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RESEARCH PAPER

Effects of pyrrophenone, an inhibitor of group IVA phospholipase A_2, on eicosanoid and PAF biosynthesis in human neutrophils

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Background and Purpose: The biosynthesis of leukotrienes (LT) and platelet-activating factor (PAF) involves the release of their respective precursors, arachidonic acid (AA) and lyso-PAF by the group IVA PLA_2 ($cPLA_2\alpha$). This paper aims at characterizing the inhibitory properties of the $cPLA_2\alpha$ inhibitor pyrrophenone on eicosanoids and PAF in human neutrophils (PMN).
Experimental Approach: Freshly isolated human PMN were activated with physiological and pharmacological agents (fMLP, PAF, exogenous AA, A23187 and thapsigargin) in presence and absence of the $cPLA_2\alpha$ inhibitor pyrrophenone and biosynthesis of LT, PAF, and PGE_2 was measured.
Key Results: Pyrrophenone potently inhibited LT, PGE_2 and PAF biosynthesis in PMN with IC_{50}s in the range of 1–20 nM. These inhibitory effects of pyrrophenone were specific (the consequence of substrate deprivation), as shown by the reversal of inhibition by exogenous AA and lyso-PAF. Comparative assessment of pyrrophenone, methyl-arachidonoyl-fluoro-phosphonate (MAFP) and arachidonoyl-trifluoromethylketone ($AACOCF_3$) demonstrated that pyrrophenone was more specific and 100-fold more potent than MAFP and $AACOCF_3$ for the inhibition of LT biosynthesis in A23187-activated PMN. The inhibitory effect of pyrrophenone on LT biosynthesis was reversible as LT biosynthesis was recovered when pyrrophenone-treated PMN were washed with autologous plasma. No alteration of phospholipase D (PLD) activity in fMLP-activated PMN was observed with up to 10 μM pyrrophenone, suggesting that the $cPLA_2\alpha$ inhibitor does not directly inhibit PLD.
Conclusions and Implications: Pyrrophenone is a more potent and specific $cPLA_2\alpha$ inhibitor than MAFP and $AACOCF_3$ and represents an excellent pharmacological tool to investigate the biosynthesis and the biological roles of eicosanoids and PAF.
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Keywords: Leukotriene; platelet-activating factor; prostaglandin; neutrophil; leukocyte; phospholipase A_2; lipid mediator; pyrrophenone; pyrrolidine-1; $cPLA_2\alpha$

Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonoyl-trifluoromethylketone; ADA, adenosine deaminase; $cPLA_2\alpha$, group IVA phospholipase A_2; EtOH, ethanol; LT, leukotriene; MAFP, methyl-arachidonoyl-fluoro-phosphonate; MeCN, acetonitrile; MeOH, methanol; PAF, platelet-activating factor; PG, prostaglandin; PLD, phospholipase D; RP, reverse phase; SPE, solid-phase extraction

Introduction

Lipid mediators such as prostaglandins (PGs), leukotrienes (LTs), lipoxins and platelet-activating factor (PAF) are important in the development and resolution of the inflammatory response. The biosynthesis of the lipid mediators of inflammation involves the activation of a phospholipase A_2 (PLA_2) and the subsequent release of arachidonic acid (AA) and lyso-PAF. The free AA is then oxygenated by the lipoxygenases or the cyclooxygenases to yield eicosanoids, whereas lyso-PAF is acetylated to PAF by the acetyl-CoA/lyso-PAF acetyltransferase. Among the PLA_2 family, the group IVA phospholipase ($cPLA_2\alpha$) is recognized as the most important PLA_2 activity implicated in the biosynthesis of PG, LT and PAF. Indeed, experiments with $cPLA_2\alpha$-deficient mice demonstrated that this enzyme was essential for the biosynthesis of eicosanoids and PAF *in vivo* (Bonventre *et al.,* 1997; Uozumi *et al.,* 1997; Fujishima *et al.,* 1999; Shindou *et al.,* 2000).

Early pharmacological studies demonstrated that the inhibition of PLA_2 activity by *p*-bromophenacyl bromide, mepacrine, arachidonoyl-trifluoromethylketone ($AACOCF_3$) and methyl-arachidonoyl-fluoro-phosphonate (MAFP) resulted in the inhibition of LT biosynthesis in activated

