Characterization of a novel inhibitor of cytosolic phospholipase A₂α, pyrrophenone

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Cytosolic phospholipase A₂α (cPLA₂α), one of the three subtypes of cPLA₂ (α, β and γ), is thought to be a rate-limiting enzyme in eicosanoid biosynthesis. We developed a novel and potent cPLA₂α inhibitor with an optically active pyrrolidine, termed pyrrophenone, and characterized this compound in detail using enzyme and cellular assay systems. Pyrrophenone, which shows strong inhibition of cPLA₂α activity, is one of the most potent cPLA₂α inhibitors reported to date. Similar inhibitory potencies for cPLA₂α were obtained from three different assays. The inhibitory activity of pyrrophenone is two or three orders of magnitude more potent than arachidonyl trifluoromethyl ketone (AACOCF₃) under the same assay conditions. Pyrrophenone shows reversible inhibition of cPLA₂α and displays no characteristics of the slow-binding inhibition observed for AACOCF₃. Pyrrophenone also inhibited the esterase and lysophospholipase activities of cPLA₂α. However, the inhibition by pyrrophenone of 14 kDa secretory PLA₂s, types IB and IIA, was over two

orders of magnitude less potent than that for cPLA₂α. Pyrrophenone strongly inhibited arachidonic acid release in calcium ionophore (A23187)-stimulated human monocytic cells (THP-1 cells) in a dose-dependent manner with an IC₅₀ value of 0.024 μM, followed by suppression of eicosanoid synthesis, and also showed dose-dependent inhibition for interleukin-1-induced prostaglandin E₂ synthesis in human renal mesangial cells with an IC₅₀ value of 0.0081 μM. The mechanism of inhibition of eicosanoid synthesis in these cell-based assays was due to inhibition of only one step of arachidonic acid release without any effect on cyclooxygenase or lipoxygenase pathways. These results suggest that pyrrophenone could be a potential therapeutic agent for inflammatory diseases.

Key words: arachidonic acid release, cPLA₂α inhibitor, cytosolic PLA₂α inhibitor.

INTRODUCTION

Phospholipase A₂ (PLA₂) hydrolyses the ester bond at the *sn*-2 position of phospholipids to produce non-esterified fatty acids and lysophospholipids. Cytosolic PLA₂ (cPLA₂) is a member of a diverse superfamily of PLA₂ enzymes, including secretory PLA₂ (sPLA₂), calcium-independent PLA₂ and other members [1]. cPLA₂ exists as three multiple isoforms, α, β and γ [2,3]. The molecular masses of cPLA₂α, cPLA₂β and cPLA₂γ are approx. 85, 114 and 61 kDa, respectively, and the sequences of the three isoforms display about 30% identity with each other. Of the three isoforms, cPLA₂α has been the best characterized with respect to the properties of the enzyme and the protein structure, with cPLA₂β and cPLA₂γ remaining to be clarified. cPLA₂α has two separate units consisting of an N-terminal C2 domain and a catalytic domain and is translocated to the phospholipid membrane via the C2 domain at the nanomolar level of Ca²⁺, where it acts on arachidonate-containing phospholipids [4]. The phosphorylation of cPLA₂α by kinases of the mitogen-activated protein kinase family results in an increase in enzyme activity [5]. Thus the functional activation of cPLA₂α is regulated by intracellular Ca²⁺ and kinase-dependent phosphorylation. Recently, a growing amount of evidence has indicated that cPLA₂α contributes directly to the biosynthesis of eicosanoids by releasing arachidonic acid from membrane phospholipids and is associated

with various inflammatory diseases, according to experiments using cPLA₂α-deficient transgenic mice [6]. In these mice, eicosanoid synthesis from inflammatory cells decreases significantly, as do allergic symptoms, post-ischaemic brain injury and acute lung injury induced by sepsis syndrome or acid aspiration [7–9], indicating the contribution of cPLA₂α to eicosanoid synthesis, leading to progression of inflammation, reperfusion injury and acute lung injury. Therefore, cPLA₂α represents a potentially useful therapeutic target to control such diseases.

Recently, the X-ray crystal structure of human cPLA₂α was solved and the structure of the catalytic domain was clarified [10]. The active site of cPLA₂α is embedded deeply in the catalytic domain and the catalytic residues Ser-228 and Asp-549 are located at the bottom of the funnel. Similar to a number of lipases, cPLA₂α has a lid region located partially over the active site, whereas the fold of cPLA₂α exhibits a novel topology distinct from that of the α/β hydrolase family, which contains lipases. Although information on the three-dimensional structure of a target protein is one of the most useful tools for the development of inhibitors, there have been no cPLA₂α inhibitors designed on the basis of such information.

Arachidonyl trifluoromethyl ketone (AACOCF₃), which has been used widely as a cPLA₂α inhibitor, is a mechanism-based inhibitor characterized by the formation of a hemiketal with a serine residue in the active site [11]. However, in spite of its

Abbreviations used: PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; sPLA₂, secretory PLA₂; AACOCF₃, arachidonyl trifluoromethyl ketone; MAFP, methyl arachidonyl fluorophosphate; PC, phosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; [¹⁴C]PAPC, 1-palmitoyl-2-[¹⁴C]arachidonoyl-*sn*-glycero-3-phosphocholine; DOG, *sn*-1,2-dioleoylglycerol; PGE₂, prostaglandin E₂; LTC₄, leukotriene C₄; ADAM, 9-anthryldiazomethane; 7-HCA, 7-hydroxycoumarinyl-arachidonate; AA-861, 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadienyl)-1,4-benzoquinone; IL-1β, interleukin-1β; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); FCS, fetal calf serum; DG lipase, diacylglycerol lipase; COX, cyclooxygenase; arachidonoyl thio-PC, 1-hexadecyl-2-arachidonoylthio-2-deoxy-*sn*-glycero-3-phosphocholine; diheptanoyl thio-PC, 1,2-bis(heptanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine; LPS, lipopolysaccharide; X_i(50), the mol fraction of inhibitor in total lipid that causes 50% inhibition.

