

Species Difference of 5-Lipoxygenase Derived from Polymorphonuclear Leukocytes on Sensitivity to Drugs†

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Abstract—Effects of 12 compounds including 5-lipoxygenase (5-LO) inhibitors and antiallergic drugs on the activity of 5-LO from the polymorphonuclear leukocytes (PMN) of guinea pigs, rats, rabbits, monkeys and humans were examined. The 5-LO activity was inhibited by the drugs according to the following orders of efficacy: Guinea pig 5-LO, 5-HDHDMF>TZI-2721>NDGA>AA861>FPL55712>isoproterenol; rat 5-LO, 5-HDHDMF>AA861>TZI-2721>NDGA>FPL55712>KP-136>MCI-826>benoxaprofen; and rabbit 5-LO, 5-HDHDMF>TZI-2721>NDGA>AA861>FPL55712>KP-136>MCI-826. The 5-LO activity from the rhesus monkey was dose-dependently inhibited by only 3 compounds, 5-HDHDMF>NDGA>TZI-2721, in this order, but the other compounds, except for AA861, did not show any effect on this activity. In humans, 5-LO activity was inhibited in the following order: TZI-2721>5-HDHDMF>NDGA>AA861>FPL55712. From these results, it was strongly suggested that there is a species difference of this enzyme in its sensitivity to drugs.

Slow reacting substance of anaphylaxis (SRS-A), which has been considered as one of important chemical mediators of allergic bronchial asthma, is now well-defined as a mixture of 3 metabolites, leukotriene (LT) C₄, LTD₄ and LTE₄, generated from the precursor arachidonic acid through the 5-lipoxygenase (5-LO) pathway.

Purification and physicochemical properties of 5-LO have been extensively investigated by Samuelsson et al. (1–3) and other investigators (4–8) since the discovery of the enzyme in the leukocytes of the rabbit peritoneal cavity by Borgeat et al. (9). On the other hand, it is hardly known whether polymorphonuclear leukocyte (PMN)-derived 5-LO has any species difference in its sensitivity to drugs.

In the present study, the effects of various drugs on the activities of 5-LO prepared from human, monkey, rabbit, rat and guinea pig PMN were compared.

Materials and Methods

Animals: Male Hartley guinea pigs weighing 550–650 g and male Wistar rats weighing 200–250 g were purchased from Shizuoka Laboratory Animal Center (Hamamatsu). Japanese white male rabbits weighing 3.0–3.5 kg (Shimizu Experimental Material Co. Ltd., Kyoto) and male rhesus monkeys weighing 7.5–9.0 kg (generously supplied by Central Research Laboratory, Nippon Shinyaku Co., Ltd., Kyoto) were also used in the experiment.

Reagents: Reagents used were casein, glutathione (GSH), adenosine-5'-triphosphate disodium salt trihydrate (ATP) and N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES) (all purchased from Wako Pure Chem. Ind. Ltd., Osaka); ethylenediaminetetraacetic acid sodium salt (EDTA,

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Nakarai Chem. Ltd., Kyoto); heparin (Kodama Chem., Tokyo); [^{14}C]-arachidonic acid (^{14}C -AA, 59.6 mCi/mmol, Amersham Int. plc, Buckinghamshire); and [^{14}C]-5-hydroxyeicosatetraenoic acid (^{14}C -5-HETE, biologically synthesized). Other commercially available reagents used in this study were of the highest grade available.

Drugs: Drugs employed were AA861 (Takeda Chem. Ind., Ltd., Osaka); 5-hexyloxy - 3',4' - dihydroxy - 6,7 - dimethoxyflavone (5-HDHDMF, generously supplied by Prof. Tokunaru Horie, Tokushima Univ., Tokushima); TZI-2721 (Teikoku Hormone Mfg. Co., Ltd., Kawasaki); nordihydroguaiaretic acid (NDGA, Sigma Chem., St. Louis, MO); benoxaprofen (synthetic compound, Kaken Pharm. Co., Ltd., Kyoto); FPL55712 (Fisons Ltd., Loughborough); MCI-826 (Mitsubishi Kasei Ltd., Inashiki); L-isoproterenol D-bitartrate (Nakarai Chem. Ltd., Kyoto); theophylline (Nakarai Chem. Ltd., Kyoto); tranilast (Kissei Pharm. Co. Ltd., Matsumoto);

KP-136 and C₄C (Kaken Pharm. Co. Ltd., Kyoto). The chemical structures of these drugs are illustrated in Fig. 1. All drugs were dissolved and diluted with dimethylsulfoxide (DMSO) before use.

