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SHORT COMMUNICATION

# A Selective Inhibitor of Arachidonate 5-Lipoxygenase Scavenging Peroxide Activator

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**ABSTRACT.** A novel compound termed YT-18 (2,3-dihydro-2,4,6,7-tetramethyl-2-[(4-phenyl-1-piperazinyl) methyl]-5-benzofuranamine) selectively inhibited 5-lipoxygenases of porcine leukocytes ($IC_{50}$ value, 7.5 μM), human leukocytes (1.5 μM), and rat basophilic leukemia cells (14 μM), which are responsible for bioactive leukotriene synthesis. In contrast, the compound up to 1 mM had almost no effect on 12-lipoxygenases of leukocytes and platelets, 15-lipoxygenase, and cyclooxygenases-1 and -2. YT-18 also inhibited the leukotriene synthesis in intact rat basophilic leukemia cells. In the 5-lipoxygenase reaction, YT-18 caused a lag phase, thereby delaying the start of the reaction. The lag was abolished by the addition of 13-hydroperoxy-linoleic acid in a dose-dependent manner, and most (but not all) of the reduced 5-lipoxygenase activity was recovered. BIOCHEM PHARMACOL **54**;4:529–532, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** arachidonic acid; leukotriene; 5-lipoxygenase; cyclooxygenase; coumaran; peroxide

Arachidonate 5-, 12-, and 15-lipoxygenases are a group of enzymes that incorporate a molecular oxygen into various positions of unsaturated fatty acids [1]. Fatty acid cyclooxygenase also catalyzes a lipoxygenase-like reaction [2]. Isozymes of the 12-lipoxygenase have recently been found, namely of the leukocyte-type and platelet-type [1]. In addition, cyclooxygenase-2 was shown to be an isozyme of the previously known enzyme cyclooxygenase-1 [2]. 5-Lipoxygenase is a bifunctional enzyme that catalyzes both oxygenation of arachidonic acid to 5-hydroperoxy-6*E*, 8*Z*, 11*Z*, 14*Z*-eicosatetraenoic acid (5-HPETE†) and dehydration of the 5-HPETE to form 5,6-epoxy-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid ($LTA_4$) [1]. The $LTA_4$ then serves as a precursor of various leukotrienes implicated in the inflammatory process and allergic reaction [3]. Therefore, selective and potent 5-lipoxygenase inhibitors have been extensively sought by many researchers.

## MATERIALS AND METHODS

2,3-Dihydro-2,4,6,7-tetramethyl-2-[(4-phenyl-1-piperazinyl) methyl]-5-benzofuranamine (YT-18) and other YT compounds (see Table 1) were synthesized by Pharmaceutical Research Laboratories I, Takeda Chemical Industries Ltd. (Osaka, Japan). [1-$^{14}$C]5-HPETE [4] and 13-hydroperoxy-9*Z*, 11*E*-octadecadienoic acid (13-HPODE) [5] were prepared enzymatically as described previously. 5-Lipoxygenase was highly purified from the 105,000 × *g* supernatant of porcine leukocyte homogenate by immunoaffinity chromatography [4]. The 105,000 × *g* supernatant of the sonicate of human leukocytes [4] and rat basophilic leukemia cells [6] were also used as the cytosol preparations of human and rat 5-lipoxygenases. Recombinant 12-lipoxygenases of porcine leukocytes [7] and human platelets [8] and rabbit reticulocyte 15-lipoxygenase [9] were purified as described previously. Cyclooxygenase-1 was solubilized with 1% Triton X-100 from the microsomal fraction of sheep seminal vesicle. Cyclooxygenase-2 was expressed in BmN4 insect cells by transfection of the recombinant baculovirus carrying mouse cyclooxygenase-2 cDNA, and the enzyme was solubilized from the microsomal fraction with 45 mM octylglucoside. Protein concentration was determined by the method of Lowry *et al.* [10] with BSA as standard.

The standard assay conditions were described previously for lipoxygenases [4, 6–9] and cyclooxygenases [11]. In view of the suicide inactivation of lipoxygenases and cyclooxygenases [1, 2], the initial velocities were determined by their reactions with [1-$^{14}$C]arachidonic acid for 1 min at 30° for 5-, 12-, and 15-lipoxygenases and at 24° for cyclooxygenases. The amount of enzyme oxygenating ca. 2.5 nmol of arachidonic acid per min was used for each enzyme assay. The ethereal extract was then subjected to TLC with a solvent system of diethyl ether/petroleum ether/acetic acid (85:15:0.1, v/v). The amount of the reaction product was determined by measuring radioactivity on the TLC plates by a Fujix Bio-image analyzer BAS 2000

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† *Abbreviations:* 5-HPETE, 5-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; 13-HPODE, 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; $LTA_4$, 5, 6-epoxy-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid; $LTB_4$, 5,12-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid; YT-18, 2,3-dihydro-2,4,6,7-tetramethyl-2-[(4-phenyl-1-piperazinyl)methyl]-5-benzofuranamine.