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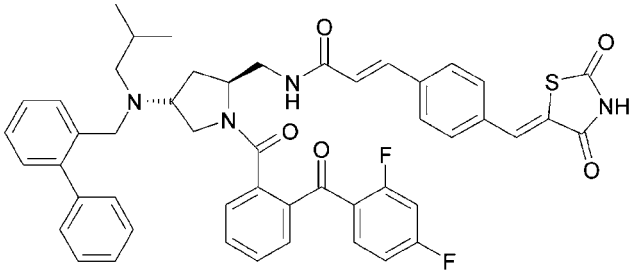
polymorphonuclear neutrophil (PMN) and other leukocytes. Although effective at blocking the biosynthesis of LT, these inhibitors demonstrated a lack of specificity. For example, MAFP, one of the most commonly used cPLA$_2\alpha$ inhibitors, also binds to the cannabinoid receptor 1 and inhibits the group VIA of PLA$_2$, fatty acid amide hydrolase and PAF acetylhydrolase (Balsinde and Dennis, 1996; Lio *et al.,* 1996; Deutsch *et al.,* 1997; Kell *et al.,* 2003). The isolation and molecular characterization of cPLA$_2\alpha$ led to the development of more potent and specific cPLA$_2\alpha$ inhibitors. Among these new cPLA$_2\alpha$ inhibitors, pyrrolidine-1 and pyrrophenone (Figure 1) developed by Shionogi Research Laboratories (Seno *et al.,* 2000, 2001; Ono *et al.,* 2002) represent a class of promising pharmacological tools to investigate the mechanisms of lipid mediator biosynthesis and to assess the role of these mediators in physiological and pathological processes such as host defense and inflammatory diseases.

Although pyrrophenone and pyrrolidine-1 have recently been used successfully in a limited number of studies, their inhibition profile and relative potencies and specificities versus other currently used cPLA$_2\alpha$ inhibitors have not been thoroughly investigated. In this study, we characterized the effects of the cPLA$_2\alpha$ inhibitor pyrrophenone on LT, prostaglandin E$_2$ (PGE$_2$) and PAF biosynthesis in human PMN stimulated under various experimental conditions and compared the potency and specificity of pyrrophenone with those of the currently used PLA$_2$ inhibitors, MAFP and AACOCF$_3$.

Methods

Isolation of human PMN

Venous blood from healthy donors was collected in 10 ml tubes containing 143 USP units of heparin and PMN were isolated as described previously (Boyum, 1968). Briefly, following centrifugation of blood, the platelet-rich plasma was discarded and erythrocytes were removed by dextran sedimentation. Mononuclear cells were then separated from the granulocytes by centrifugation on Ficoll–Paque cushions and a hypotonic lysis was performed on the granulocyte cell pellets to remove the remaining erythrocytes. The granulocyte suspensions contained mainly PMN ($\geqslant$95%) and cell viability was always greater than 98% as measured by Trypan blue exclusion. PMN were finally re-suspended in Hank's balanced salt solution (HBSS) containing 1.6 mM $CaCl_2$ at 5 or 10×10^6 cells ml^{-1}, as indicated. In all experimental settings, incubation volume was 1 ml.



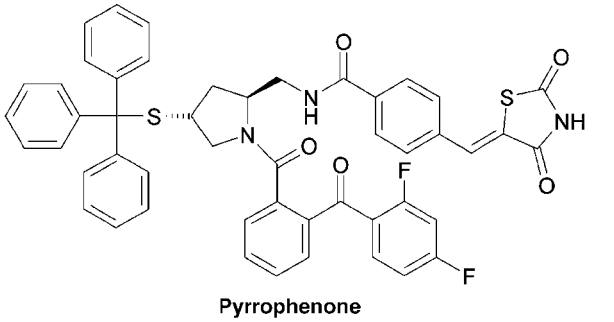


Figure 1 Structures of the cPLA$_2\alpha$ inhibitors pyrrolidine-1 and pyrrophenone.

Stimulation of LT and PAF biosynthesis

(A) In experiments involving stimulation with PAF or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), PMN suspensions in HBSS containing 1.6 mM $CaCl_2$ (37°C, 5×10^6 cells ml^{-1}) were primed with 1.5 nM tumor necrosis factor-α (TNF-α), 700 pM granulocyte macrophage colony-stimulating factor (GM-CSF) and 10 μM cytochalasin B for 30 min, then stimulated with 300 nM of either fMLP or PAF for 10 min. (B) In experiments involving stimulation with thapsigargin, AA and A23187, unprimed PMN suspensions in HBSS containing 1.6 mM $CaCl_2$ (37°C, 5×10^6 cells ml^{-1}) were stimulated with either thapsigargin (100 nM, 10 min), AA (3 μM, 5 min) or A23187 (100 nM, 5 min). In these experimental settings (A and B), 0.3 U ml^{-1} adenosine deaminase (ADA) was added to incubation media 5 min prior stimulation to eliminate the inhibitory constraint exerted by extracellular adenosine (Krump *et al.,* 1997). (C) In experiments performed with human blood, freshly drawn human blood samples containing $\sim$14 U ml^{-1} heparin were incubated at 37°C in the presence of increasing concentrations of pyrrophenone, then stimulated with 10 μM ionomycin for 20 min. In all experimental settings, the PLA$_2$ inhibitors MAFP, AACOCF$_3$ and pyrrophenone were always added 10 min before stimulation with AA, A23187, thapsigargin, PAF or fMLP. Inhibitors and stimulatory agents were solubilized in ethanol (EtOH) or dimethylsulfoxide (DMSO) for addition to PMN suspensions. The final concentration of these solvents never exceeded 0.2% in incubation media.