Preparation of guinea pig 5-LO: Guinea pig 5-LO was prepared according to the method of Ochi et al. (6). Two % casein-containing saline was i.p. injected at a dose of 10 ml/100 g body weight. After 16 hr, the animal was sacrificed under ether anesthesia and 5 ml/animal of saline containing 300 U heparin was i.p. injected. The peritoneal fluid containing leukocytes was obtained after laparotomy. The peritoneal cavity was once rinsed with saline, 10 ml/animal, combined with the previous peritoneal fluid, and filtered on gauze. The filtrate was centrifuged at 1,000×g for 5 min at 4°C. The pellet was subjected to hypotonic shock with 0.2% saline (1 ml/animal) for 30 sec for hemolysis. After the same volume of 1.6% saline was added and centrifuged again under the above

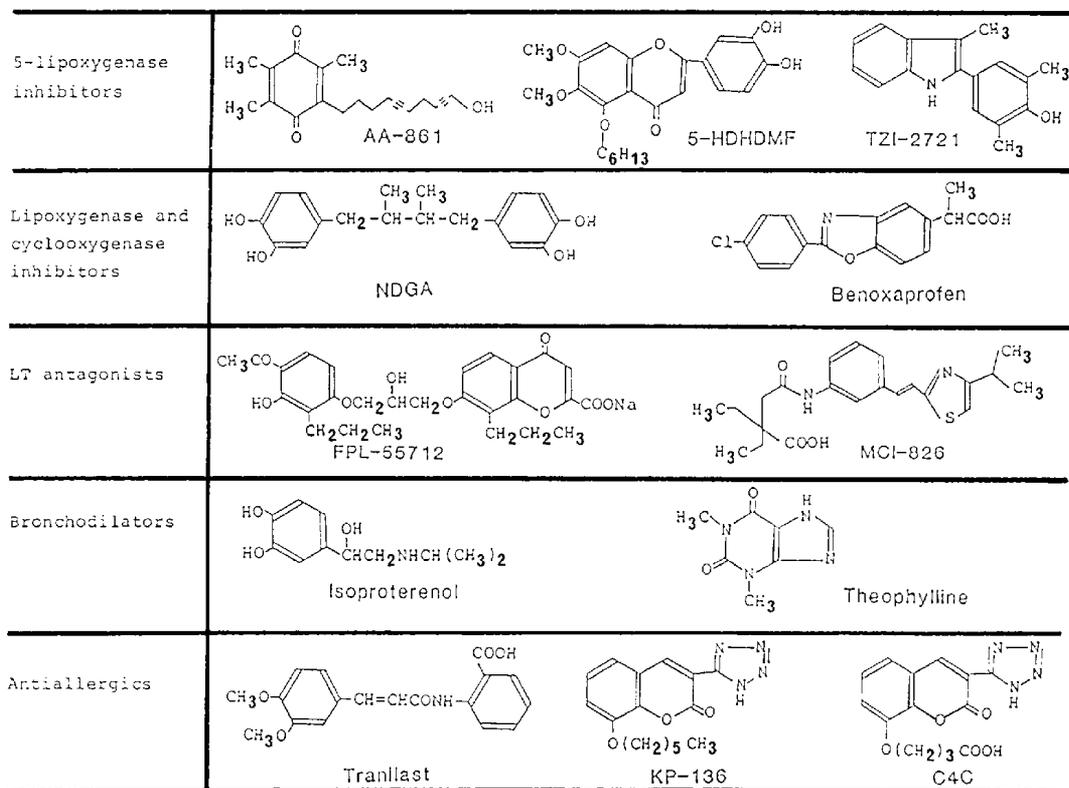


Fig. 1. Chemical structures of drugs used.

condition, the pellet was suspended with 50 mM HEPES buffer containing 0.5 mM EDTA (pH 8.0, EDTA/HEPES solution) at 2×10^8 PMN/ml. Then, the cell suspension was ultrasonicated at 20 kHz for 30 sec under ice-cooling and centrifuged at $10,000 \times g$ for 10 min at 4°C . The supernatant was further centrifuged at $105,000 \times g$ for 60 min at 4°C . To 19.5 ml of the resultant supernatant were added 9 ml of 50% polyethylene glycol 6,000 and 1.5 ml of 2 M KCl. The mixture was allowed to stand for 30 min under ice-cooling and then centrifuged at $30,000 \times g$ for 10 min at 4°C . The precipitate was dissolved in 5 ml of EDTA/HEPES solution and recentrifuged at $30,000 \times g$ for 10 min at 4°C . Crude 5-LO of the supernatant was poured into a column (25 \times 50 mm) of DEAE-Sephacel (Pharmacia), washed with EDTA/HEPES solution, and then eluted with EDTA/HEPES solution containing 0.5 M KCl. The 5-LO fraction was concentrated using Mol Cut (M.W.=30,000, Nippon Millipore Ltd.) and used as the partially purified 5-LO enzyme reference of guinea pigs.