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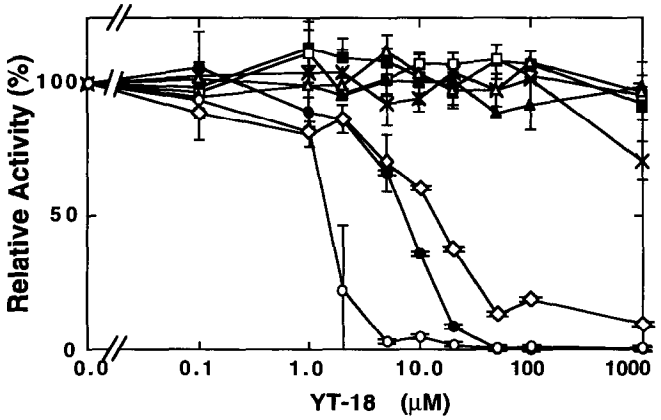



FIG. 1. Effects of YT-18 on various lipoxygenases and cyclooxygenases. Reactions were carried out under the standard conditions in the presence of YT-18 at various concentrations. Porcine 5-lipoxygenase (9 μg, closed circles), human 5-lipoxygenase (425 μg cytosol, open circles), and rat 5-lipoxygenase (134 μg cytosol, open lozenges), leukocyte 12-lipoxygenase (3.3 μg, open triangles), platelet 12-lipoxygenase (0.6 μg, closed triangles), 15-lipoxygenase (2.6 μg, crosses), cyclooxygenase-1 (8.2 μg, open squares), and cyclooxygenase-2 (47.4 μg, closed squares) are shown. Each plot represents the mean ± SD of three runs.

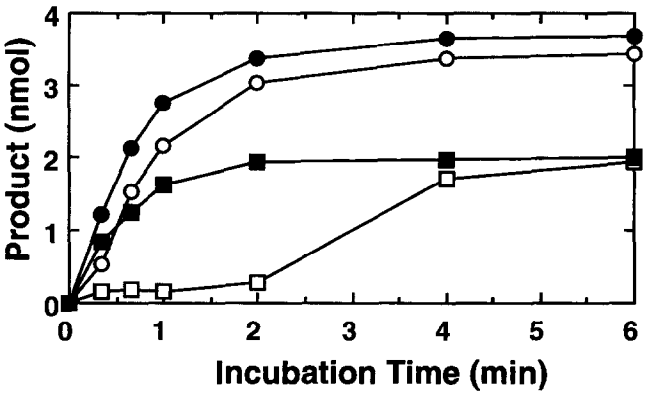



FIG. 2. Effects of YT-18 and 13-HPODE on the time course of porcine leukocyte 5-lipoxygenase reacting with arachidonic acid. Reactions were carried out at 30° in the presence of none (open circles), 6 μM 13-HPODE (closed circles), 50 μM YT-18 (open squares), or both (closed squares).

(Fuji Photo Film, Tokyo, Japan). Inhibitory effects of YT compounds on lipid peroxidation were determined and expressed as $IC_{50}$ values or as % inhibition by 1 μM compounds by Dr. Shigenori Ohkawa of Takeda Research Laboratories. Rat liver microsome (0.3 mg of protein) was incubated with 0.25 mM $FeCl_2$ and 3 mM NADPH in the presence of YT compounds for 1 h at 37°. Peroxide production was determined by the thiobarbituric acid method [12].

## RESULTS AND DISCUSSION

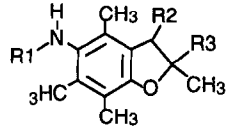
As shown in Fig. 1, YT-18 inhibited the 5-lipoxygenase of porcine leukocytes with an $IC_{50}$ value of 7.5 μM. Preincubation of the enzyme with 5 μM YT-18 at room temperature for 20 min did not affect the degree of inhibition. YT-18 also inhibited 5-lipoxygenases from human leukocytes and rat basophilic leukemia cells with $IC_{50}$ values of 1.5 μM and 14 μM, respectively. In contrast, the compound at 1 mM did not significantly inhibit the other enzymes, i.e., leukocyte and platelet 12-lipoxygenases, 15-lipoxygenase, cyclooxygenase-1, and cyclooxygenase-2. Preincubation of intact rat basophilic leukemia cells with YT-18 decreased the 5-lipoxygenase products dose dependently, with the $IC_{50}$ value being ca. 10 μM.

YT-18 changed the time-course of the 5-lipoxygenase reaction. As shown by open squares in Fig. 2, YT-18 brought about a lag phase in the initiation of the enzyme reaction, and the reaction velocity after the lag was considerably lower. The addition of 13-HPODE abolished the lag phase (Fig. 2, closed squares). The control 5-lipoxygenase activity in the absence of YT-18 was almost unaffected by 13-HPODE (Fig. 2, closed circles). A typical result out of three experiments is presented in Fig. 2. In the presence of 50 μM YT-18, the effect of 13-HPODE was dose-dependent, and the enzyme was saturated with ca. 4 μM 13-HPODE. As assayed by a 1-min enzyme reaction, 13-HPODE at 6 μM considerably (but not totally) reduced the inhibitory effect of 50 μM YT-18 on porcine (from 96.3 ± 2.1% to 58.3 ± 3.9%, $n = 3$) and rat (from 93.4 ± 2.7% to 50.8 ± 3.0%, $n = 3$) 5-lipoxygenases. In contrast, 13-HPODE or YT-18 or both did not significantly affect the activities of other lipoxygenases and cyclooxygenases under our assay conditions.

