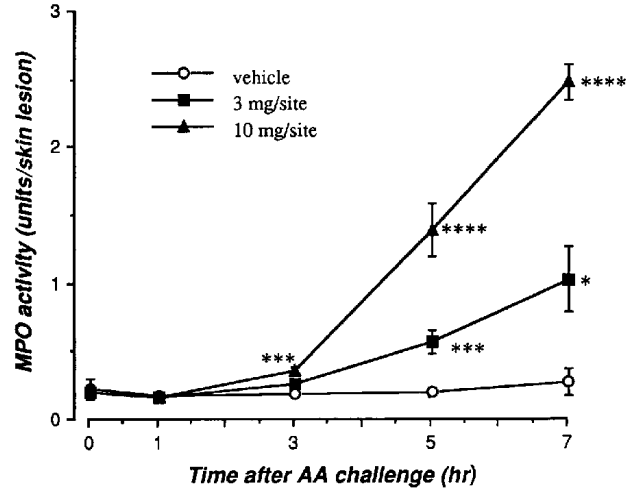

Fig. 3. Time course of changes in MPO activity at the lesion of AA-induced skin inflammation. The rats were sacrificed at the indicated time after AA injection. The MPO activity at the skin lesion was measured. Each point represents the mean±S.E.M. of 3 to 4 rats. The data were statistically analyzed by Student's *t*-test (*$P<0.05$, ***$P<0.01$, ****$P<0.001$).

Effects of oral or intraperitoneal treatment with known inhibitors of 5-lipoxygenase and cyclooxygenase on the skin inflammation

The dual inhibitor phenidone was administered at a

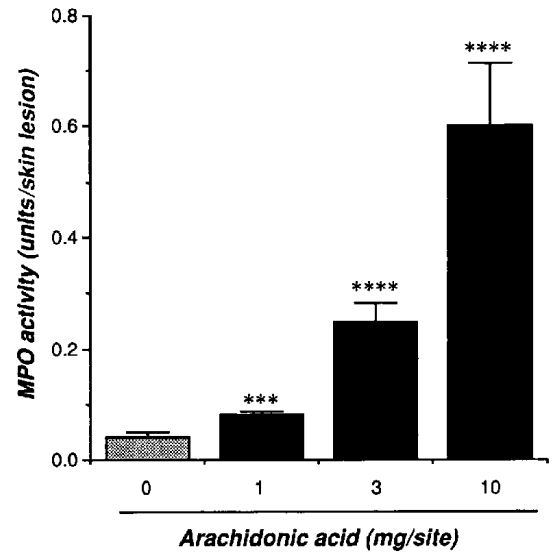

Fig. 4. MPO activity at the skin lesion of AA-induced skin inflammation. The suspension of AA was injected intradermally. Five hours later, the MPO activity of the inflamed skin was measured. Each column represents the mean±S.E.M. of 5 rats. The data were statistically analyzed by Student's *t*-test (***$P<0.01$, ****$P<0.001$).

dose of 100 mg/kg intraperitoneally or orally 0.5 hr before and 2.5 hr after AA injection. As shown in Fig. 5, phenidone showed a significant inhibition of MPO activity by either route of administration, suggesting a good bioavailability of the compound.

A 5-mg/kg dose of the cyclooxygenase inhibitor indomethacin (0.5 hr before AA injection) or 100 mg/kg of the lipoxygenase inhibitor AA-861 (0.5 hr before and 2.5 hr after AA injection) was intraperitoneally administered. As shown in Fig. 6, AA-861 inhibited the MPO activity under the conditions used, while indomethacin was without effect.

When 100 mg/kg of AA-861 or 50 mg/kg of the dual inhibitor BW-755C was orally administered, AA-861 was not effective while BW-755C inhibited the MPO activity as shown in Fig. 7.

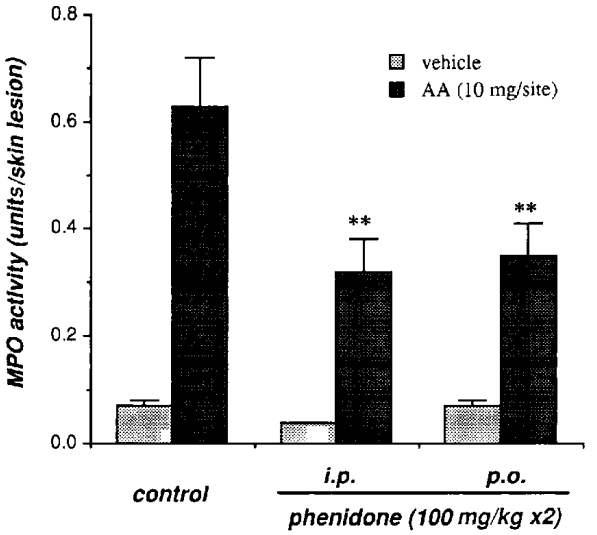

Fig. 5. Effect of phenidone on MPO activity at the skin lesion. Phenidone was administered intraperitoneally or orally 0.5 hr before and 2.5 hr after AA injection. Five hours after induction, MPO activity at the skin lesion was measured. Each column represents the mean±S.E.M. of 7 rats. The data were statistically analyzed by Student's *t*-test (**P<0.02).