5-LO product and AA analysis

For the determination of 5-LO product biosynthesis, cell incubations were stopped by the addition of 0.5. volume of cold (4°C) methanol/acetonitrile (MeOH/MeCN) (1/1, v/v) containing 12.5 ng of each of prostaglandin B$_2$ (PGB$_2$) and 19-OH-PGB$_2$ as internal standards. The denatured samples were centrifuged (600 *g*, 10 min at 22°C) and the supernatants were analyzed by reverse phase-high-performance liquid chromatography (RP-HPLC) using an online extraction procedure as described previously (Borgeat *et al.,* 1990). In experiments with whole human blood, incubations were stopped by placing the samples in an ice-water bath. The plasma samples obtained by centrifugation (300 *g*, 20 min at 4°C) were denatured with 10 volumes of a cold stop solution (4°C, MeOH/MeCN, 1/1 (v/v)) containing 12.5 ng of both 19-OH-PGB$_1$ and PGB$_1$ as internal standards. The denatured samples were centrifuged (600 *g*, 20 min at 4°C) and the

supernatants were then evaporated to a volume of ~1 ml using a stream of nitrogen (in a water bath at 22°C) and analyzed by RP-HPLC as described previously (Surette *et al.,* 1993). LTB_4, 20-COOH-LTB_4, 20-OH-LTB_4, 6(*E*)-LTB_4, 6(*E*)-12-epi-LTB_4 and 5(*S*)-HETE are collectively referred to as 5-LO products; these six compounds represent the major 5-LO metabolites of AA detectable by RP-HPLC and ultraviolet detection in human PMN. Quantitation of the various metabolites was achieved by using the internal standard PGB_2 for normalization and authentic standards of 20-OH-LTB_4 (also used for quantitation of 20-COOH-LTB_4), LTB_4 (also used for quantitation of 6(*E*)-LTB_4 and 6(*E*)-12-epi-LTB_4) and 5-HETE for calibration.

For the analysis of AA, incubations were stopped by the addition of 0.5 volume of a cold (4°C) stop solution (MeOH/MeCN, 1/1, v/v) containing 12.5 ng of both 19-OH-PGB_2 and PGB_2, and 20 ng of $^2H_8$-AA. The denatured incubation media were centrifuged (600 *g*, 10 min at 22°C) and the supernatants were analyzed by RP-HPLC. The AA-containing fractions were collected, evaporated to dryness under reduced pressure using a Savant Speed-Vac concentrator model SCIIOA (Thermo Electro Corp., Milford, MA, USA) (drying rate set at 'low') and redissolved in 50 μl MeCN for analysis by LC/MS using electrospray ionization in the negative mode as described previously (Surette *et al.,* 1993).

PAF and lyso-PAF analysis

For the determination of PAF and lyso-PAF, cell incubations were stopped by the addition of 1 volume of cold (4°C) EtOH containing 5 ng of $^2H_4$-PAF as internal standard. The denatured samples were then centrifuged (600 *g*, 20 min at 22°C), and PAF and lyso-PAF were recovered from the supernatants and analyzed as described previously (Harrison *et al.,* 1999), with minor modifications. Briefly, the samples were loaded on a 60 mg C_{18} solid-phase extraction (SPE) cartridge and successively washed with 4 ml water and 2 ml EtOH/water (50/50, v/v). PAF and lyso-PAF were then eluted from the C_{18} cartridge with 2 ml EtOH/water (98/2, v/v), which were directly loaded onto an EtOH-conditioned 100 mg silica SPE cartridge. The silica cartridge was then washed with 2 ml EtOH and PAF and lyso-PAF were eluted with 1.1 ml MeCN/water (60/40, v/v). Samples were evaporated to dryness under reduced pressure in a Speed-Vac concentrator (drying rate set at 'low') and re-suspended in 50 μl of the HPLC mobile phase (hexane/isopropanol, 20 mM aqueous ammonium acetate, 3/4/0.7 (v/v/v)). Analysis of PAF and lyso-PAF was then performed by LC-MS/MS by the measurement of the PAF/$^2H_4$-PAF ratio ((*m/z* 508→59)/(*m/z* 512→59)) and lyso-PAF/$^2H_4$-PAF ratio ((*m/z* 466→377)/(*m/z* 512→59)), respectively. Quantitation was achieved using standard curves generated by analysis (ratio determination) of solutions containing increasing amounts of PAF or lyso-PAF and a fixed amount of $^2H_4$-PAF.