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Figure 1 Structures of pyrrophenone and compound 1f used in this study

potent inhibition of $\text{cPLA}_2\alpha$, AACOCF_3 also inhibits type VI calcium-independent PLA_2 and any enzyme located downstream of the arachidonic acid release in the eicosanoid biosynthetic pathway [12,13]. In addition, since AACOCF_3 is easily metabolized to its reduction products by incubation with some cells [12], it is difficult to use in experiments requiring a long incubation time with such cells and for investigating pharmacological efficacy *in vivo*.

In a recent report [14], we described the identification of potent $\text{cPLA}_2\alpha$ inhibitors having the 1,2,4-trisubstituted pyrrolidine framework. The most potent compound among them (compound 4d) inhibited $\text{cPLA}_2\alpha$ about 230-fold more strongly than AACOCF_3 . Compound 4d strongly inhibited arachidonic acid release from calcium ionophore (A23187)-stimulated human monocytic cells (THP-1 cells) followed by suppression of prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4) synthesis. However, it is difficult to obtain numerous pyrrolidine inhibitors, including compound 4d, in crystalline form, which offers the advantages of stability and productivity to yield clinically active agents. We therefore carried out further follow-up studies and, as a result, were able to synthesize a potent $\text{cPLA}_2\alpha$ inhibitor (termed pyrrophenone) in crystalline form, as shown in Figure 1 [15]. Pyrrophenone can be synthesized more economically with fewer reaction steps than compound 4d. For example, the substituent at the 4-position of the pyrrolidine ring of pyrrophenone can be introduced by a one-step reaction using triphenylmethanethiol, while the substituent at the 4-position of compound 4d is constructed by stepwise addition of two different amino substituents [14,15]. Although the inhibitory activities of pyrrophenone for $\text{cPLA}_2\alpha$ and arachidonic acid release from A23187-stimulated THP-1 cells are almost the same as those of compound 4d, pyrrophenone inhibited arachidonic acid release from A23187-stimulated human whole blood with about three times more potency than compound 4d [14,15]. In summary, pyrrophenone has more advantages in terms of production, handling and

efficacy in whole blood. Information on the X-ray crystal structure of pyrrophenone should be helpful for exploring the interaction between the pyrrolidine inhibitor and $\text{cPLA}_2\alpha$. Therefore, we selected pyrrophenone for the characterization of the pyrrolidine inhibitor. In this paper, we provide more detailed characterization of the inhibition of $\text{cPLA}_2\alpha$ by pyrrophenone.

MATERIALS AND METHODS

Materials

The human promonocytic cell line THP-1 was purchased from the A.T.C.C. (Manassas, VA, U.S.A.). Human glomerular mesangial cells were obtained from BioWhittaker (Walkersville, MD, U.S.A.). Purified recombinant group IB sPLA_2 was expressed and purified as described previously [16]. Recombinant human group IIA sPLA_2 and $\text{cPLA}_2\alpha$, and anti-human $\text{cPLA}_2\alpha$ IgG were generous gifts from Dr Ruth Kramer (Eli Lilly, Indianapolis, IN, U.S.A.). Porcine pancreatic lipase was purchased from Elastin Products (Pacific, MI, U.S.A.) and purified further. Pyrrophenone and compound 1f were synthesized according to methods described previously [14,15]. Methyl arachidonoyl fluorophosphonate (MAFP), indomethacin and NS-398 were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). 9-Anthryldiazomethane (ADAM) was obtained from Funakoshi Co., Tokyo, Japan. 1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), 1-myristoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, *sn*-1,2-dioleoylglycerol (DOG) and 1-stearoyl-2-arachidonoyl-*sn*-glycerol were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). 1-Palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (^{14}C PAPC; 50 mCi/mmol) was purchased from DuPont-New England Nuclear Life Science Products (Boston, MA, U.S.A.). 1-Palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine was purchased from Bachem (Bubendorf, Switzerland). Triolein was obtained from NuChek Prep (Elysian, MN, U.S.A.). 7-Hydroxycoumarinyl-arachidonate (7-HCA) and 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadienyl)-1,4-benzoquinone (AA-861) were obtained from Biomol (Plymouth Meeting, PA, U.S.A.). A23187 and AACOCF_3 were purchased from Calbiochem (San Diego, CA, U.S.A.). 1-Hexadecyl-2-arachidonoylthio-2-deoxy-*sn*-glycero-3-phosphocholine (arachidonoyl thio-PC) was synthesized as described previously [17]. 1,2-Bis(heptanoylthio)-1,2-dideoxy-rac-*sn*-glycero-3-phosphocholine (diheptanoyl thio-PC) was synthesized according to the procedure of Reynolds et al. [18]. Interleukin-1 β (IL-1 β) was purchased from Genzyme (Cambridge, MA, U.S.A.).

Measurement of $\text{cPLA}_2\alpha$ activity

Standard assays for $\text{cPLA}_2\alpha$ activities were performed using three systems, as follows. The phosphatidylcholine (PC)/Triton assay was carried out according to the method described previously [11], with some modifications. Briefly, the mixed micelles consisting of 100 μM PAPC and 200 μM Triton X-100 were used as substrate and the assay mixture contained 50 mM Hepes (pH 7.5), 30% glycerol, 5 mM CaCl_2 , 1 mg/ml BSA and 1 mM dithiothreitol (DTT). The assay was incubated for 20 min at 37 $^\circ\text{C}$ in a final volume of 200 μl and terminated by Dole's reagent consisting of a mixture of heptane/propan-2-ol/1 M H_2SO_4 (10:40:1, by vol.). The released fatty acids were extracted by Dole's extraction procedure [19], labelled with ADAM, and quantified by the HPLC method described previously [20]. The chromogenic assay was carried out according to the method described previously by Reynolds et al. [17]. The mixed micelles consisting of 1 mM arachidonoyl thio-PC and 2 mM Triton X-

100 were used as substrate and the assay mixture contained 80 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM 2,6-di-*t*-butyl-4-methylphenol ('BHT'), 1 mg/ml BSA, 10 mM CaCl₂ and 30 % glycerol. After reaction at 37 °C for 60 min in a final volume of 230 µl, 20 µl of the solution, consisting of 12.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 475 mM EGTA, was added to the reaction mixture and the absorbance at 405 nm was measured.