Various analogues of YT-18 with substitutions on the coumaran ring were prepared as listed in Table 1, and their effects on porcine leukocyte 5-lipoxygenase activity and lipid peroxidation were examined. YT-18 was one of the most potent inhibitors of both 5-lipoxygenase activity and lipid peroxidation. YT-16, YT-19, YT-21, and YT-23 were as potent as YT-18 in inhibiting both 5-lipoxygenase and lipid peroxidation. In contrast, YT-14, YT-17, and YT-22 showed less inhibitory effect on lipid peroxidation and 5-lipoxygenase activity. YT-19 also inhibited 15-lipoxygenase and cyclooxygenase-1.

That such a lag may be abolished by hydroperoxy unsaturated fatty acids was reported for various oxygenases reacting with arachidonic acid as substrate, namely, the cyclooxygenase of ovine vesicular gland [2] and 5-, 12-, and 15-lipoxygenases [1]. Some antioxidants were reported to inhibit soybean lipoxygenase by reducing the active ferric enzyme to the inactive ferrous enzyme, and the reduced inactive enzyme was oxidized back to the active enzyme with the consumption of 13-HPODE [13, 14]. As shown in Table 1, YT-18 and its analogues also showed antioxidant activity in inhibiting lipid peroxidation, and the 5-lipoxygenase inhibitions by YT-18 and its analogues appear to be correlated with the antioxidant activity. Their inhibitory effects may be attributable either to the reduction of the enzyme or to the scavenging of the peroxide activator for 5-lipoxygenase or both.

TABLE 1. Effects of YT-18 and its analogues on various lipoxygenase activities and lipid peroxidation. Enzyme reactions were carried out as described in Fig. 1 in the presence of YT-18 and its analogues. Lipid peroxidation was assessed by the formation of 2-thiobarbituric acid-reactive substance with rat liver microsome. The inhibitor concentration required for a 50% decrease in enzyme activity or lipid peroxidation was determined ($IC_{50}$).




| | R1 | R2 | R3 | IC50 (μM) for enzyme activities | | | | | | Inhibition of lipid peroxidation | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 5-LO | Leukocyte 12-LO | Platelet 12-LO | 15-LO | COX-1 | COX-2 | IC50 (μM) | %inhibition |
| YT-14 | $CH_3$ | H | $CH_3$ | 36.0 | >100 | >100 | >100 | >100 | >100 | | 18% at 1 μM |
| YT-15 | H | H | $CH_3$ | 23.5 | >100 | >100 | >100 | >100 | >100 | 0.76 | |
| YT-16 | H | $C_6H_5$ | $CH_3$ | 6.5 | >100 | >100 | >100 | >100 | >100 | 0.29 | |
| YT-17 | H | H | $CH_2N$⬡ | 35.0 | >100 | >100 | >100 | >100 | >100 | | 18% at 1 μM |
| YT-18 | H | H | $CH_2N$⬡$NC_6H_5$ | 7.5 | >100 | >100 | >100 | >100 | >100 | 0.07 | |
| YT-19 | H | H | $CH_2SC_6H_5$ | 2.3 | >100 | >100 | 28 | 98 | >100 | 0.27 | |
| YT-20 | H | H | $CH_2SO_2C_6H_5$ | 29.0 | >100 | >100 | >100 | >100 | >100 | 0.80 | |
| YT-21 | H | H | $CH_2S$-⬡N | 4.0 | >100 | >100 | 97.5 | >100 | >100 | 0.63 | |
| YT-22 | H | H | $CH_2S(CH_2)_2COOH$ | >100 | >100 | >100 | >100 | >100 | >100 | | 0% at 1 μM |
| YT-23 | H | H | $CH_2NHCH_2C_6H_5$ | 10.5 | >100 | >100 | >100 | >100 | >100 | 0.29 | |

The high specificity of 5-lipoxygenase inhibition by YT-18 can be explained neither by the reduction of the ferric enzyme nor by its nonspecific scavenging of the peroxide activators because, as mentioned above, other lipoxygenases and cyclooxygenases were not inhibited by YT-18. The 5-lipoxygenase activity, which was inhibited by YT-18, was not fully reversed by the addition of a saturating amount of 13-HPODE, as shown in Fig. 2. Approximately one-fourth of the activity remained inhibited, which must be attributed to a certain function of YT-18 other than antioxidant activity. When porcine 5-lipoxygenase was allowed to react with various concentrations of arachidonic acid for 1 min for determination of initial velocity in the absence or presence of 5 μM YT-18, an apparently competitive inhibition by YT-18 was shown by Lineweaver-Burk plot analysis. The activity of YT-18 as a selective inhibitor of 5-lipoxygenase may be due both to its antioxidant activity and to its specific binding to the enzyme active site.

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