Preparation of rat and rabbit 5-LO: Similarly to guinea pigs, casein-treated rats were exsanguinated and 20 ml/animal of saline containing heparin (40 U/ml), 10 mM Na_2HPO_4 and 1 mM EDTA was i.p. injected. After collection of the peritoneal fluid containing leukocytes, the peritoneal cavity was rinsed with saline (10 ml/animal). The washings were combined with the previously collected peritoneal fluid and then filtered through gauze. The filtrate was centrifuged at $1,000 \times g$ for 10 min at 4°C to obtain the PMN pellet.

Under pentobarbital anesthesia, 600 ml of 0.2% oyster glycogen/animal was i.p. injected to rabbits, and the peritoneal fluid was collected 16–18 hr later. Following filtration of the fluid through gauze, the filtrate was centrifuged at $600 \times g$ for 5 min at 4°C to obtain the PMN pellet.

Similarly to the guinea pig PMN pellet, hypotonic treatment and centrifugation were applied to the rat and rabbit PMN pellets. Either rat or rabbit PMN was suspended at 5×10^8 PMN and 2.5×10^8 PMN/ml with EDTA/HEPES solution for rats and rabbits, respectively.

Both rat and rabbit PMN were ultrasonicated at 20 kHz for 30 and 10 sec, respectively, under ice-cooling. The supernatants obtained by centrifugation at $5,000 \times g$ for 10 min at 4°C were used as rat and rabbit 5-LO enzyme references, respectively.

Preparation of rhesus monkey and human 5-LO: A one-fifth volume of 6% dextran was added to heparinized peripheral blood samples (10 units heparin/ml blood) drawn from the rhesus monkey or a healthy human volunteer, and each mixture was subjected to upside-down mixing. After allowing them to stand at room temperature, the upper layer containing leukocytes was aspirated.

The leukocyte layer of the rhesus monkey was centrifuged at $500 \times g$ for 5 min at 4°C . The human leukocyte layer was layered on an equivolume of a 9% Ficoll-400/33.4% Conray-400 (24/10) solution and centrifuged at $500 \times g$ for 30 min at 4°C . Both of the pellets were suspended at 5×10^7 PMN/ml with EDTA/HEPES solution following hypotonic treatment and centrifugation. Rhesus monkey and human PMN suspensions were ultrasonicated at 20 kHz for 40 and 10 sec, respectively, under ice-cooling. The supernatants obtained by centrifugation at $5,000 \times g$ for 10 min at 4°C were used as the respective 5-LO references.

All procedures described above were carried out at 4°C , and all of the 5-LO references were kept at -80°C until use.

Measurement of the 5-LO activities: A mixture of 100 μl of an enzyme (guinea pig, rat, rabbit, rhesus monkey and human 5-LO contained 10.1, 24.4, 67.3, 16.3–32.6 and 24.5 μg as protein, respectively), 20 μl of 250 mM phosphate buffer (pH 7.3) containing 10 mM GSH and 20 mM ATP, 18 μl of EDTA/HEPES solution and 2 μl of either drug solution or DMSO was preincubated at 30°C for 10 min. Subsequently, 20 μl of saline containing 50 mM CaCl_2 and 40 μl of ^{14}C -AA of 3×10^4 d.p.m. were added, followed by incubation at 30°C for 20 min. The reaction was terminated by adding 200 μl of an ice-cooled mixture of ethyl acetate/methanol/0.2 M citric acid (30/4/1). Following vigorous stirring for 1 min, the reaction solution was centrifuged at $1,700 \times g$ for 10 min at 4°C . One hundred μl of the organic layer was

spotted on a TLC plate. After saturation of the TLC plate with a developing solvent consisting of a mixture of diethylether/petroleum ether/acetic acid (50/50/4) at room temperature, the TLC was developed under ice-cooling. After completion of the development, the portion corresponding to ^{14}C -5-HETE reference, which was spotted on a site differing from the test sample site and developed simultaneously, was scraped out to measure its radioactivity.