The PC/DOG assay was performed using liposomes containing 2.5 µM [¹⁴C]PAPC and 1.25 µM DOG according to the method described by Kramer et al. [21], with some modifications. The assay mixture contained 50 mM Hepes buffer (pH 7.5), 150 mM NaCl, 0.1 mg/ml BSA, 1 mM CaCl₂ and 1 mM DTT. Following the incubation for 15 min at 37 °C in a final volume of 200 µl, the reaction was stopped by the addition of Dole's reagent, and the radioactivity of the released [¹⁴C]arachidonic acid was measured by scintillation counting. In all procedures, inhibitors were added to the assay mixture as DMSO solutions (final concentration, 5 %) and the control reacted with DMSO alone. $X_1(50)$ (the mol fraction of inhibitor in total lipid that causes 50 % inhibition) values were determined by dividing IC₅₀ values calculated as bulk concentration units by the total lipid concentrations in the assay (substrate plus Triton X-100 or DOG).

Measurement of other enzyme activities of cPLA₂α

Lysophospholipase activity of cPLA₂α was measured using the mixed micelles of 100 µM 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine and 200 µM Triton X-100 as substrate. The assay mixture contained 100 mM Hepes (pH 7.5), 150 mM NaCl, 1 mg/ml BSA, 30 % glycerol and 1 mM DTT. The reaction was initiated by adding cPLA₂α (15 ng/ml) to the assay mixture containing the substrate and inhibitor in a final volume of 200 µl. After the enzyme reaction for 20 min at 37 °C, the reaction was terminated by Dole's reagent. The released fatty acids were extracted using Dole's extraction method, labelled with ADAM and quantified by HPLC as described above. The esterase activity of cPLA₂α was measured using 80 µM sonicated 7-HCA vesicles as substrate, according to the method described by Huang et al. [22], with some modifications. The assay mixture contained 10 mM Hepes (pH 7.4), 100 µM EDTA, 1 mM CaCl₂ and 0.1 mg/ml BSA. The reaction was initiated by adding cPLA₂α (1.25 µg/ml) to the assay mixture containing the substrate and inhibitor, in a final volume of 200 µl, in a 96-well microplate. After incubation for 15 min at 37 °C, the increase in the fluorescence intensity at 460 nm (excitation, 355 nm) was monitored. $X_1(50)$ values were determined by dividing IC₅₀ values calculated as bulk concentration units by the total lipid concentrations in the assay (substrate or substrate plus Triton X-100).

Measurement of sPLA₂ and lipase activities

sPLA₂ activities were measured using diheptanoyl thio-PC as substrate according to the method of Reynolds et al. [18]. The mixed micelles consisting of 1 mM diheptanoyl thio-PC and 0.3 mM Triton X-100 were used as substrate. The assay mixture contained 25 mM Tris/HCl buffer (pH 7.5), 0.12 mM DTNB, 10 mM CaCl₂, 0.1 M KCl and 1 mg/ml BSA. The reactions were initiated by addition of 450 and 300 ng/ml of purified human group IB and group IIA sPLA₂, respectively, to the assay mixture containing the substrate and inhibitor in a final volume of 200 µl. The reaction was monitored by the absorbance at 405 nm, with a microplate reader. Porcine pancreatic lipase activity was measured using mixed micelles of 0.5 mM triolein and 3 mM sodium deoxycholate as substrate. The assay mixture contained 100 mM Tris/HCl buffer (pH 8.0), 150 mM NaCl,

5 mM CaCl₂ and 1 mg/ml BSA in a final volume of 200 µl. After addition of the enzyme solution (10 ng/ml) to the assay mixture containing the substrate and inhibitor, the enzyme reaction was performed for 20 min at 37 °C and terminated by Dole's reagent. The released fatty acids were extracted by Dole's extraction method, labelled with ADAM and quantified by HPLC as described above.

In all procedures, test compounds were added to the assay mixture as DMSO solution (5 % of total reaction volume) and the control reacted with DMSO alone. $X_1(50)$ values were determined by dividing IC₅₀ values calculated as bulk concentration units by the total lipid concentrations in the assay (substrate plus Triton X-100 or sodium deoxycholate).

Measurement of arachidonic acid release and eicosanoid production from THP-1 cells

THP-1 cells were maintained in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate and 0.29 mg/ml L-glutamine, and pretreated with 1.3 % DMSO for 2 days before the assay. Following washing with PBS, the cells were resuspended in Hanks' balanced salt solution containing 0.1 % BSA. The cell suspension (5 × 10⁶ cells/ml) was preincubated with the inhibitor for 15 min at 37 °C and then incubated with 3 µM A23187 for 20 min at 37 °C. The reaction was terminated using Dole's reagent. Fatty acids containing arachidonic acid, extracted with Dole's extraction method, were labelled with ADAM and quantified by HPLC as described previously [16,20]. For measurements of PGE₂ and LTC₄, the reaction was stopped by cooling on ice. The supernatant was collected after centrifugation at 1000 *g* for 5 min at 4 °C, and then PGE₂ and LTC₄ levels were measured using a radioimmunoassay kit (DuPont-New England Nuclear Life Science Products) and an enzyme immunoassay kit (Cayman Chemicals Co.), respectively.

The effects of inhibitor on eicosanoid production by arachidonic acid addition were examined by the following methods. For measurements of PGE₂, after preincubation of inhibitor with the cells in Hanks' balanced salt solution containing 0.1 % BSA for 15 min at 37 °C, 50 µM arachidonic acid was added, and then the reaction mixture was incubated for 20 min at 37 °C. For measurement of LTC₄, the cells suspended in Hanks' balanced salt solution containing 0.1 % BSA were preincubated with 200 µM aspirin for 60 min at 37 °C, and the cells were washed with PBS. Following preincubation with the inhibitor for 15 min at 37 °C, 250 µM arachidonic acid was added and the reaction mixture was incubated for 20 min at 37 °C. The reactions were terminated by cooling on ice, and PGE₂ and LTC₄ levels were measured as described above. The cytotoxicity of the inhibitor was checked by measuring the activity of lactate dehydrogenase.