Induction of cyclooxygenase-2 and stimulation of PGE_2 biosynthesis

In experiments where PGE_2 biosynthesis was investigated, PMN suspensions in HBSS containing 1.6 mM $CaCl_2$ (37°C, 10^7 cells ml^{-1}) were pre-incubated 4 h with 700 pM GM-CSF, 1.5 nM TNF-α and 10 μM cytochalasin B (to induce cyclooxygenase-2 (COX-2) expression), then stimulated with 100 nM A23187 for 5 min. Incubations were stopped by placing the samples in an ice-water bath. Cell suspensions were immediately centrifuged (600 *g*, 5 min at 22°C), and PGE_2 was measured in the resulting supernatants using a PGE_2 ELISA according to the manufacturer's instructions.

Stimulation and analysis of phospholipase D activity

phospholipase D (PLD) activity was measured as described previously (Marcil *et al.,*. 1999). Briefly, PMN were pre-labeled with 1-*O*-[^{3}H]alkyl-2-lyso-phosphatidylcholine (2 μCi for 10^7 PMN) for 90 min at room temperature. The PMN suspensions in HBSS containing 0.8 mM $CaCl_2$ (10^7 cells ml^{-1}) were warmed up to 37°C, then treated with increasing concentrations of pyrrophenone and 10 μM cytochalasin B for 10 and 5 min, respectively. The suspensions were then stimulated for 10 min with 1 nM fMLP or 100 nM thapsigargin in the presence of 1% EtOH. Incubations were stopped by the addition of 3.6 volumes of a cold (4°C) stop solution (chloroform/MeOH/hydrochloric acid (50/100/1, v/v/v)) containing unlabeled phosphatidylethanol as internal standard. The lipids were extracted (Bligh and Dyer, 1959), the organic phase was evaporated to dryness under a stream of nitrogen and samples were spotted on pre-washed silica gel 60 thin layer chromotography (TLC) plates. Phosphatidylethanol was resolved from the other lipids using the solvent mixture chloroform/MeOH/acetic acid (65/15/2, v/v/v). Lipids were visualized by Coomassie Brilliant Blue staining and the different lipid classes were scraped off the plates. Radioactivity in the phosphatidylethanol fraction was measured by liquid scintillation counting and the results were corrected for background radioactivity and quenching. In all experimental settings, 0.3 U ml^{-1} ADA was added to the incubation media to eliminate the inhibitory constraint exerted by extracellular adenosine (Krump *et al.,* 1997).

Data analysis

Average results are presented as means ± s.e.m.

Materials

$^2H_4$-PAF (1-*O*-(7,7,8,8-$^2H_4$)hexadecyl-2-acetyl-glyceryl-3-phosphorylcholine), $^2H_8$-AA ((5,6,8,9,11,12,14,15-$^2H_8$)-arachidonic acid), LTB_4, 20-OH-LTB_4, 5-HETE and the PGE_2 ELISA kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). A23187, ionomycin, AA, cytochalasin B, DMSO, PAF, PGB_1, PGB_2, 19-OH-PGB_1 and 19-OH-PGB_2 were obtained from the Sigma Chemical Co. (Saint Louis, MO, USA). ADA was purchased from Roche Applied Science (Indianapolis, IN, USA). Thapsigargin was purchased from Research Biochemicals International (Natick, MA, USA). Pyrrophenone was a generous gift from Dr Kaoru Seno (Shionogi Research Laboratories, Osaka, Japan). Ficoll–Paque medium and Trypan blue were purchased from Wisent Laboratories (St-Bruno, Québec, Canada). C_{18} SPE cartridges (60 mg, 3 ml capacity) were obtained form Waters Corporation (Milford, MA, USA) and Bond Elut SPE silica cartridges (100 mg, 2 ml) were purchased from Varian (Harbor City, CA, USA).

