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Suppression of inflammatory responses to 12-*O*-tetradecanoylphorbol-13-acetate and carrageenin by YM-26734, a selective inhibitor of extracellular group II phospholipase A_2

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1 YM-26734 [4-(3,5-didodecanoyl-2,4,6-trihydroxyphenyl)-7-hydroxy-2-(4-hydroxyphenyl)chroman] dose-dependently inhibited the activities of extracellular phospholipase A_2 (PLA_2): rabbit platelet-derived group II and porcine pancreas-derived group I PLA_2, with IC_{50} values of 0.085 (0.056–0.129, $n = 5$) and 6.8 (5.0–9.6, $n = 5$) μM, respectively.

2 In contrast, YM-26734 did not reduce the activity of intracellular PLA_2 prepared from mouse macrophages, which preferentially hydrolyzed arachidonoyl phospholipids at concentrations up to 50 μM. YM-26734 also showed no effect against either sheep seminal vesicle cyclo-oxygenase or rat leukocyte 5-lipoxygenase.

3 Lineweaver-Burk analysis showed that YM-26567-1 behaved as a competitive inhibitor of group II PLA_2 derived from rabbit platelets, with a K_i value of 48 nM.

4 In mice, YM-26734 inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 1 μg/ear)-induced ear oedema in a dose-dependent manner, with ED_{50} values of 45 (30–67) μg/ear ($n = 5$) and 11 (4–32) mg kg^{-1}, i.v. ($n = 5$), but did not decrease arachidonic acid (4 mg/ear)-induced ear oedema at 1 mg/ear and 30 mg kg^{-1}, i.v.

5 In rats, the accumulation of exudate fluids and leukocytes in the pleural cavity in response to carrageenin injection (2 mg) was significantly less in a group treated with YM-26734 (20 mg kg^{-1}, i.v.) than in the control group (0.43 ± 0.02 vs 0.59 ± 0.03 g per cavity and 3.8 ± 0.2 vs 4.9 ± 0.3 × 10^7 cells per cavity, respectively; $n = 5$).

6 These results suggest that YM-26734 is a potent and competitive inhibitor of extracellular PLA_2 with selectivity for group II PLA_2, and that the inhibition of group II enzymes activity may cause the suppression of inflammatory responses to TPA and carrageenin.

Keywords: YM-26734; phospholipase A_2; mouse ear oedema; rat pleurisy

Introduction

Phospholipase A_2 [EC3.1.1.4] (PLA_2) catalyzes the hydrolysis of the acyl-ester bound to the *sn*-2 position of membrane phospholipids, resulting in the formation of fatty acids and lysophospholipids. Arachidonic acid (AA) and 1-*O*-aklyl-2-lyso-glycero-3-phosphorylcholine are two such products which can be converted into potent proinflammatory lipid mediators: the eicosanoids (prostaglandin and leukotriene) and platelet activating factor (PAF), respectively (Waite 1985). These mediators (Salmon & Higgs 1987; Chan *et al.*, 1985; Braquet *et al.*, 1987) each promote the inflammatory process by inducing leukocyte infiltration (PAF and leukotriene B_4 (LTB_4)), epidermal proliferation (LTB_4 and 12-hydroxyeicosatetraenoic acid (HETE)), vascular permeability (PAF, LTC_4 and LTD_4), and vasodilatation (prostaglandin E_2 (PGE_2) and PGI_2).

Mammalian cells contain multiple forms of PLA_2 which can be classified into extracellular and intracellular forms. Extracellular PLA_2 can be further divided into two groups based on their amino acid sequence (Waite, 1987). In mammals, group I PLA_2 occurs mainly in the pancreas, whereas group II PLA_2 is distributed in cells such as platelets (Kramer *et al.*, 1989) and neutrophils (Wright *et al.*, 1990). Group II enzyme is released by platelets into the extracellular space in response to thrombin and PAF (Horigome *et al.*, 1987), and is found in the soluble form at inflammatory sites such as in human synovial fluid from patients with rheumatoid arthritis (Kramer *et al.*, 1989). In addition to these findings, purified group II PLA_2 elicits or exacerbates inflammatory responses when injected into the tissue of mice (Chang *et al.*, 1989), rats (Murakami *et al.*, 1990) and rabbits (Bomalaski *et al.*, 1991). Regulation of group II enzyme may therefore achieve important therapeutic effects, particularly in inflammatory disease. Regarding intracellular PLA_2, Clark *et al.* (1991) recently reported the cloning of a cDNA encoding a novel PLA_2 in human monocytic cell line U937. This PLA_2 is distributed in the cytosol of macrophages (Wijkander & Sundler 1989; Clark *et al.*, 1990), platelets (Takayama *et al.*, 1991) and the kidney (Gronich *et al.*, 1990), and preferentially hydrolyzes phospholipids containing an arachidonoyl residue at the *sn*-2 position. The finding that this cytosolic PLA_2 is activated by intracellular concentrations of Ca^{2+} in response to receptor occupancy suggests that this PLA_2 type may operate intracellularly and regulate eicosanoid production in cells exposed to inflammatory stimuli. It remains to be determined which of the PLA_2 isoforms are biologically significant in inflammatory processes, particularly with regard to extracellular group II or intracellular PLA_2. Selective inhibitors of each isoform are required to solve this problem.