Measurement of lipolytic enzyme activities in THP-1 cells

DMSO (1.3 %)-treated THP-1 cells, described above, were collected, washed with PBS and centrifuged at 1000 *g* for 5 min at 4 °C. The cells were collected and resuspended at a cell density of 5 × 10⁷ cells/ml in 50 mM Hepes buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 µM leupeptin, 50 µM pepstatin A, 100 mM NaF, 200 µM sodium orthovanadate, 1 µM microcystin LR, 10 µM genistein and 1 µM staurosporin. The cells were broken by sonication and used for each assay. cPLA₂α activity was measured using liposomes containing 2.5 µM [¹⁴C]PAPC and 1.25 µM DOG in 100 mM glycine/NaOH buffer (pH 9.5) containing 1 mM DTT, 150 mM NaCl and 1 mM

CaCl₂ in a final volume of 200 μ l. Calcium-independent PLA₂ activity was measured using liposomes of 100 μ M 1-myristoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine in 100 mM Tris/HCl (pH 8.0) containing 150 mM NaCl and 5 mM EDTA in a final volume of 200 μ l. Diacylglycerol lipase (DG lipase) activity was measured using the mixed micelles consisting of 500 μ M 1-stearoyl-2-arachidonoyl-*sn*-glycerol and 1 mM sodium deoxycholate in 100 mM Tris/HCl buffer (pH 8.0) containing 150 mM NaCl and 5 mM CaCl₂ in a final volume of 200 μ l. Inhibitor (10 μ l) and enzyme sample (20 μ l) were added and the assay was performed for 30 min at 37 °C. For MAFP, after it was preincubated with the enzyme sample in each reaction buffer for 15 min at 37 °C, the substrate was added. The reaction was terminated by adding Dole's reagent and the released arachidonic acid was quantified by scintillation counting for cPLA₂ α and HPLC for calcium-independent PLA₂ and DG lipase as described above.

Measurement of PGE₂ production from IL-1 β -stimulated human mesangial cells

Human mesangial cells were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin G, 100 μ g/ml streptomycin sulphate and 0.29 mg/ml L-glutamine. Confluent human mesangial cells in 96-well culture plates were washed with PBS and then added to the culture medium described above. Following preincubation with the inhibitor for 15 min at 37 °C, 50 units/ml IL-1 β was added and the reaction mixture was incubated for 24 h at 37 °C in the presence of 5% CO₂. The reactions were terminated by withdrawing the medium, and PGE₂ levels in the medium were measured as described above. The effect of inhibitor on PGE₂ production by arachidonic acid addition was examined by the following method. Human mesangial cells were stimulated with 50 units/ml IL-1 β for 24 h in Dulbecco's modified Eagle's medium containing 10% FCS. The medium was removed from the wells and the cells were washed with PBS to remove PGE₂ produced during IL-1 β stimulation. After preincubation with inhibitor in Hanks' balanced salt solution containing 0.1% BSA for 15 min at 37 °C, 50 μ M arachidonic acid was added, and then the assay was incubated for 20 min at 37 °C. PGE₂ levels in the medium were measured as described above. Human mesangial cells in passages 5–7 were used for the experiment. The cytotoxicity of the inhibitor was checked by measuring the activity of lactate dehydrogenase.

RESULTS

Effect of pyrrophenone on cPLA₂ α enzyme

Figure 2 shows the dose–response curves for the inhibition of cPLA₂ α by pyrrophenone. Pyrrophenone showed similar inhibition curves for cPLA₂ α in a dose-dependent manner under three different sets of assay conditions. The $X_{1/2}$ (50) values (mole fraction) of pyrrophenone were 0.00041 for the chromogenic assay, 0.0011 for the PC/DOG assay and 0.00026 for the PC/Triton assay, as shown in Table 1. AACOCF₃ also inhibited cPLA₂ α with similar $X_{1/2}$ (50) values of 0.013–0.081 under the three sets of assay conditions. Comparison of the $X_{1/2}$ (50) values for each assay shows that the inhibitory potency of pyrrophenone was about 32–235 times stronger than that of AACOCF₃.

In addition to PLA₂ activity, cPLA₂ α is known to possess lysophospholipase activity, transacylase activity and esterase activity [23–25]. In particular, the specific activity for lysophospholipids of cPLA₂ α was shown to be the same or higher than

Figure 2 Dose-dependent inhibition of cPLA₂ α by pyrrophenone

PLA₂ activity was measured using the chromogenic assay (●), PC/DOG assay (△) and PC/Triton assay (○). The assay mixtures containing the substrate and the inhibitor at the indicated mol fractions were incubated with cPLA₂ α (1000 ng/ml for the chromogenic assay, 25 ng/ml for the PC/DOG assay and 100 ng/ml for the PC/Triton X-100 assay). The concentrations of inhibitor are represented as the mole fraction of the total lipid concentration in the assay (substrate plus Triton X-100 or DOG). The data are presented as means $\pm$ S.D. from three independent determinations.

that for diacylphospholipids [23,26]. In fact, the lysophospholipase activity of cPLA₂ α was about 10-fold higher than the PLA₂ activity under similar assay conditions with the substrate/Triton X-100 (100 μ M/200 μ M) mixed micelles. We examined the effect of pyrrophenone on lysophospholipase and esterase activities (Table 1). Pyrrophenone and AACOCF₃ inhibited the lysophospholipase activity with $X_{1/2}$ (50) values of 0.0023 and 0.032, respectively, with pyrrophenone showing approx. 14-fold more potent inhibition. Furthermore, pyrrophenone showed inhibitory activity with an $X_{1/2}$ (50) value of 0.00066 for esterase activity measured using the arachidonic acid ester of 7-hydroxycoumarin as substrate and exhibited approx. 18-fold more potent inhibition than AACOCF₃ [$X_{1/2}$ (50) value = 0.012].