Triplicate measurements were carried out for each of the species-derived 5-LO activities, and the effect of each drug was expressed by the mean formation rate (%) of ^{14}C -5-HETE vs. the control or IC_{50} value obtained by the dose-reaction relationship.

Statistical analysis: IC_{50} values were tested statistically by the linear regression analysis of the concentration-reaction relation using the method of least squares.

Results

Effect on the guinea pig 5-LO activity: Table 1 and Fig. 2 show effects of the examined drugs on the guinea pig 5-LO activity. In the control, which was incubated after addition of ^{14}C -AA to 5-LO in the absence of drugs, ^{14}C -5-HETE of 9.1×10^3 d.p.m. was formed. The treatment with AA861, 5-HDHDMF, TZI-2721 or NDGA inhibited 5-HETE formation in a dose-dependent manner,

and the IC_{50} values of the drugs were 0.47, 0.01, 0.045 and $0.27 \mu\text{M}$, respectively.

At $100 \mu\text{M}$, FPL55712 and isoproterenol produced almost complete inhibition and an approximately 50% inhibitory effect, respectively; however, neither had an effect at $10 \mu\text{M}$. Other drugs had no effect, even at $100 \mu\text{M}$.

Effect on the rat 5-LO activity: The effects of drugs on the rat 5-LO activity are summarized in Table 1 and Fig. 3. ^{14}C -5-HETE of 9.4×10^3 d.p.m. was formed in the control. AA861, 5-HDHDMF, TZI-2721 and NDGA dose-dependently inhibited the 5-LO activity, and their IC_{50} values were 0.12, 0.01, 0.23 and $0.43 \mu\text{M}$, respectively. FPL55712, MCI-826, KP-136 and benoxaprofen also had dose-dependent inhibitory effects, and their IC_{50} values were 8.0, 30.0, 20.7 and $78.6 \mu\text{M}$, respectively. Other drugs had no effect at $100 \mu\text{M}$.

Effect on the rabbit 5-LO activity: Table 1 and Fig. 4 show the effects of drugs on the rabbit 5-LO activity. The control produced ^{14}C -5-HETE of 1.1×10^4 d.p.m. AA861, 5-HDHDMF, TZI-2721, NDGA, FPL55712 and KP-136 dose-dependently inhibited the activity and their IC_{50} values were 1.17, 0.05, 0.34, 0.72, 15.9 and $47.5 \mu\text{M}$, respectively. At $100 \mu\text{M}$, MCI-826 showed 45% inhibition on the rabbit 5-LO activity, but the other drugs had no effect.

Table 1. Effect of examined drugs on the 5-lipoxygenase activities derived from polymorphonuclear leukocytes of the guinea pig, rat, rabbit, rhesus monkey and human

Compound	$\text{IC}_{50} \mu\text{M}$				
	Guinea pig	Rat	Rabbit	Rhesus monkey	Human
AA861	0.47	0.12	1.17	not tested	2.3
5-HDHDMF	0.01	0.01	0.05	0.07	0.22
TZI-2721	0.045	0.23	0.34	3.1	0.15
NDGA	0.27	0.43	0.72	1.2	0.35
Benoxaprofen	no effect*	78.6	no effect*	no effect*	no effect*
FPL55712	≥ 10	8.0	15.9	no effect*	32.6
MCI-826	no effect*	30.0	≈ 100	no effect*	no effect*
Isoproterenol	≈ 100	no effect*	no effect*	no effect*	no effect*
Theophylline	no effect*	no effect*	no effect*	no effect*	no effect*
Tranilast	no effect*	no effect*	no effect*	no effect*	no effect*
KP-136	no effect*	20.7	47.5	no effect*	no effect*
C_4C	no effect*	no effect*	no effect*	no effect*	no effect*

*no effect at $100 \mu\text{M}$

Effect on the rhesus monkey 5-LO activity: LO activity are listed and depicted in Table 1 and Fig. 5, respectively. The effect of drugs on the rhesus monkey 5-