Previously we reported that YM-26567-1 [(+)-*trans*-4-(3-dodecanoyl-2,4,6-trihydroxyphenyl)-7-hydroxy-2-(4-hydroxyphenyl)chroman], a natural product isolated from the fruit of *Horsefieldia amygdaline*, competitively inhibits extracellular group II PLA_2 prepared from rabbit platelets (Miyake *et al.*, 1992). We screened YM-26567-1 derivatives to find an inhibitor which selectively targets the group II isoform. YM-26734 (Figure 1) was the result of this process. In the present

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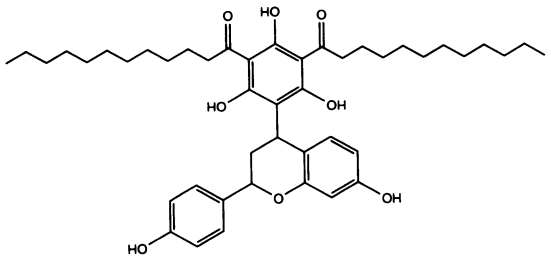

Figure 1 Chemical structure of YM-26734, [4-(3,5-didodecanoyl-2,4,6-trihydroxyphenyl)-7-hydroxy-2-(4-hydroxyphenyl)chroman].

paper, we characterize the effects of YM-26734 on not only extracellular (group I and II) but also intracellular PLA_2. In addition, we also describe the effects of this compound on enzymes related to AA cascade and inflammatory responses in animal models.

Methods

Assay of PLA_2

Extracellular PLA_2 activities were measured by established methods (Pepinsky *et al.*, 1986) using the substrate [^{3}H]-oleic acid-labelled autoclaved *Escherichia coli* (*E. coli*) which was prepared according to the method of Elsbach & Weiss (1990). Phospholipids in radiolabelled *E. coli* were quantified on the basis of inorganic phosphate according to the method of Bartlett (1959). The specific radioactivity of these lipids was approximately $1-2 \times 10^5$ c.p.m. nmol^{-1} inorganic phosphate. The incubation mixture for standard assay of PLA_2 activity contained 150 µl of Tris-HCl buffer (100 mM, pH 8.0), Ca^{2+} (10 mM), bovine serum albumin (BSA, 0.2 mg ml^{-1}), and [^{3}H]-oleic acid-labelled autoclaved *E. coli* (5 µM). Test compounds were dissolved in methanol and added to the reaction mixture just before the addition of the enzyme solution. The final concentration of methanol in the reaction mixture was less than 1% which showed no effect on the enzyme activities. The reaction was started by adding the enzyme solution and stopped after 10 min incubation at either 6°C or 37°C by adding 25 µl of 4 N HCl and 25 µl of 40 mg ml^{-1} BSA. As enzyme sources, rabbit platelet and porcine pancreas PLA_2 were added at 130 and 1.0 ng for incubation at 6°C, or at 1.0 and 0.01 ng for incubation at 37°C, respectively. Tubes were kept on ice for 30 min and then *E. coli* was pelleted by centrifugation for 5 min at 10,000 *g*. Radioactivity of each supernatant was counted with a liquid scintillation counter. For experiments in which substrate concentration-dependence was determined, the reaction was performed at 6°C for 5 min in the presence of 65 ng of rabbit platelet enzyme.

Intracellular PLA_2 activity was measured as the release of radiolabeled AA from 1-palmitoyl-2-[^{14}C]-arachidonoyl phosphatidylcholine according to the methods of Clark *et al.* (1990). 1-Palmitoyl-2-[^{14}C]-arachidonoyl phosphatidylcholine (10 µM) was dried under nitrogen, then suspended in 0.1 ml of 100 mM glycine buffer, pH 9.0, containing 200 µM Triton X-100, 10 mM $CaCl_2$, 0.25 mg ml^{-1} BSA, and 40% glycerol. The suspension was then sonicated to form mixed micellea of phospholipid and Triton X-100. The reaction was started by adding the enzyme solution (approximately 5 µg protein of cytosolic fraction from macrophages) and stopped after a 60 min incubation period at 37°C by mixing with 0.5 ml of isopropyl alcohol:heptane:0.5 M H_2SO_4 (10:5:1). Heptane (0.3 ml) and water (0.2 ml) were then added, and the solution was vigorously mixed for 15 s. The heptane phase was mixed with silica (40 mg) and centrifuged, and the radioactivity in each supernatant was counted by liquid scintillation spectrometry.