AACOCF₃ was recognized to be a slow-binding inhibitor for cPLA₂ α and to take a long time to express full inhibitory activity [11,27]. To clarify the modes of inhibition for cPLA₂ α by pyrrophenone, we analysed the reaction progress curve with pyrrophenone and the effect of preincubation time with pyrrophenone on cPLA₂ α inhibition. Figure 3 shows the reaction progress curve for cPLA₂ α with 80 nM pyrrophenone (corresponding to the IC₅₀ value of pyrrophenone in the PC/Triton assay). Constant inhibition was observed for all indicated reaction times on the reaction progress curve in the presence of pyrrophenone. Pyrrophenone exhibited constant inhibition of cPLA₂ α that was independent of preincubation time, as shown in Figure 4. Therefore, preincubation was not required to achieve the full inhibitory activity of pyrrophenone for cPLA₂ α . These results reveal that pyrrophenone binds rapidly to cPLA₂ α and is not a slow-binding inhibitor different from AACOCF₃.

We next tested whether pyrrophenone is a reversible or irreversible inhibitor (Figure 5). cPLA₂ α was preincubated in the presence or absence of pyrrophenone, then diluted 1500-fold by transferring aliquots into the assay mixture, and the enzyme activity was measured. cPLA₂ α treated with pyrrophenone exhibited a rapid- and dose-dependent recovery of enzyme

Table 1 Effect of pyrrophenone on multiple enzyme activities of cPLA₂α

The data represent means from three independent experiments. Lyso-PL, lysophospholipase.

Compound	X _i (50) (mole fraction)				
	PLA ₂				
	Chromogenic	PC/DOG	PC/Triton	Lyso-PL	Esterase
Pyrrophenone	0.00041	0.0011	0.00026	0.0023	0.00066
AACOCF ₃	0.013	0.081	0.061	0.032	0.012

Figure 3 Reaction progress curves of cPLA₂α in the presence of pyrrophenone

cPLA₂α activity was measured using the PC/Triton assay without (●) or with (○) 80 nM pyrrophenone. Values are expressed as means ± S.D. from three independent experiments.

Figure 4 Effect of preincubation of pyrrophenone with cPLA₂α on PLA₂ activity

cPLA₂α (65 ng/ml) was preincubated without or with 80 nM pyrrophenone in the assay mixture for the indicated times at 37 °C, followed by addition of the PC/Triton X-100 mixed micellar solution. The enzyme reaction was performed at 37 °C for 20 min and PLA₂ activity was determined using the PC/Triton assay. Data represent the percentage of control values without inhibitor; values are expressed as means ± S.D. from three independent experiments.

activity by dilution and inhibitory potency similar to that shown in Figure 2 at each final concentration after dilution, indicating that pyrrophenone is a reversible inhibitor.

Figure 5 Reversible inhibition of cPLA₂α by pyrrophenone

cPLA₂α (170 μg/ml) was preincubated in 25 mM Tris/HCl (pH 8.0), 0.5 M NaCl and 0.25 mg/ml BSA for 60 min at 37 °C without (●) or with 10 (○) and 125 (△) μM pyrrophenone, and then diluted approx. 1500-fold into the assay mixture containing PC/Triton X-100 mixed micelles. The enzyme reaction was performed at 37 °C. PLA₂ activity was determined using the PC/Triton assay. Values are expressed as means ± S.D. from three independent experiments.

MAFP has been reported to be an active-site-directed, irreversible inhibitor [28]. AACOCF₃ was shown to prevent cPLA₂α from inactivation by MAFP, indicating that AACOCF₃ competes with MAFP at least partially at the same site on cPLA₂α as described previously [28]. To anticipate the possible interaction site of pyrrophenone with cPLA₂α, a protection assay was carried out. In this experiment, compound 1f (Figure 1), described in the previous report [14], was used as a negative compound. This compound has a 4-benzoyl-benzoyl group at the 1-position of pyrrolidine instead of a 2-benzoyl-benzoyl group, which proved to be essential for the improvement of its inhibition of cPLA₂α in our study of the structure–activity relationship [14], and was found to have at least 2380-fold weaker inhibition for cPLA₂α than pyrrophenone [14]. No protective activity for the inhibition of cPLA₂α by 25 μM MAFP was observed for compound 1f at 25 μM, whereas pyrrophenone at 25 μM attenuated the inhibition by MAFP, as shown in Figure 6. These findings suggest that pyrrophenone interacts with sites on cPLA₂α that are similar to those used by MAFP, because the protection from inhibition by MAFP is dependent on cPLA₂α inhibitory potency.

Effect of pyrrophenone on other enzymes

To verify the selectivity of cPLA₂α inhibition by pyrrophenone, we examined its inhibitory potency towards sPLA₂s (group

Figure 6 Effect of pyrrophenone and compound 1f on MAFP inactivation for cPLA₂α

MAFP (25 μ M) was preincubated with 180 μ g/ml cPLA₂α in 25 mM Tris/HCl (pH 8.0), 0.5 M NaCl and 0.25 mg/ml BSA in the absence (closed bars) or presence of 25 μ M pyrrophenone (open bars) or 25 μ M compound 1f (hatched bars) at 40 °C for the indicated times. An aliquot was diluted approx. 1500-fold into the assay mixture containing PC/Triton X-100 mixed micelles. The enzyme reaction was performed at 37 °C for 20 min and PLA₂ activity was determined using the PC/Triton assay. Controls were measured by preincubation with DMSO alone (closed bars), pyrrophenone (open bars) or compound 1f (hatched bars). The remaining activities for the respective controls were determined. Values are expressed as means $\pm$ S.D. from three independent experiments. Data were analysed using Student's *t* test; **P* < 0.05, ***P* < 0.01 for comparison of MAFP plus pyrrophenone with MAFP alone.