Results

Pyrrophenone inhibits LT, PAF and PGE_2 biosynthesis in PMN

A first series of experiments was undertaken to characterize the inhibitory effect of the $cPLA_2\alpha$ inhibitor pyrrophenone on 5-LO products and PAF biosynthesis in PMN activated by either the natural agonists fMLP and PAF or the pharmacological agent thapsigargin. As shown in Figure 2a, pyrrophenone suppressed, in a concentration-dependent manner, the fMLP-, PAF- and thapsigargin-induced biosynthesis of 5-LO products in PMN with IC_{50}s of 1–10 nM. In each experimental condition, the concentration–inhibition curves of the different 5-LO products by pyrrophenone showed similar IC_{50}s (data not shown). Figure 2b shows that thapsigargin-induced PAF biosynthesis is similarly inhibited by pyrrophenone with an IC_{50} of 1–2 nM. In separate experiments, PMN were exposed to GM-CSF, TNF-α and cytochalasin B for induction of COX-2 expression, and PGE_2 synthesis was stimulated with the ionophore A23187; as shown in Figure 2c, pyrrophenone also inhibited PGE_2 biosynthesis in PMN with an IC_{50} of 3–4 nM. In other experiments, pyrrophenone similarly inhibited PGE_2 biosynthesis in PMN treated with the priming agents (to induce COX-2 expression) and stimulated with fMLP (data not shown). In the experiments shown in Figure 2, the average amount ($\pm$s.e.m.) of PAF biosynthesized following activation with thapsigargin was 5.2 ($\pm$0.3) pmol $(10^6$ PMN$)^{-1}$. The average amounts of 5-LO products generated by thapsigargin-, fMLP- and PAF-activated PMN were 114 ($\pm$35), 55 ($\pm$13), and 41 ($\pm$5) pmol $(10^6$ PMN$)^{-1}$, respectively. The average amount of PGE_2 formed by A23187-treated PMN was 3.4 ($\pm$0.4) pmol $(10^6$ PMN$)^{-1}$. Finally, addition of pyrrophenone 30 min before the stimulation of whole heparinized human blood with 10 μM ionomycin resulted in dose–inhibition curves of the biosynthesis of 5-LO products with IC_{50}s around 1 μM (data not shown).

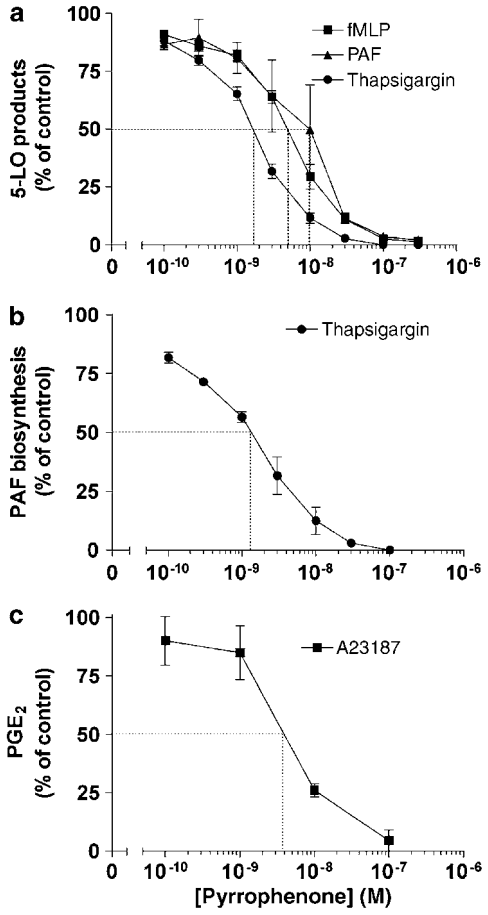


Figure 2 Inhibitory effects of pyrrophenone on lipid mediator biosynthesis in activated human PMN. (**a**) PMN suspensions (37°C, 5×10^6 cells ml^{-1}) were treated with increasing concentrations of pyrrophenone and stimulated with either 300 nM fMLP, 300 nM PAF or 100 nM thapsigargin as described in Methods. Incubations were stopped by the addition of 0.5 volume of a cold (4°C) stop solution (MeOH/MeCN, 1/1) containing 12.5 ng of both 19-OH-PGB_2 and PGB_2 (as internal standards) and 5-LO products were analyzed by RP-HPLC as described in Methods. (**b**) PMN suspensions were treated with pyrrophenone and stimulated with 100 nM thapsigargin as described above. Incubations were stopped by the addition of 1 volume of EtOH containing 5 ng of $^2H_4$-PAF. PAF and lyso-PAF were extracted and analyzed by LC/MS/MS as described in Methods. (**c**) PMN suspensions (37°C, 10^7 cells ml^{-1}) were treated 4 h with 700 pM GM-CSF, 1.5 nM TNF-α and 10 μM cytochalasin B for optimal COX-2 expression. Cell suspensions were incubated with increasing concentrations of pyrrophenone, then stimulated with 100 nM A23187 for 5 min. Incubations were stopped and PGE_2 was analyzed by ELISA in PMN supernatants as described in Methods. In all experimental settings, ADA (0.3 U ml^{-1}) and pyrrophenone were added 10 min before the addition of the agonists. Data represent the mean ($\pm$s.e.m.) of three separate experiments, each performed in duplicate.