Phospholipid hydrolysis was expressed as velocity (micromol of free fatty released per minute per milligram protein), calculated from the specific activities of the radiolabelled phospholipids and the protein concentration of the enzyme. The percentage of enzyme inhibition was obtained by comparison with vehicle control hydrolysis.

Preparation of extracellular PLA_2

Extracellular group I PLA_2 derived from porcine pancreas was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Extracellular group II PLA_2 was prepared from rabbit platelets according to a modification of the methods of Horigome *et al.* (1987). Blood from rabbits anaesthetized with sodium pentobarbitone (20 mg kg^{-1}, i.v.) was collected in a plastic syringe containing 3.8% (w v^{-1}) sodium citrate, and centrifuged for 10 min at 270 *g* at room temperature to prepare platelet-rich plasma. The platelets were pelleted from the plasma by further centrifugation at 1,200 × *g* for 10 min, resuspended at 2×10^9 cells ml^{-1}, and incubated at 37°C for 5 min with 2.5 units ml^{-1} thrombin in the presence of 2 mM Ca^{2+}. The mixture was centrifuged at 3,000 *g* for 10 min at 4°C, and the supernatant was applied to a heparin-Sepharose CL-6B column. After extensive washing, the column was eluted with a linear concentration gradient of NaCl and the eluted fractions were assayed for PLA_2 activity. The PLA_2 activity was eluted at a molarity of approximately 0.8 to 1.1 M NaCl. Fractions showing high PLA_2 activity were pooled and condensed by ultrafiltration with a centricon-10 and stored at − 80°C until use.

Preparation of intracellular PLA_2

Intracellular PLA_2 was prepared from mouse peritoneal resident macrophages according to the method of Wijkander & Sundler (1989). Resident macrophages were collected from mice by peritoneal lavage, plated onto culture dishes (2×10^5 cells cm^{-2}) and allowed to adhere for 2 h at 37°C. The adherent cells were collected by scraping, then homogenized with a Dounce homogenizer in 10 mM HEPES buffer, pH 7.4, containing 80 mM KCl, 5 mM dithiothreitol and 1 mM EGTA. The homogenate was centrifuged at 700 *g* for 5 min and the resulting supernatant was further centrifuged at 100,000 *g* for 60 min to obtain the cytosolic fraction. The cytosolic fraction was stored at 4°C in the presence of 10% (v v^{-1}) glycerol and used within 1 week.

Assay of cyclo-oxygenase

Cyclo-oxygenase activity was measured in 0.1 ml incubations with sheep seminal vesicle microsome (4 mg ml^{-1}) and [^{14}C]-AA (50 µM) in 0.1 M Tris-HCl buffer, pH 7.6, containing 2 mM tryptophan, 4 mM reduced glutathione and the test compound. After incubation at 37°C for 10 min, the reaction was terminated by adding 0.3 ml of diethyl ether:methanol: 1 M citric acid (30:4:1). The samples were centrifuged at 1,000 *g* for 1 min, then dehydrated by the addition of 0.5 *g* of Na_2SO_4. The organic phases were analyzed by thin layer chromatography (t.l.c.) on silica gel 60 plates using benzene:dioxane:acetic acid (50:50:2.5) as solvent. The amount of radioactivity migrating at the AA and PGE_2 positions was determined by liquid scintillation spectrometry. Cyclo-oxygenase activity was expressed as the percentage of conversion of AA to PGE_2. The percentage of enzyme inhibition was obtained by comparison with vehicle controls.

Assay of 5-lipoxygenase

The activity of 5-lipoxygenase was measured from the conversion of [^{14}C]-AA to 5-HETE using t.l.c. to resolve the products of the reaction (Skoog *et al.*, 1986). 5-Lipoxygenase was prepared from polymorphonuclear leukocytes in rat peritoneal exudates collected 18–20 h after a 10 ml injection