IB and IIA sPLA₂s) and porcine pancreatic lipase. sPLA₂ activities and lipase activity were measured by assay using diheptanoyl thio-PC/Triton X-100 mixed micelles and triolein/sodium deoxycholate mixed micelles as substrates, respectively. Pyrrophenone did not inhibit group IB sPLA₂ even at a concentration of 250 μ M (mole fraction, 0.192) and the *X*_I(50) value for group IIA sPLA₂ was only approx. 0.192. Furthermore, pyrrophenone showed no inhibition of porcine pancreatic lipase, even at 250 μ M (mole fraction, 0.071). These results suggest that pyrrophenone is a selective inhibitor for cPLA₂α compared with sPLA₂s and pancreatic lipase, and that its inhibition is not due to interference with the enzyme's access to the substrate interface by non-specific membrane perturbation.

Effect of pyrrophenone on arachidonic acid release and eicosanoid production from A23187-stimulated THP-1 cells

The increase of intracellular calcium is an important factor in the translocation of cPLA₂α to the membrane, i.e. the nuclear envelope. To verify the efficacy of pyrrophenone in a whole-cell system, we examined the effect of pyrrophenone on arachidonic acid release and eicosanoid production from A23187-stimulated THP-1 cells (Table 2). Pyrrophenone showed dose-dependent inhibition of arachidonic acid release by A23187 stimulation with an IC₅₀ value of 0.024 μ M. PGE₂ and LTC₄ productions were also inhibited with similar IC₅₀ values (0.025 and 0.014 μ M, respectively) to arachidonic acid release. No lactate dehydrogenase release was observed at 100 μ M. AACOCF₃ and MAFP showed inhibition of arachidonic acid release from THP-1 cells with IC₅₀ values of 86 and 3.3 μ M, respectively, and pyrrophenone showed about 3583- and 138-fold more potent inhibition than AACOCF₃ and MAFP, respectively. To determine whether pyrrophenone has inhibitory activities for any steps of eicosanoid synthesis other than arachidonic acid release, the effect of pyrrophenone on PGE₂ and LTC₄ production by direct addition of arachidonic acid to THP-1 cells was examined. The standard cyclo-oxygenase (COX) inhibitors, indomethacin and NS-398, and the standard 5-lipoxygenase inhibitor, AA-861, inhibited the production of PGE₂ and LTC₄, respectively, as with A23187 stimulation, whereas pyrrophenone did not show any inhibition of eicosanoid synthesis, demonstrating that pyrrophenone only inhibited the arachidonic acid-release step in A23187-stimulated THP-1 cells, leading to the suppression of PGE₂ and LTC₄ production. Comparison between a non-selective inhibitor of COX-1, indomethacin, and a selective inhibitor of COX-2, NS-398, showed that indomethacin displayed 139-fold more potent inhibition of PGE₂ production with the direct addition of arachidonic acid than NS-398. This suggests that COX-1 mainly contributes to PGE₂ synthesis from THP-1 cells and that pyrrophenone has no effect on COX-1 activity.

Besides cPLA₂ activity, calcium-independent PLA₂ activity and DG lipase activity, hydrolysing *sn*-2 arachidonic acid of glycerolipids, were found in THP-1 cells. To examine the effect of pyrrophenone on these enzyme activities that may lead to release arachidonic acid from the membrane, 1-myristoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine was used as the substrate for calcium-independent PLA₂ and 1-stearoyl-2-arachidonoyl-*sn*-glycerol for DG lipase, and both enzyme activities were measured under optimized conditions. To compare inhibitory potencies under the same conditions, cPLA₂α activity in THP-1 cell lysates was also measured using PAPC/DOG liposomes.

Table 2 Effect of pyrrophenone on arachidonic acid release and eicosanoid production in THP-1 cells

Values are expressed as means $\pm$ S.D. from three independent experiments. ND, not done.

Compound	IC ₅₀ (μ M)				
	A23187			Arachidonic acid	
	Arachidonic acid	PGE ₂	LTC ₄	PGE ₂	LTC ₄
Pyrrophenone	0.024 $\pm$ 0.002	0.025 $\pm$ 0.019	0.014 $\pm$ 0.007	> 100	> 100
AACOCF ₃	86 $\pm$ 15	ND	41 $\pm$ 30	ND	> 100
MAFP	3.3 $\pm$ 0.87	ND	6.7 $\pm$ 3.5	ND	ND
Indomethacin	> 10	0.0036 $\pm$ 0.0043	> 10	0.013 $\pm$ 0.007	> 100
NS-398	> 10	0.21 $\pm$ 0.08	> 10	1.8 $\pm$ 2.4	> 100
AA-861	> 10	ND	0.0047 $\pm$ 0.001	ND	0.068 $\pm$ 0.026

Table 3 Effect of pyrrophenone on lipolytic enzymes in THP-1 lysates

Values are expressed as the means from two independent experiments.

Compound	IC ₅₀ (μM)		
	cPLA ₂ activity	Ca ²⁺ -independent PLA ₂ activity	DG lipase activity
Pyrrophenone	≈ 0.001	37	> 100
AACOCF ₃	0.4	> 100	> 100
MAFP	0.92	0.67	0.49

Table 4 Effect of pyrrophenone on PGE₂ production in human mesangial cellsValues are expressed as means ± S.D. from three independent experiments. The amounts of PGE₂ produced in response to IL-1β and arachidonic acid were 38.87 ± 4.88 and 11.59 ± 4.38 ng/ml, respectively.

Compound	IC ₅₀ (μM)	
	IL-1β stimulation	Arachidonic acid
Pyrrophenone	0.0081 ± 0.0031	> 10
AACOCF ₃	2.3 ± 0.59	> 10
Indomethacin	0.004 ± 0.0021	0.037 ± 0.015
NS-398	0.0059 ± 0.0039	0.075 ± 0.04

PLA₂ activity measured under this assay condition decreased by approx. 20 % of the control with the specific antibody raised against cPLA₂α (results not shown). As presented in Table 3, the inhibitory activities of pyrrophenone for calcium-independent PLA₂ (IC₅₀, 37 μM; mole fraction, 0.37) and DG lipase (IC₅₀, > 100 μM; mole fraction, > 0.067) were much less potent than for cPLA₂α (IC₅₀, approx. 0.001 μM; mole fraction, approx. 0.00027). AACOCF₃ also displayed a tendency for selective inhibition of cPLA₂α under the assay conditions employed. On the other hand, MAFP showed similar IC₅₀ values in the three enzyme assays.