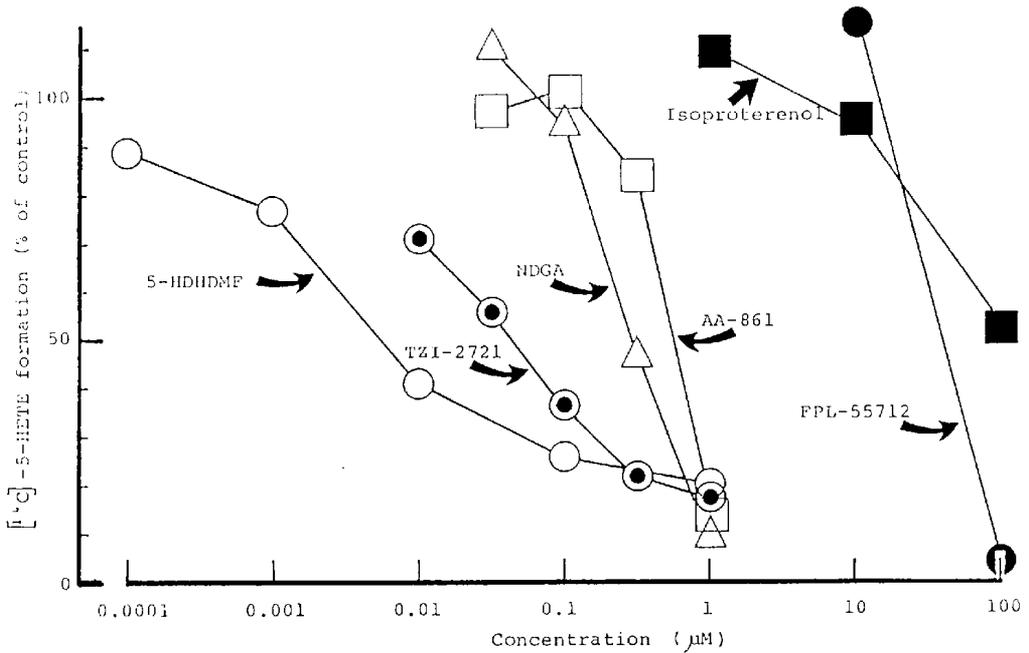


Fig. 2. Effects of various drugs on the 5-lipoxygenase activity from guinea pig polymorphonuclear leukocytes.

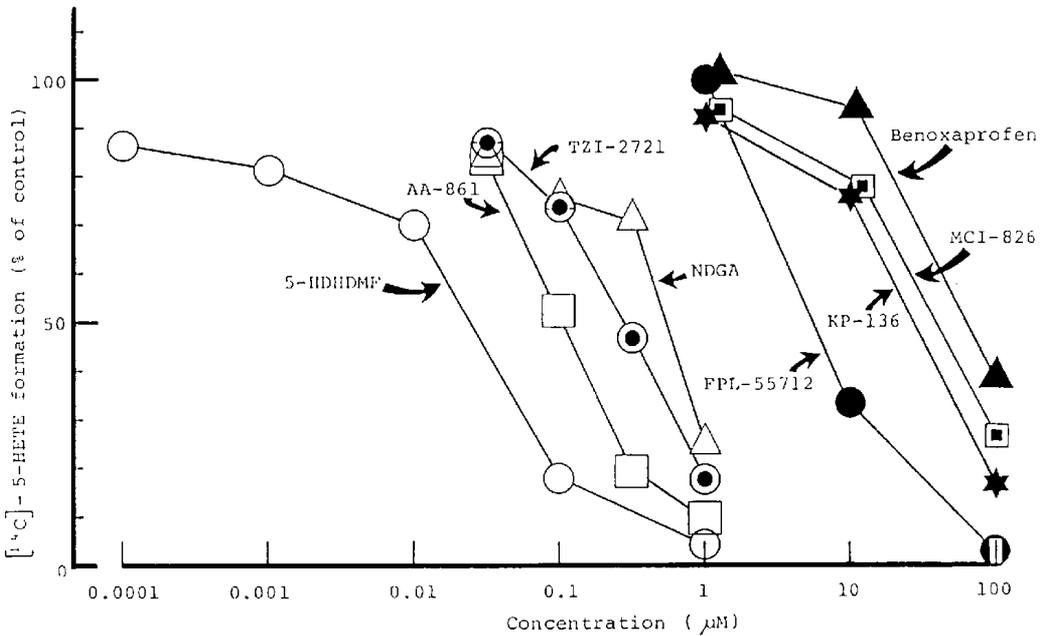


Fig. 3. Effects of various drugs on the 5-lipoxygenase activity from rat polymorphonuclear leukocytes.