of 8% (w v^{-1}) casein. The leukocytes were lysed at 4°C by sonication at a concentration of 2×10^8 cells ml^{-1} in 10 mM HEPES buffer, pH 7.3, containing 2 mM EDTA and 1 mM mercaptoethanol. The soluble fraction (100,000 *g* supernatant) was used as the enzyme preparation. The incubation mixture of the assay of 5-lipoxygenase activity contained 0.1 ml of 25 mM phosphate buffer, pH 7.3, 1 mM ATP, 1 mM Ca^{2+}, the enzyme preparation (0.6 mg ml^{-1}), and test compound. The enzyme was preincubated at 37°C for 2 min before initiation of the reaction by the addition of [^{14}C]-AA (final 5 μM). After incubation for 10 min at 37°C, the reaction was stopped by adding 0.4 ml of diethyl ether:methanol: 1 M citric acid (30:4:1). The samples were centrifuged at 1,000 *g* for 1 min, then dehydrated by the addition of 0.5 *g* of Na_2SO_4. The organic phases were analyzed by t.l.c. on silica gel 60 plates using ethyl acetone:iso-octane:acetic acid:H_2O (100:50:20:100) as solvent. The amount of radioactivity migrating at the positions of AA and 5-HETE was determined with a liquid scintillation spectrometer. 5-Lipoxygenase activity was expressed as the percentage of conversion of AA to 5-HETE. The percentage of enzyme inhibition was obtained by comparisons with vehicle control conversion.

Induction of mouse ear oedema

A modification of the methods of Young *et al.* (1983) was used. 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and AA were dissolved in acetone at concentrations of 100 μg ml^{-1} and 400 mg ml^{-1} respectively, and applied to the right ears of mice by an automatic pipette in a volume of 10 μl; vehicle was applied to the left ears. After the indicated time, mice were killed and the ears were excised and weighed. For topical evaluation, all drugs were dissolved in acetone and applied 30 min prior to TPA or AA application, except prednisolone which was applied 3 h beforehand. For systemic evaluation, YM-26734 was dissolved in equimolar NaOH with 0.1 N solution, diluted with saline, and injected into the saphenous vein 30 s before application of either TPA or AA. Ear oedema was calculated from the formula, (R-L)/L × 100, where R and L were the weight of the right and left ears. The percentage inhibition was calculated by comparing individual values in treatment groups to the mean value of the control group.

Induction of rat pleurisy

Pleurisy was induced in rats by intrapleural injection of 0.1 ml of 2% (w v^{-1}) λ-carrageenin under light anaesthesia with ether (Miyasaka & Mikami, 1982). Four hours later, rats were killed with chloroform. The pleural cavity was lavaged twice with 2.0 ml of saline containing 2 units ml^{-1} heparin and the exudate harvested on ice. The weight of exudate fluids was measured and the number of migrated leukocytes was counted in a Coulter Counter (Coulter Electronics). YM-26734 was dissolved as described before and injected into the saphenous vein 30 s before carrageenin injection. Indomethacin was suspended in 0.5% methylcellulose solution and administered orally 60 min before carrageenin injection.

Materials and animals

YM-26734 was chemically synthesized in our laboratories. Other materials were purchased from the following sources: λ-carrageenin, bovine serum albumin (BSA, fatty acid-free), indomethacin and TPA from Sigma Chemical Co. (St. Louis, MO, U.S.A.); heparin from Novo Industry (Denmark); thrombin from Mochida Pharmaceutical Co. Ltd. (Japan); heparin-Sepharose CL-6B from Pharmacia (Sweden); Centricon-10 from Amicon (Danvers, MA, U.S.A.); AA from Nakarai Tesque Co. (Japan); casein sodium, reduced glutathione, manoalide, prednisolone and phenidone (1-phenyl-3-pyrazolidone) from Wako Chemical Co. (Japan); sheep seminal vesicle microsomes from Funakoshi Co. (Japan); and [^{3}H]-oleic acid, [^{14}C]-AA, 1-palmitoyl-2-[^{14}C]-arachidonoyl phosphatidylcholine and 1-palmitoyl-2-[^{14}C]-oleoyl phosphatidylcholine from New England Nuclear (Boston, MA, U.S.A.). Male ICR mice (25–35 g) and male Wistar rats (140–180 g) were purchased from Japan SLC Co. (Japan), and female Japanese white rabbits (3.0–3.5 kg) were purchased from Clean Experimental Animal Center (Japan), they were maintained on a standard pellet chow and distilled water *ad libitum*.

Statistical analysis

Data are expressed as the mean ± s.e.mean or the mean with 95% confidence limits. Statistical differences were determined by ANOVA. The level of significance was set at 5% ($P<0.05$). The ED_{50} or IC_{50} values were determined by probit analysis.