Effect of pyrrophenone on PGE₂ production from IL-1β-stimulated human glomerular mesangial cells

Pyrrophenone inhibited arachidonic acid release and eicosanoid production from A23187-stimulated THP-1 cells. However, since A23187 is not a naturally occurring biological stimulant, it is important to clarify the efficacy of a potent cPLA₂α inhibitor on eicosanoid synthesis induced by natural pro-inflammatory mediators when exploring its possible use as a therapeutic agent for cPLA₂α inhibition. IL-1β is a pro-inflammatory cytokine produced by macrophages and monocytes and plays an important role in the mediation of inflammatory responses. IL-1β can induce expression and protein synthesis of cPLA₂α with the induction of COX-2 synthesis, leading to prostaglandin production in various cultured cell types [29,30]. We therefore assessed the effect of pyrrophenone on PGE₂ synthesis by IL-1β stimulation in human mesangial cells. As shown in Table 4, pyrrophenone strongly inhibited IL-1β-induced PGE₂ production from human mesangial cells with an IC₅₀ value of 0.0081 μM, comparable with those of indomethacin and NS-398. No lactate dehydrogenase release was observed after incubation for 24 h at 10 μM pyrrophenone and the inhibition for PGE₂ production by pyrrophenone did not involve a cytotoxic effect. AACOCF₃ showed about 284-fold less potent inhibition than pyrrophenone. To examine whether the observed inhibition of pyrrophenone for

PGE₂ production involves action against the PGE₂ biosynthetic step located downstream of arachidonic acid release, the effect of pyrrophenone on the production of PGE₂ by direct addition of arachidonic acid to human mesangial cells was examined. Our Western-blot analysis showed that human mesangial cells constitutively express COX-1, but not COX-2, whereas IL-1β stimulation markedly induced COX-2 expression alone. In resting mesangial cells, PGE₂ production by arachidonic acid addition was hardly observed, whereas the COX-2-induced cells by IL-1β stimulation exhibited marked PGE₂ production on arachidonic acid addition (results not shown). Therefore, after preincubation with IL-1β for 24 h and then washing of the cells to remove PGE₂ produced during IL-1β stimulation, arachidonic acid was added and the produced PGE₂ was measured. Pyrrophenone did not inhibit PGE₂ production on addition of arachidonic acid to the cells, indicating that the inhibition of PGE₂ production by pyrrophenone is not due to suppression of the PGE₂ biosynthetic step located downstream of arachidonic acid release. Comparison of indomethacin and NS-398 showed that NS-398 caused almost the same inhibition of PGE₂ production as indomethacin on direct addition of arachidonic acid, unlike in the case of THP-1 cells. The profile of these COX inhibitors for PGE₂ production is consistent with the finding of marked PGE₂ production only when cells were stimulated with IL-1β, as described above, suggesting that pyrrophenone has no effect on COX-2 activity because COX-2 contributes mainly to PGE₂ synthesis from IL-1β-stimulated human mesangial cells.

DISCUSSION

We characterized the cPLA₂α inhibitor pyrrophenone by enzyme assay and cell-based assay. Here we offer evidence that pyrrophenone inhibits the interaction between the substrate and the active site of the enzyme in the interface without affecting the interfacial quality of the substrate. If the inhibitor binds to the interface of substrate aggregates, where it would compete with the substrate for the active site of the enzyme, each $X_1(50)$ value should be similar, independent of the total concentration of substrate in different assay systems [26,31]. When we measured the cPLA₂α inhibitory activity of compounds using three separate assay systems distinct from the total lipid-surface concentration in the assay mixture, the $X_1(50)$ values of pyrrophenone were similar for all assays. AACOCF₃ also inhibited cPLA₂α with similar $X_1(50)$ values under the three sets of assay conditions. Furthermore, pyrrophenone exhibited selective inhibition that was over two orders of magnitude more for cPLA₂α than for other lipolytic enzymes, sPLA₂s and pancreatic lipase, based on comparison of their $X_1(50)$ values. Although we did not show it in this report, another pyrrolidine inhibitor (compound 4d or pyrrolidine-1), which we developed, was reported to exhibit much less potent inhibition of cPLA₂γ than of cPLA₂α [32]. In competition assay with MAFP, an irreversible inhibitor of cPLA₂α, pyrrophenone offered protection against MAFP inhibition, as in the case of AACOCF₃ described previously [28]. On the other hand, compound 1f, which showed much less potent inhibition [$X_1(50)$ value of > 2.7] for cPLA₂α in the PG/DOG assay than pyrrophenone [14], could not prevent MAFP action, suggesting that pyrrophenone could inhibit the PLA₂ activity by interacting with cPLA₂α in the region near the binding site of MAFP. Pyrrophenone also showed inhibitory activity for esterase activity and lysophospholipase activity of cPLA₂α with potencies similar to that for PLA₂ activity. The mutation of Ser-228 of cPLA₂α has been reported to abolish PLA₂ activity, lysophospholipase activity and esterase activity [33–35], suggesting that Ser-228 is essential for the three hy-

hydrolytic activities of cPLA₂α and that pyrrophenone can interact with cPLA₂α in the vicinity of Ser-228. Taken together, these findings indicate that the inhibition of cPLA₂α by pyrrophenone is due to interference with the interaction between the substrate and the active site of the enzyme and not due to a change in the interfacial quality of the substrate by non-specific membrane perturbation.