Inhibition of LT and PAF biosynthesis correlates with the inhibition of AA and lyso-PAF release

We next directly assessed the effects of pyrrophenone on the release of the products of $cPLA_2\alpha$-mediated phospholipid hydrolysis, namely AA and lyso-PAF. As shown in Figures 3a and b, treatment of PMN with pyrrophenone also led to the concentration-dependent inhibition of both AA and lyso-PAF release. Moreover, the pyrrophenone-mediated inhibition of AA and lyso-PAF release closely paralleled the dose–inhibition curves (similar IC_{50} values) observed for LT and PAF biosynthesis in both agonist- and thapsigargin-activated PMN (Figure 2), supporting that the inhibition by pyrrophenone of LT and PAF biosynthesis is the consequence of its ability to inhibit $cPLA_2\alpha$ catalysis and AA and lyso-PAF release. In these experiments, the average amounts of free AA measured in PAF- and thapsigargin-stimulated PMN suspensions were 110$\pm$15 and 338$\pm$75 pmol $(10^6$ PMN$)^{-1}$.

Reversal of the inhibitory effect of pyrrophenone on LT biosynthesis by exogenous AA

The conclusive demonstration that pyrrophenone inhibits LT biosynthesis in activated human PMN through substrate deprivation (rather than through a nonspecific effect on the 5-LO-pathway) implies that LT biosynthesis can be restored by substrate replenishment. As shown in Figure 4, the addition of AA fully restored the biosynthesis of LT in thapsigargin-activated PMN treated with pyrrophenone. Similar results were also obtained with fMLP-activated PMN (data not shown).

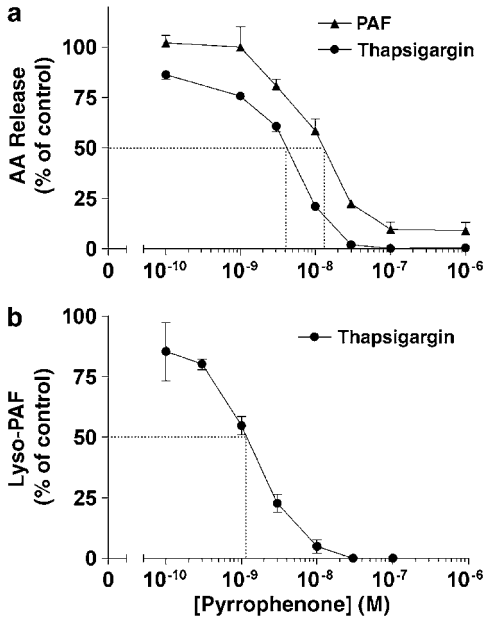


Figure 3 Inhibitory effects of pyrrophenone on AA and lyso-PAF release in activated human PMN. (**a**) PMN suspensions (37°C, 5×10^6 cells ml^{-1}) were incubated in the presence of increasing concentrations of pyrrophenone, then stimulated with either 100 nM thapsigargin or 300 nM PAF for 5 and 2 min, respectively, as described in Methods. Incubations were stopped by the addition of 0.5 volume of a cold (4°C) stop solution (MeOH/MeCN, 1/1, v/v) containing 12.5 ng of both 19-OH-PGB_2 and PGB_2, and 20 ng of $^2H_8$-AA as internal standards. Supernatants were collected and analyzed for AA content by LC/MS as described in Methods. (**b**) PMN suspensions (37°C, 5×10^6 cells ml^{-1}) were stimulated with 100 nM thapsigargin for 10 min as described in Methods. Incubations were stopped by the addition of 1 volume of EtOH containing 5 ng of $^2H_4$-PAF. Supernatants were collected and lyso-PAF was extracted and analyzed as described in Methods. In all experimental settings, 0.3 U ml^{-1} ADA and pyrrophenone were added 10 min before the addition of the stimuli. Data represent the mean (±s.e.m.) of three separate experiments, each performed in duplicate.

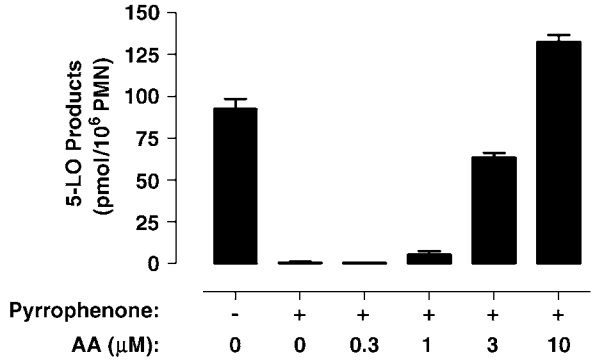


Figure 4 Reversal of the inhibitory effect of pyrrophenone on thapsigargin-induced LT biosynthesis. PMN suspensions (37°C, 5×10^6 cells ml^{-1}) were stimulated with 100 nM thapsigargin for 10 min as described in Methods. AA was added simultaneously with thapsigargin. ADA (0.3 U ml^{-1}) and 100 nM pyrrophenone were added 10 min before the stimulation of the cells with thapsigargin. Incubations were stopped by the addition of 0.5 volume of a cold (4°C) stop solution (MeOH/MeCN, 1/1) containing 12.5 ng of both 19-OH-PGB_2 and PGB_2 as internal standards and 5-LO products were analyzed as described in Methods. Data represent the mean (± s.e.m.) of three separate experiments, each performed in duplicate.