Results

Inhibitory effects of YM-26734 on extracellular PLA_2 activities

Figure 2 shows the effect of YM-26734 on the initial rates of PLA_2 hydrolysis of *E. coli* phospholipids at 6°C. YM-26734 dose-dependently inhibited both rabbit platelet and porcine pancreas PLA_2. In the presence of a fixed concentration of phospholipids, IC_{50} values for hydrolysis of rabbit platelet and porcine pancreas PLA_2 were 0.085 (0.056–0.129) and 6.8 (5.0–9.6) μM, respectively. YM-26734 showed approximately 100 fold more potent inhibition against group II than group I PLA_2 in mammals. To determine whether the action of YM-26734 against PLA_2 was affected by incubation temperature, we examined its inhibitory effects on extracellular PLA_2 activities at 37°C. YM-26734 inhibited rabbit platelet and porcine pancreas PLA_2 activity at 37°C, with IC_{50} values of 0.12 (0.08–0.19) and 7.6 (5.5–10.5) μM, respectively. Thus, the potency of YM-26734 for each enzyme was independent of incubation temperature.

Effect of YM-26734 on intracellular PLA_2

Cytosolic fraction prepared from mouse peritoneal resident macrophages was used as a source of intracellular PLA_2. In contrast to extracellular PLA_2, the cytosolic fraction prepared from mouse peritoneal macrophages showed PLA_2 activity with an approximately 11 fold higher preference for 1-palmitoyl-2-arachidonoyl- than for 1-palmitoyl-2-oleoyl-phosphatidylcholine, and was insensitive to the reductive

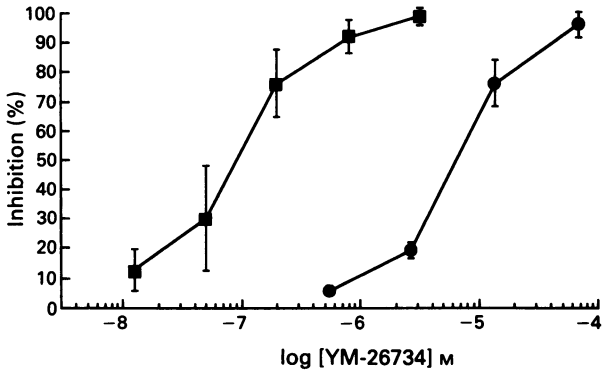

Figure 2 Dose-response curves for YM-26734 on activities of phospholipase A_2 (PLA_2) prepared from rabbit platelets (■) and porcine pancreas (●). Each value represents the mean ± s.e.mean of five independent experiments.

agent, dithiothreitol (data not shown). These findings accord with information published by Wijkander & Sundler (1989) and Clark et al. (1990). Manoalide, used as a reference compound, inhibited intracellular PLA_2 activity (81% inhibition at 50 μM), whereas YM-26734 did not affect enzyme activity (− 14% inhibition at 50 μM).

Lineweaver-Burk analysis of inhibition by YM-26734 of group II PLA_2

The dependence of inhibition on substrate concentration was examined for PLA_2 hydrolysis. Figure 3 shows double-reciprocal plots of kinetic data for the hydrolysis of phospholipids in *E. coli* plasma membrane by rabbit platelet PLA_2 at different concentrations of YM-26734. In the concentration-range used here, rabbit platelet PLA_2 was shown to give linear double-reciprocal plots that conformed well to Michaelis-Menten kinetics ($\gamma = 0.991$). Similar kinetics have been demonstrated with group II PLA_2 in human synovial fluid (Jacobson et al., 1990). Lineweaver-Burk analysis revealed that YM-26734 behaved as a competitive inhibitor of rabbit platelet PLA_2, with K_i values of 48 nM.

Effect of YM-26734 on cyclo-oxygenase and 5-lipoxygenase

The inhibitory selectivity of YM-26734 for enzymes associated with metabolic pathways leading from phospholipids to eicosanoids was evaluated by assessing its activity against sheep seminal vesicle microsomal cyclo-oxygenase and rat leukocyte 5-lipoxygenase. Indomethacin and phenidone were used as reference inhibitors of cyclo-oxygenase and 5-lipoxygenase, respectively. Table 1 shows that YM-26734 inhibited neither sheep seminal vesicle cyclo-oxygenase nor rat leukocyte 5-lipoxygenase.

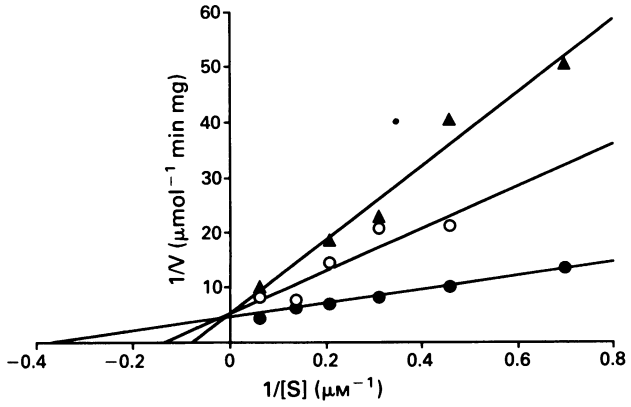

Figure 3 Lineweaver-Burk analysis of phospholipase A_2 (PLA_2) derived from rabbit platelet incubated with YM-26734. Rabbit platelet PLA_2 activity was measured in the presence of 0 (●), 0.1 (○) and 0.2 μM (▲) YM-26734. Each value represents the mean of two determinations.