Effects of oral treatment with the 4-acylaminophenol derivatives T-0799 and T-0757 on the skin inflammation

The 4-acylaminophenol derivatives T-0799 and T-0757 were orally administered 0.5 hr before and 2.5 hr after AA injection at doses of 10, 30 or 100 mg/kg. As shown in Fig. 8, both compounds significantly inhibited the inflammation at doses above 30 mg/kg.

DISCUSSION

There are no suitable experimental systems for studying leukocyte infiltration except for the techniques using a body cavity (i.e., pleural or peritoneal cavity) or artificial body space (i.e., air pouch). Since these systems are not disease models, one of the purposes of the present investigation was to establish a simpler and more quantitative

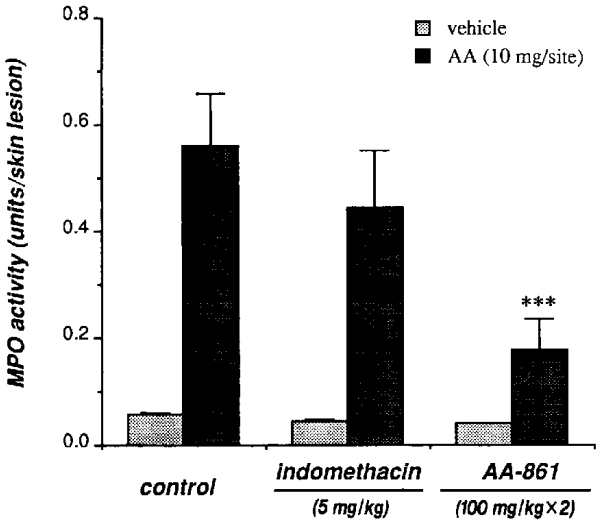

Fig. 6. Effects of indomethacin and AA-861 on MPO activity at the skin lesion. Indomethacin was administered intraperitoneally 0.5 hr before induction, and AA-861 was administered intraperitoneally 0.5 hr before and 2.5 hr after induction. Five hours after induction, MPO activity at the skin lesion was measured. Each column represents the mean±S.E.M. of 5 rats. The data were statistically analyzed by Student's *t*-test (***P<0.01).

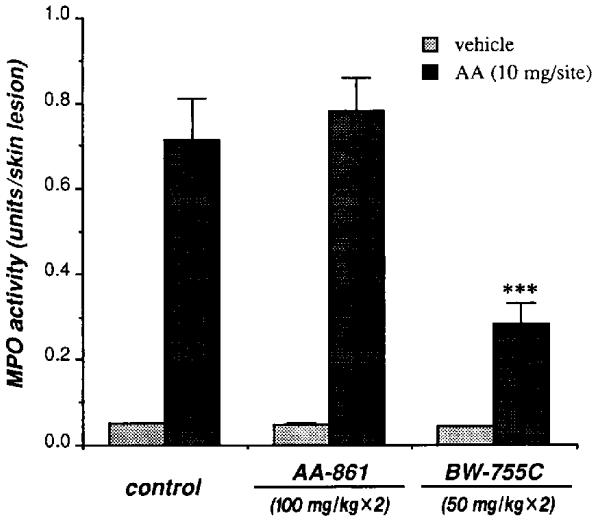

Fig. 7. Effects of oral treatment with AA-861 and BW-755C on MPO activity at the skin lesion. AA-861 and BW-755C were administered orally 0.5 hr before and 2.5 hr after AA-injection. Five hours after induction, MPO activity at the skin lesion was measured. Each column represents the mean±S.E.M. of 5 rats. The data were statistically analyzed by Student's *t*-test (***P<0.01).