Pyrrophenone is more potent and specific than MAFP and AACOCF$_3$

In the next series of experiments, we undertook to compare the ability of pyrrophenone and two other frequently used cPLA$_2\alpha$ inhibitors, MAFP and AACOCF$_3$, on LT biosynthesis in PMN. The drugs were tested in two distinct experimental conditions, that is, in A23187-stimulated PMN, in which model AA release and LT biosynthesis involve the cPLA$_2\alpha$; and in AA-stimulated PMN, in which condition LT biosynthesis occurs from exogenous AA and is therefore independent of cPLA$_2\alpha$ activity (Surette *et al.,* 1999). In these experiments, as expected, PAF biosynthesis was important in A23187-stimulated PMN and undetectable in AA-stimulated PMN (data not shown), confirming the involvement of cPLA$_2\alpha$ in the former but not the latter condition. As shown in Figure 5a, pyrrophenone inhibited 5-LO product biosynthesis in A23187-stimulated PMN with an IC_{50} (5 nM) similar to those observed with other stimuli of LT biosynthesis (Figure 2a); pyrrophenone also inhibited AA-induced LT biosynthesis, however, at a 100-fold higher concentration, clearly indicating that pyrrophenone also exerts unspecific inhibitory effects on the 5-LO pathway when used at concentrations greater than 100 nM. In contrast, both MAFP and AACOCF$_3$ proved to be much less potent than pyrrophenone (IC_{50}s $\geqslant 1\,\mu$M) at inhibiting A23187-induced LT biosynthesis. The most striking and unexpected observation was, however, that MAFP and AACOCF$_3$ showed little if any difference in their abilities to inhibit LT biosynthesis in the two experimental conditions tested, clearly demonstrating that these two inhibitors, at the concentrations required to inhibit AA release by the cPLA$_2\alpha$ also exerted unspecific effects on the 5-LO pathway (possibly by a direct inhibition of the 5-LO and/or FLAP).

The inhibitory effect of pyrrophenone on LT biosynthesis is reversible

MAFP has been shown to irreversibly inhibit cPLA$_2\alpha$ by forming a stable complex with the enzyme (Ghomashchi *et al.,*. 1999), whereas AACOCF$_3$ has been shown to slowly but tightly associate with cPLA$_2\alpha$ (Trimble *et al.,* 1993). The occupation of the catalytic site of cPLA$_2\alpha$ in a reversible manner is the suggested mechanism by which pyrrophenone inhibits AA release (Ono *et al.,* 2002). In order to assess the reversibility of LT biosynthesis inhibition by pyrrophenone in activated PMN, we performed a series of experiments in which pyrrophenone-treated PMN were washed with incubation buffer or with autologous plasma. Table 1 shows that washing of the pyrrophenone-treated PMN with incubation buffer (HBSS) before stimulation with thapsigargin does not restore LT biosynthesis. In contrast, washing of PMN with autologous plasma fully restored the ability of thapsigargin-activated PMN to generate LT upon stimulation with thapsigargin, demonstrating that the pyrrophenone-mediated inhibition of cPLA$_2\alpha$ is a reversible process.

Pyrrophenone does not inhibit PLD activity

In the next experiments, we further assessed the specificity of pyrrophenone by investigating the putative inhibitory