Table 1 Effect of YM-26734 and reference compounds on cyclo-oxygenase and 5-lipoxygenase

Compound	IC_{50} (μM)[a] Cyclo-oxygenase	5-Lipoxygenase
YM-26734	− 12% at 100[b]	3% at 10[b]
Indomethacin	6.3	NT
Phenidone	NT	1.1

[a]Each value is the mean of two independent determinations.
[b]Percentage of inhibition at the highest concentration tested.
NT: not tested.

Inhibitory effect of YM-26734 on TPA-induced mouse ear oedema

The anti-oedema activity of YM-26734 was evaluated by use of a TPA-induced ear oedema model. Data for YM-26734 against TPA-induced ear oedema are presented in Figure 4. Topical administration of YM-26734 caused a dose-dependent inhibition of swelling with an ED_{50} value of 45 (31–67) μg/ear (Figure 4a). As shown in Table 2, YM-26734 was approximately 3, 5 and 40 fold more potent than the irreversible PLA_2 inhibitor manoalide, the cyclo-oxygenase inhibitor, indomethacin and the eicosanoid synthesis inhibitor, phenidone, respectively, and about one tenth as active as prednisolone, a strong anti-inflammatory steroid which induces PLA_2-inhibitory protein (Flower 1988; Suwa et al., 1990). Further, its effectiveness in systemic administration was evaluated using TPA-induced mouse ear oedema. Intravenous administration of YM-26734 inhibited ear oedema in response to TPA application in a dose-dependent manner ($ED_{50} = 11$ mg kg^{-1}, Figure 4b).

Effect of YM-26734 on AA-induced mouse ear oedema

The anti-oedema selectivity of YM-26734 was evaluated by assessing its activity against another ear oedema model, AA-induced ear oedema. The ear oedema was measured at 60 min after application of AA at 4 mg/ear, generating a submaximal response. Topical application (1 mg/ear) of the eicosanoid synthesis inhibitor, phenidone and the cyclo-oxygenase inhibitor, indomethacin, significantly inhibited AA-induced ear oedema (Table 2). In contrast, YM-26734

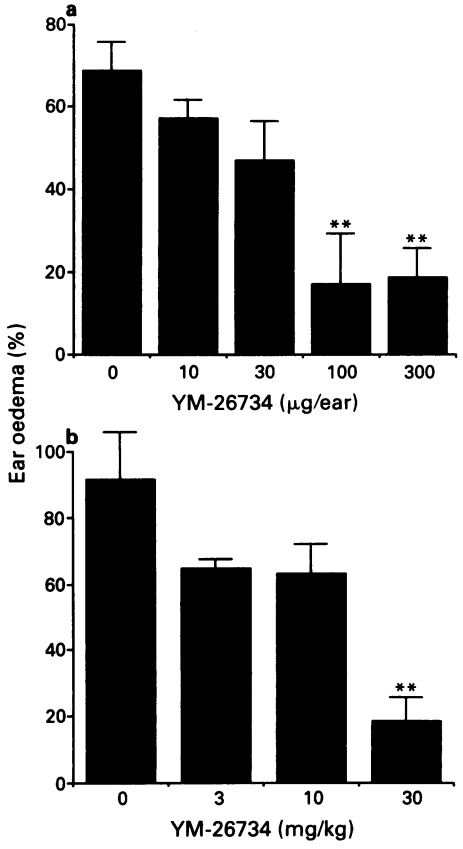

Figure 4 Inhibitory effect of YM-26734 on TPA-induced mouse ear oedema. YM-26734 was topically administrated 30 min (a), or intravenously injected 30 s (b), prior to the application of TPA at 1 μg/ear. Ear oedema was measured 4 h after TPA application. Each value represents the mean ± s.e.mean of 5 mice. $*P<0.05$, $**P<0.01$ compared with respective controls.