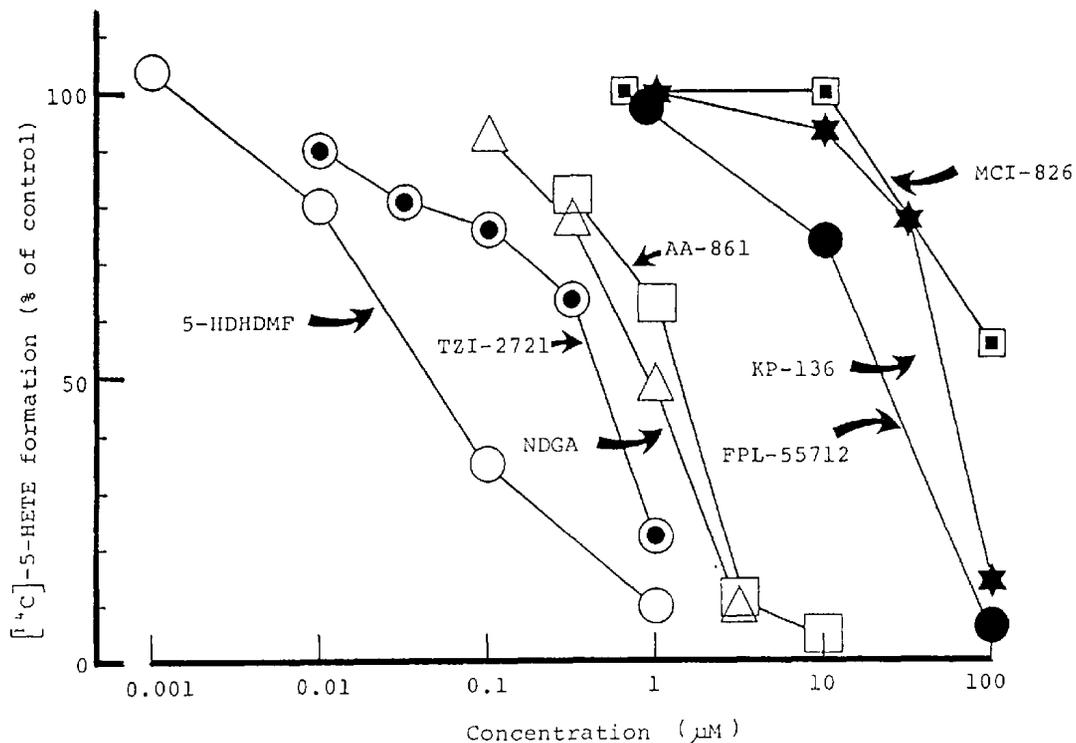


Fig. 4. Effects of various drugs on the 5-lipoxygenase activity from rabbit polymorphonuclear leukocytes.

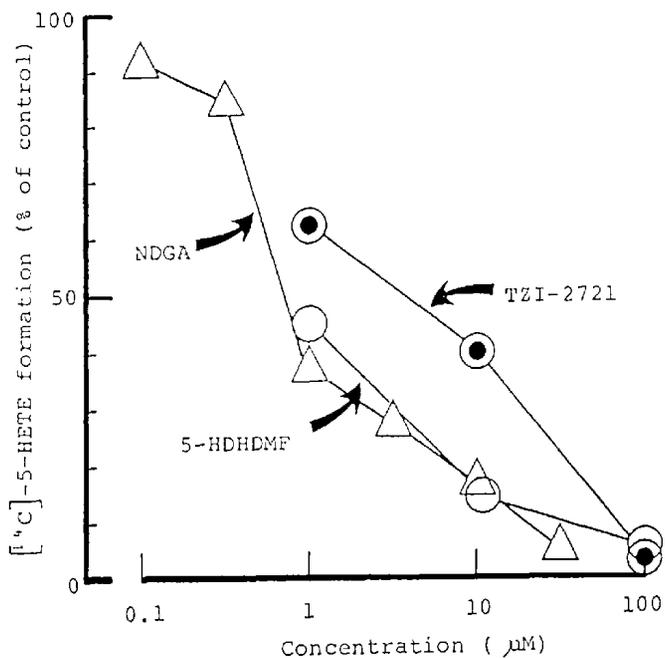


Fig. 5. Effects of various drugs on the 5-lipoxygenase activity from rhesus monkey polymorphonuclear leukocytes.