AACOCF₃ is a slow-binding inhibitor, thus requiring a long incubation time to exhibit its full inhibitory potency by binding tightly to the enzyme [11,27]. On the other hand, pyrrophenone is not a slow-binding inhibitor, because the reaction progress curve with pyrrophenone exhibited constant inhibition throughout the enzyme reaction and preincubation had no effect on its inhibitory potency. Therefore, pyrrophenone is more suitable than AACOCF₃ for examining the efficacy of cPLA₂α inhibition for inflammatory responses, including arachidonic acid release caused within a short period after stimulation. Our previous report [15] showed that pyrrophenone inhibited arachidonic acid release in a dose-dependent manner with concomitant inhibition of PGE₂, thromboxane B₂ and LTB₄ production in a whole-blood assay that closely reflected *in vivo* conditions, and their inhibitory potencies of pyrrophenone for eicosanoid synthesis were equal to those of indomethacin and AA-861 [15]. Similarly, pyrrophenone also exhibited potent inhibition of arachidonic acid release and eicosanoid production from A23187-stimulated rat and dog whole blood (results not shown). These findings indicate that pyrrophenone is stable in plasma irrespective of the animal species and can penetrate the intact cell membrane in whole blood. On the other hand, AACOCF₃ does not display any inhibition of arachidonic acid release in the whole-blood assay [15], suggesting that there are some problems with the stability in the plasma component and the potency of penetration into intact cells with plasma protein. Accordingly, pyrrophenone would be more advantageous than AACOCF₃ for use in various cell-based assays and pharmacological experiments.

In cPLA₂α-deficient mice, the production of PGE₂ and cysteinyl leukotrienes from A23187-stimulated peritoneal cells decreased markedly compared with those in wild-type mice [7]. As described previously [14], the inhibition of A23187-induced arachidonic acid release by pyrrolidine derivatives paralleled the inhibitory activity against cPLA₂α enzyme in the process of chemical modification to improve the inhibitory activity. Finally, pyrrophenone strongly inhibited arachidonic acid release without affecting enzymes located downstream of the arachidonic acid release. Furthermore, pyrrophenone had much lower inhibitory potency against calcium-independent PLA₂ and DG lipase, having hydrolysing activities for arachidonyl-glycerolipids found in THP-1 cells, than against cPLA₂α activity, as shown in Table 3. When we measured the content of total phospholipids in THP-1 cells, it was about 100 nmol/5 × 10⁶ cells per ml, corresponding to 100 μM (results not shown). Since an IC₅₀ value of 0.024 μM in this cell-based assay corresponded to a mole fraction of about 0.00024 for total phospholipids in THP-1 cells, the inhibitory potency of arachidonic acid release of pyrrophenone in the cell-based assay appears to be well correlated with those in the enzyme assay. These results indicate that pyrrophenone can inhibit arachidonic acid release and eicosanoid production by acting as a selective cPLA₂α inhibitor in A23187-stimulated THP-1 cells.

In rat mesangial cells, IL-1β enhanced the synthesis of both group IIA sPLA₂ and cPLA₂α [36]. In addition to cPLA₂α, group IIA sPLA₂ was also suggested to contribute to PGE₂ synthesis by IL-1β and/or tumour necrosis factor-α stimulation from experiments using sPLA₂ inhibitor and antisense oligonucleotides against group IIA sPLA₂ in various cell types [37,38]. In order to

determine whether IL-1β induces the synthesis of sPLA₂ in human mesangial cells, we performed Western-blot analysis using group IIA sPLA₂ antibody and measurement of enzyme activity using phosphatidylglycerol, a preferred substrate for sPLA₂s [16]. However, we could not detect group IIA sPLA₂ protein in either control cells or stimulated cells by Western blotting in the culture medium or in the cell fraction and found no increases of IL-1β-induced PLA₂ activity measured with phosphatidylglycerol (results not shown). The sPLA₂ inhibitor indoxam, displaying a broad spectrum of inhibition for sPLA₂ isozymes [39], showed no apparent inhibitory activity at 10 μM for PGE₂ synthesis from IL-1β-stimulated human mesangial cells, whereas pyrrophenone completely blocked PGE₂ synthesis from IL-1β-stimulated human mesangial cells at the concentration of 1 μM (results not shown). Additionally, no inhibition by pyrrophenone was observed for PGE₂ synthesis by direct arachidonic acid addition. These results indicate that pyrrophenone inhibited only the step of arachidonic acid release and that cPLA₂α contributed mainly to IL-1β-induced PGE₂ synthesis in human mesangial cells.

AACOCF₃, in addition to its direct inhibition of cPLA₂α, exhibits interference with lipopolysaccharide (LPS)-induced nuclear factor-κB activation (DNA binding of nuclear factor-κB) in human peripheral blood mononuclear cells by preventing inhibitor-κB degradation [40]. Addition of exogenous lipid mediators, i.e. arachidonic acid, platelet-activating factor and leukotriene B₄, to LPS-treated cells prevents the inhibition of DNA binding of nuclear factor-κB by AACOCF₃, suggesting that this kind of lipid mediator, which would be produced via cPLA₂α action, could contribute to nuclear factor-κB activation. Similarly, AACOCF₃ was shown to prevent activation of mitogen-activated protein kinase [41]. IL-1β activates nuclear factor-κB and mitogen-activated protein kinase pathways in the same manner as LPS, by which cPLA₂α activation and COX-2 expression are induced, leading to PGE₂ synthesis [5,42]. IL-1β-mediated signalling utilizes an analogous molecular framework to LPS signalling via signalling molecules coupled to its receptor [43], suggesting that cPLA₂α-generated lipid mediators promote the IL-1β signalling cascade. Therefore, in addition to blocking the supply of arachidonic acid for COX-2, pyrrophenone may attenuate the IL-1β signalling cascade and also inhibit IL-1β-induced cPLA₂α activation and COX-2 expression in human mesangial cells.

In conclusion, pyrrophenone is a potent and reversible inhibitor of cPLA₂α and also exhibits the inhibition of multiple enzyme activities of cPLA₂α, lysophospholipase and esterase activities. On the other hand, this compound showed no or much less potent inhibition of sPLA₂s and pancreatic lipase compared with that of cPLA₂α. In A23187-stimulated THP-1 cells and IL-1β-stimulated human mesangial cells, pyrrophenone inhibited eicosanoid synthesis by specifically inhibiting the arachidonic acid step without any effect on downstream enzymes. Our findings indicate that pyrrophenone is a promising cPLA₂α inhibitor for clinical use.

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