Table 2 Inhibition of mouse ear oedema induced by application of either 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or arachidonic acid (AA)

Compound[b]	ED_{50} (μg/ear)[a] *TPA*	*AA*
YM-26734	45 (31–67)	NS at1000[c]
Prednisolone	3.3 (1.8–6.2)[d]	NT
Manoalide	119 (90–158)[d]	NT
Indomethacin	201 (174–231)[d]	70% at 1000[d,e]
Phenidone	1900 (1600–2300)[d]	62% at 1000[d,e]

[a]Ear oedema was measured 1 and 4 h after application of AA 4 mg/ear and TPA 1 μg/ear, respectively. Numbers in parentheses show 95% confidence limits ($n \geq 5$ per dose, dose = 3,4 points). [b]Compounds were applied to the mouse ear 30 min prior to TPA or AA application, except prednisolone which was applied 3 h beforehand. [c]No significant inhibition at the highest concentration tested. [d]Data from Miyake *et al.* (1992). [e]Percentage of statistically significant inhibition. NT: not tested.

did not significantly inhibit ear swelling caused by AA at 1 mg/ear. Moreover, intravenous administration of YM-26734 with 30 mg kg^{-1} also showed no effect on the oedema.

Inhibitory effect of YM-26734 on carrageenin-induced rat pleurisy

The anti-inflammatory action of YM-26734 was further investigated in a carrageenin-induced rat inflammation model. The pleural cavity was selected as the inflammatory site, in which accumulation of both exudate fluids and leukocytes can be observed as inflammatory responses to carrageenin. As shown in Figure 5, intrapleural carrageenin

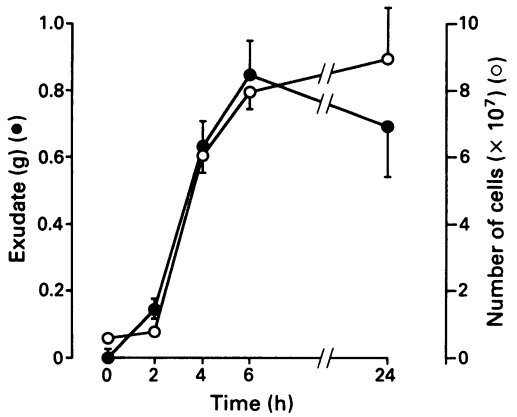

Figure 5 Time course of accumulation of exudate fluid (●) and leukocytes (○) in the pleural cavity in response to carrageenin injection in rats; 2 mg carrageenin was injected intrapleurally. Each value represents the mean ± s.e.mean of 5 rats.

(2 mg) rapidly induced exudate and leukocyte accumulation in the cavity 2 to 4 h after injection, with levels plateauing at 6 to 24 h. The effects of YM-26734 on these responses were measured at 4 h after injection, using the cyclo-oxygenase inhibitor indomethacin as a reference. As shown in Table 3, intravenous administration of YM-26734 at 20 mg kg^{-1} significantly inhibited the accumulation of both exudate fluids and leukocytes in the pleural cavity in response to carrageenin injection.

Discussion

In this study, we found that YM-26734 inhibited extracellular group II PLA_2 from rabbit platelets 100 fold more potently than extracellular group I PLA_2 from porcine pancreas, and had no effect on intracellular PLA_2 from mouse peritoneal macrophages. The high selectivity of YM-26734 for extracellular over intracellular PLA_2 may be explained by the difference in amino acid sequence of the enzyme, as reported by Clark *et al.* (1991). It has been shown that intracellular PLA_2 preferentially hydrolyzes arachidonoyl phospholipids, whereas phospholipids containing two saturated fatty acids, such as dipalmitoyl phosphatidylcholine, are poor substrates for the enzyme. YM-26734 has two saturated alkyl chains which may hinder access to the catayltic site of the intracellular PLA_2. Against this, the 100 fold preferential inhibition of group II over group I PLA_2 is surprising, since the extracellular PLA_2s are reported to share a highly conserved amino acid sequence as the catalytic site: His^{48}, Asp^{99}, Tyr^{62} and Tyr^{73} (Kramer *et al.*, 1989), and all PLA_2 inhibitors reported so far show poor selectivity between the two types of extracellular PLA_2. In this regard, YM-26734 may represent a unique tool in the investigation of differences in catalytic site between the two enzymes, as well as of the physiological significance of extracellular group II PLA_2.

Several inhibitors of PLA_2 activity prepared from various sources (snake venom, bee venom or porcine pancreas) and their anti-inflammatory effects have been reported. However, it is not clear which type of enzyme plays a role in inflammatory responses, as none of these inhibitors has been selective. Moreover, many reported PLA_2 inhibitors affect enzymes metabolizing AA to eicosanoids, which are involved in the inflammatory process. Manoalide, a natural marine product isolated from the sponge *Luffariella variabilis*, inhibits not only extracellular group II PLA_2 purified from human synovial fluid (Jacobson *et al.*, 1990) and rabbit platelets (Miyake *et al.*, 1992), but also intracellular PLA_2 isolated from the cytosol of human monocytic cell line, U937 (Marshall *et al.*, 1991b). Although manoalide shows an anti-inflammatory effect on TPA-induced mouse ear oedema (Burley *et al.*, 1982), this compound also potently inhibits 5-lipoxygenase prepared from rat basoleukaemia cell line RBL-1 (De Vries *et al.*, 1988) in addition to its PLA_2 inhibitory effects. Nordihydroguaiaretic acid also inhibits human synovial fluid PLA_2 (Marshall *et al.*, 1991a) and acts

Table 3 Inhibitory effect of YM-26734 on accumulation of exudate fluid and leukocytes in rat carrageenin-induced pleurisy.