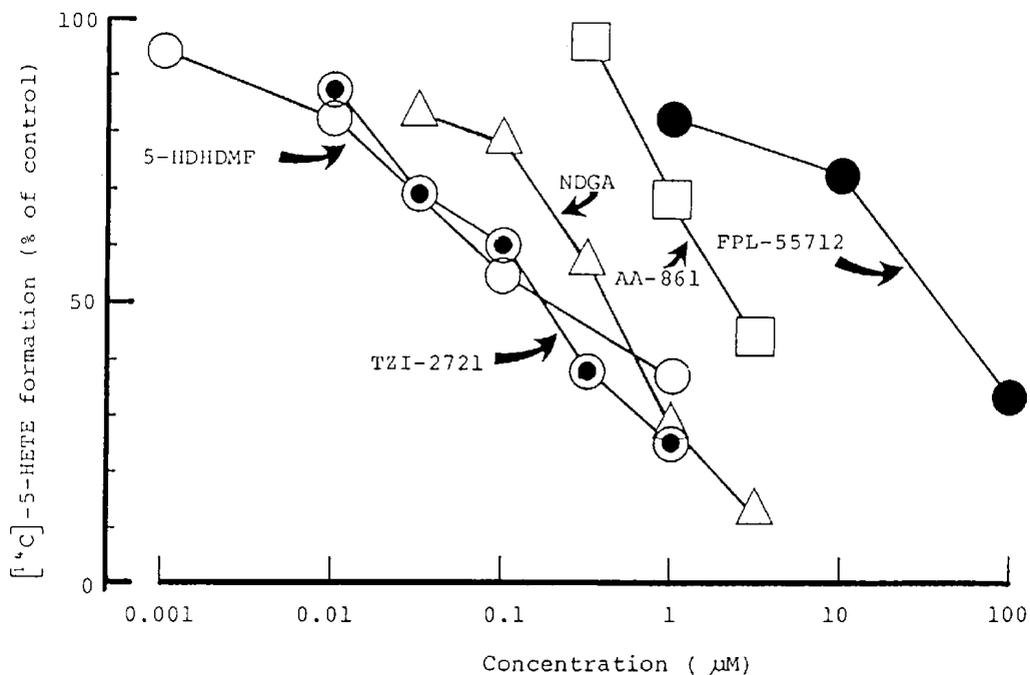


Fig. 6. Effects of various drugs on the 5-lipoxygenase activity from human polymorphonuclear leukocytes.

^{14}C -5-HETE of 2.5×10^3 d.p.m. The activity was dose-dependently inhibited by 5-HDHDMF, TZI-2721 and NDGA, of which the IC_{50} values were 0.07, 3.1 and 1.2 μM , respectively. Other drugs had no inhibitory effect even at 100 μM .

Effect on the human 5-LO activity: Table 1 and Fig. 6 show the effects of drugs on the human 5-LO activity. ^{14}C -5-HETE of 5.0×10^3 d.p.m. was produced in the control. AA861, 5-HDHDMF, TZI-2721, NDGA and FPL-55712 inhibited the human 5-LO activity in a dose-dependent manner, and their IC_{50} values were 2.3, 0.22, 0.15, 0.35 and 32.6 μM , respectively. Other drugs did not inhibit the activity even at 100 μM .

Discussion

LTC_4 , LTD_4 and LTE_4 that are formed from arachidonic acid have been proven to induce a strong constriction of the airway smooth muscle, while LTB_4 has been shown to have potent chemotactic activity on leukocytes. The considerable contribution of LTs to the Schultz-Dale reaction of isolated human bronchial muscle *in vitro* has been also suggested (10). Furthermore, it has been re-

ported that the PMN derived from patients with allergic bronchial asthma produced more 5-HETE than those from healthy subjects (11). These data strongly suggest that LTs play important roles in provoking allergic bronchial asthma.

Meanwhile, it has been described that the mouse auricle inflammations elicited by calcium ionophore A23187 (12), arachidonic acid (13) or arachidonic acid plus tetradecanoylphorbol acetate (14) were all inhibited by 5-LO inhibitors. Aked et al. (15) also demonstrated that arachidonic acid-induced leukocyte infiltration and plasma transduction in the rabbit skin could be strongly inhibited by 5-LO inhibitors.

Therefore, metabolites produced by 5-LO are suggested to be closely related to the onset of not only allergic asthma but also various inflammatory disorders. From these observations, it is conceivable that an evaluation of the inhibitory effect against the 5-LO activity in terms of 5-HETE formation may be a useful index to measure the efficacy of new therapeutic drugs for the treatment of the above disorders.