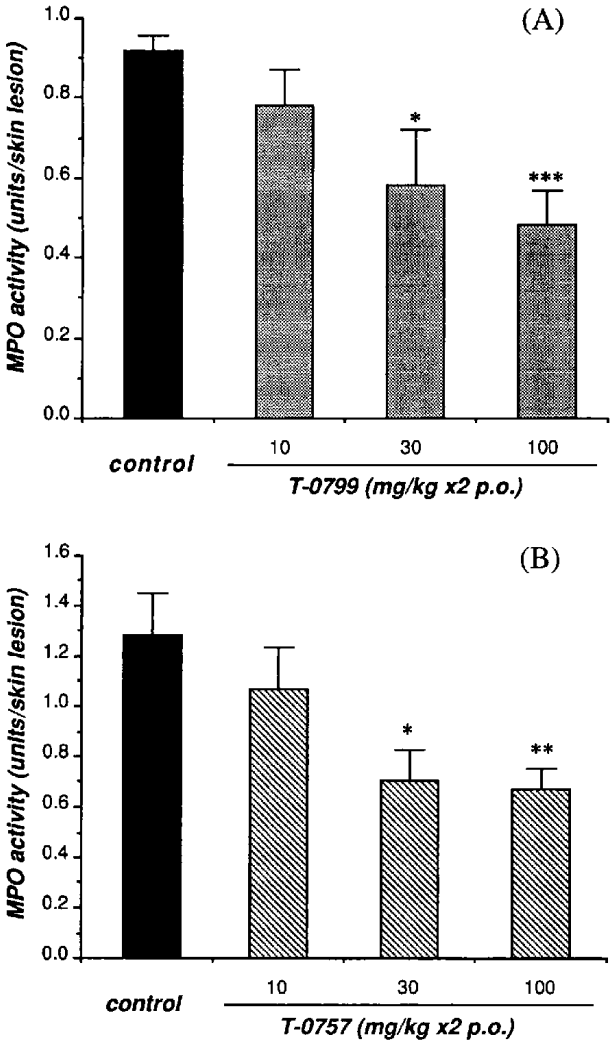

Fig. 8. Effects of 4-acylaminophenol derivatives on MPO activity at the skin lesion. T-0799 (A) and T-0757 (B) were administered orally 0.5 hr before and 2.5 hr after AA injection. Five hours after induction, MPO activity at the skin lesion was measured. Each point represents the mean ± S.E.M. of 4 to 5 rats. The data were statistically analyzed by Student's *t*-test (*$P<0.05$, **$P<0.02$, ***$P<0.01$).

method to measure leukocyte infiltration than histological examination, in a setting similar to inflammatory skin diseases such as psoriasis. Instead of histological examination which requires complicated procedures, we tried to use tissue MPO activity as an indicator of neutrophil infiltration according to Bradley et al. (12) and Lundberg and Arfors (13). However, their method to measure MPO activity is time-consuming, and hence inconvenient to assay many samples, because it is necessary to read the change of absorbance with time (rate assay). Thus, we modified the method to an endpoint assay by using 0.1 N NaOH to stop the reaction and make the endpoint color stable (see Materials and Methods). This modification enabled us to deal with many samples in a shorter time.

In the time course study, MPO activity was significantly increased 3 to 7 hr after induction (Fig. 3). These changes in MPO activity were similar to the data by DiMartino et al. (15), and the MPO activity of the time course study in the present study was higher than in the dose-response study because of fluctuation between experiments from unknown reasons. Although we did not measure the MPO activity beyond the 7th hour after AA injection, the MPO activity may most likely level off, because the neutrophil infiltration is not considered to be a lasting biological response in inflammation.

We have already reported that some of the 4-acylaminophenol derivatives are relatively selective 5-lipoxygenase inhibitors (14). The IC_{50} values of T-0799 for 5-lipoxygenase and cyclooxygenase were 0.81 and 140 μM, respectively, and those of T-0757 were 0.23 and 7.0 μM, respectively. Another purpose of the present study was to evaluate their effects on the in vivo leukocyte infiltration system. To clarify first how AA metabolites are involved in this neutrophil infiltration system, the effects of phenidone (a dual inhibitor of cyclooxygenase and lipoxygenase) (16), indomethacin (a cyclooxygenase inhibitor) and AA-861 (a lipoxygenase inhibitor) (17) on the system were tested. Phenidone, which has been reported to inhibit AA-induced paw edema reaction (1) and ex vivo synthesis of LTB_4 in rats (18), inhibited the skin inflammation, suggesting that prostaglandins (PGs) and/or LTs are involved in this model. However, the fact that AA-861 but not indomethacin inhibited the reaction suggests that production of lipoxygenase metabolite(s) induced by AA injection is likely to be involved in this inflammation model as reported by DiMartino et al. (15).

In this in vivo model of LT-mediated neutrophil infiltration, our novel lipoxygenase inhibitors (14), the 4-acylaminophenol derivatives T-0799 and T-0757, were shown to be effective (Fig. 8). Since these compounds are relatively selective inhibitors of 5-lipoxygenase and capable of inhibiting LTB_4 production (14), one of the chemotactic factors involved in the model is likely to be LTB_4. It has been reported that 5-lipoxygenase inhibitors of the redox type, such as NDGA, were inactive when administered orally (19). However, in our evaluation by oral treatment with AA-861, BW-755C (20) and the 4-acylaminophenol derivatives, all of which are of the redox type, the latter two, but not AA-861, were effective (Figs. 7 and 8). These results suggest that the oral efficacy of a lipoxygenase inhibitor may not depend on the type of inhibition mechanism, but simply on its bioavailability. The fact that the 4-acylaminophenol derivatives are orally active suggests that these compounds may be useful as therapeutic agents for the treatment of various LT-mediated inflammatory diseases.

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