Compound	*Dose*[a] (mg kg^{-1})	*n*	*Exudate fluid* *Weight* (mg)	*Inhibition* (%)	*Leukocytes migration* *Number* (× 10^7)	*Inhibition* (%)
Control		5	587 ± 27		4.91 ± 0.31	
YM-26734	5	5	566 ± 11	4	4.57 ± 0.20	7
	10	5	545 ± 28	7	4.20 ± 0.29	14
	20	5	429 ± 23**	27	3.81 ± 0.21*	22
Control		9	639 ± 31		4.66 ± 0.44	
Indomethacin	3	9	362 ± 25**	43	3.10 ± 0.25**	34

[a]YM-26734 was intravenously injected 30 s prior to carrageenin injection. Indomethacin was administered orally 60 min prior to carrageenin injection. *$P < 0.05$, **$P < 0.01$ compared with respective controls.

as an anti-inflammatory agent, but this agent also potently inhibits cyclo-oxygenase and 5-lipoxygenase. The anti-inflammatory effects of these inhibitors may therefore be due to inhibition of PLA_2, of other enzymes within the AA cascade or both. In contrast, YM-26734 does not inhibit sheep seminal vesicle cyclo-oxygenase or rat leukocyte 5-lipoxygenase as enzymes within the AA cascade, indicating it to be a selective inhibitor of extracellular group II PLA_2, which can be used to investigate the significance of extracellular group II PLA_2 in inflammatory responses and pathology.

Two animal models were used to investigate extracellular group II PLA_2 in inflammatory responses, the TPA-induced mouse ear oedema model and the carrageenin-induced rat pleurisy model. The former is widely used to evaluate the anti-inflammatory activity of PLA_2 inhibitors, TPA induces AA release and eicosanoids synthesis in cultured macrophages *in vitro* (Humes *et al.*, 1982), and TPA challenge to the mouse ear causes PGE_2 to accumulate in parallel with the swelling of the ear (Inoue *et al.*, 1989). However, it is not clear if this inflammation is regulated primarily by PLA_2 activity, or more specifically by extracellular group II enzymes. The present observation that the selective group II PLA_2 inhibitor, YM-26734, strongly inhibited TPA-induced ear oedema suggests a positive relationship between group II PLA_2 activity and inflammatory responses to TPA. As a control study, we evaluated the effect of YM-26734 in an AA-induced mouse ear oedema model, in which both cyclo-oxygenase (PGE_2) and lipoxygenase products (LTC_4/D_4) are implicated (Opas *et al.*, 1985). As expected, YM-26734 failed to inhibit AA-induced ear oedema.

We next examined the effect of YM-26734 on a carrageenin-induced rat pleurisy model. This model allows direct assessment of the effects of various classes of anti-inflammatory agents on plasma exudation and leukocyte migration. The accumulation of exudate fluid and leukocytes into the pleural cavity in response to carrageenin injection appears to be regulated by metabolites of AA for the following reasons. Carrageenin injection causes accumulation of PGE_2 (Katori *et al.*, 1978), LTB_4 (Flower *et al.*, 1986) and LTC_4/D_4 (Ueno *et al.*, 1983), and many inhibitors of cyclo-oxygenase, 5-lipoxygenase, or both suppress both plasma exudation and leukocyte migration induced by carrageenin (Ashida *et al.*, 1983; Ku *et al.*, 1988). YM-26734 also inhibited accumulation of exudate fluid and leukocytes into the pleural cavity, suggesting that extracellular group II PLA_2 may play a role in this inflammatory process. As for inflammatory factors other than eicosanoids, it is possible that such factors may also be involved in the anti-inflammatory effect of YM-26734 in this study. Although we cannot rule out such a possibility, it is reasonable to say, based on our present study, that the anti-inflammatory effect of YM-26734 can be explained, at least in part, by its action on the group II PLA_2.

In conclusion, we have demonstrated that YM-26734 is a potent and competitive PLA_2 inhibitor which is selective for extracellular group II PLA_2 and that inhibition of group II PLA_2 activity may suppress inflammatory responses to TPA and carrageenin by decreasing substrates for cyclo-oxygenase and 5-lipoxygenase.

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