5-LOs derived from different animal

species including rat basophilic leukemia cells (16–18), guinea pig PMN (19–21), rat PMN (18), rabbit PMN (22) and human PMN (17, 18) have been separately and independently used for evaluating the inhibitory effect of compounds on 5-LO activity. However, we have as yet very little information about whether there is an animal species difference in the sensitivity of 5-LO to drugs.

In this paper, the effects of 5-LO inhibitors (AA861, 5-HDHDHF, TZI-2721), LT-antagonists (FPL55712, MCI-826), anti-allergic agents (tranilast, KP-136 which is under development and its active metabolite C₄C), bronchodilators (isoproterenol, theophylline) and dual inhibitors of arachidonic acid metabolism (NDGA, benoxaprofen) on the 5-LO activities derived from guinea pig, rat, rabbit, monkey and human PMN were evaluated.

All 5-LO inhibitors could inhibit the 5-LO activity derived from all animal species in a dose-dependent manner, but the inhibitory effects were considerably different among the different animal species and drugs: AA861 had the most potent inhibitory effect on rat 5-LO, but the inhibitory effects on the guinea pig, rabbit and human 5-LO activities were approximately 1/4, 1/10 and 1/20 of that on rat 5-LO activity, respectively. The IC₅₀ value (0.47 μ M) of AA861 on the guinea pig 5-LO activity was nearly equal to that reported by Yoshimoto et al. (21)

The inhibitory effects of 5-HDHDHF on the guinea pig and rat 5-LO activities were very similar, but the effect on the human 5-LO activity was much weaker (approximately 1/20).

The inhibitory effect of TZI-2721 on the guinea pig 5-LO activity was approximately 70 times stronger than that on the rhesus monkey 5-LO activity.

On the other hand, NDGA had a concentration-dependent and nearly equal effect to inhibit the guinea pig, rat, rabbit and human 5-LO activities. Although benoxaprofen showed a weak inhibitory effect on only the rat 5-LO activity, it had no effect on the 5-LO activities derived from other animal species.

MCI-826 and KP-136 did not inhibit the human, monkey and guinea pig 5-LO activities even at concentrations as high as 100

μ M. However, MCI-826 and KP-136 exerted concentration-dependent inhibitory effects on the rat 5-LO activity and both rat and rabbit 5-LO activities, respectively.

These results strongly suggest that there are significant differences in the sensitivities of 5-LO derived from guinea pig, rat, rabbit, monkey and human PMN to various drugs.

In addition, the 5-LO (activity) in rat PMN was largely localized in the microsome fraction, but scarcely detected in the cytosol fraction. Therefore, at least in the rat PMN, localization of 5-LO is different from other animal species PMN used.

It has been reported that tranilast, an anti-allergic drug currently developed for clinical use, showed inhibition of the immunological release of chemical mediators and this mechanism must mainly contribute to its therapeutic effect (23, 24). More recently, it has been reported that the inhibitory effect of the mediator release by tranilast is more potent in LTB₄ and LTC₄, LTD₄ or LTE₄ release than in histamine release from both human lung fragments and PMN induced by immunological stimulation (25). More potent inhibition of both LTB₄ and peptide LTs (LTC₄, LTD₄ and LTE₄) suggested that tranilast might influence the 5-LO activity. As clearly shown in this paper, however, the drug does not inhibit the 5-LO activity of human PMN as well as those of other species used. Therefore, it is conceivable that other than inhibiting the common pathway leading to histamine and LT release, a possible mechanism responsible for the more potent inhibition of LT release by tranilast may be its ability to prevent a mediator releasing process prior to LT formation and subsequent to histamine release.

A higher concentration (1.2 mM) of epinephrine has been reported to inhibit the 5-LO activity (26) and enhance the cyclooxygenase activity (27, 28). In our study, however, isoproterenol had no effect on the rat, rabbit, monkey and human 5-LO activities. Although the guinea pig 5-LO activity was inhibited by approximately 50% with 100 μ M of isoproterenol, this inhibitory concentration was almost 100 times as high as that against the SRS-A release by the immunological reaction according to the study by

Ishizaka et al. (29). Therefore, isoproterenol is considered to rarely participate in direct LT formation by the immunological reaction.

Walker and Dawson (22) reported that benoxaprofen inhibited the rabbit neutrophil-derived 5-LO activity. Harvey and Osborne (19) also demonstrated that the activity of 5-LO derived from guinea pig PMN was inhibited by the drug. On the contrary, McMillan et al. (17) proved that the human 5-LO activity was not inhibited by the drug. These results strongly support our experimental data indicative of a species difference in the sensitivity of 5-LO to drugs.

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