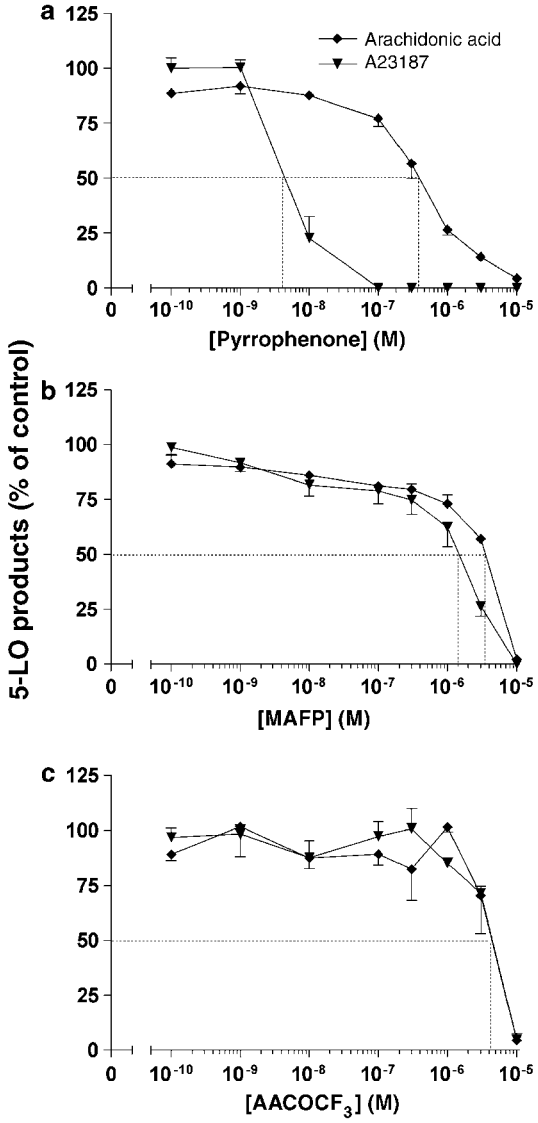


Figure 5 Comparative effects of (**a**) pyrrophenone, (**b**) MAFP and (**c**) $AACOCF_3$ on A23187- and AA-induced 5-LO product biosynthesis in human PMN. PMN suspensions (37°C, 5×10^6 cells ml^{-1}) were treated with ADA (0.3 U ml^{-1}) and increasing concentrations of cPLA$_2\alpha$ inhibitors 10 min before stimulation with either 100 nM A23187 or 2.5 μM AA for 5 min as described in Methods. Incubations were stopped by the addition of 0.5 volume of cold (4°C) stop solution (MeOH/MeCN, 50/50, v/v) containing 12.5 ng of both 19-OH-PGB_2 and PGB_2 as internal standards. 5-LO products were analyzed as described in Methods. Data represent the mean ($\pm$s.e.m.) of three separate experiments, each performed in duplicate.

effect of the drug in fMLP- and thapsigargin-induced activation of PLD in human PMN. As shown in Figure 6, pyrrophenone did not inhibit fMLP-induced PLD activity at concentrations up to 1 μM. However, pyrrophenone progressively inhibited PLD activity in PMN activated with thapsigargin. The observed inhibition of PLD activity by pyrrophenone in thapsigargin-activated PMN was completely reversed by the addition of AA to the incubation media (data not shown).

Table 1 Reversibility of pyrrophenone-induced inhibition of 5-LO products biosynthesis in human PMN

	5-LO products biosynthesis (% of control)		
	Not washed	*Washing*	
		HBSS	*Plasma*
Thapsigargin	100	75 $\pm$ 7	76 $\pm$ 8
Thapsigargin + Pyrrophenone	0.8 $\pm$ 0.2	0.5 $\pm$ 0.2	75 $\pm$ 7

Abbreviations: HBSS, Hank's balanced salt solution; PMN, polymorphonuclear neutrophil.
Pre-warmed human PMN suspensions (37°C, 5×10^6 cells ml^{-1}) were incubated 10 min in the presence of 100 nM pyrrophenone. The cell suspensions were then centrifuged (600 *g*, 2 min) and the cell pellets were re-suspended and incubated with pre-warmed (37°C) HBSS containing 1.6 mM $CaCl_2$ or human plasma for 20 min. All PMN suspensions were then washed once again with HBSS containing 1.6 mM $CaCl_2$ then re-suspended in HBSS containing 1.6 mM $CaCl_2$, 0.3 U ml^{-1} ADA and 100 nM thapsigargin for 10 min. Incubations were stopped by the addition of 0.5 volume of a cold (4°C) stop solution (MeOH/MeCN, 50/50, v/v) containing 12.5 ng of both 19-OH-PGB_2 and PGB_2 as internal standards. Supernatants were collected and analyzed for 5-LO products as described in Methods.

Discussion and conclusions

Thromboxanes, PG, LT and lipoxins represent families of metabolites of AA implicated in numerous physiological and pathophysiological processes including host defense and inflammatory diseases. Major efforts have therefore been invested in the past three decades to develop PLA_2 inhibitors and investigate the mechanisms of lipid mediator biosynthesis and to assess their biological functions. Mepacrine and *p*-bromophenacyl bromide were precious tools to evaluate the role of eicosanoids in early studies. However, the high IC_{50} values of these compounds for the inhibition of AA release and their lack of specificity has limited their utilization, and other more potent PLA_2 inhibitors such as $AACOCF_3$ and MAFP have been frequently used in eicosanoid research (Meyer *et al.,* 2005).

It has recently been clearly established that cPLA$_2\alpha$ is the major PLA_2 implicated in the biosynthesis of LT, PG and PAF. Indeed, studies implicating cPLA$_2\alpha$-deficient mice demonstrated an essential role for this enzyme in the biosynthesis of eicosanoids and PAF, as well as in the regulation of the expression of enzymes involved in their biosynthesis (Bonventre *et al.,* 1997; Uozumi *et al.,* 1997; Fujishima *et al.,* 1999; Shindou *et al.,* 2000). Therefore, the availability of potent and selective inhibitors of cPLA$_2\alpha$ was critical for future development in lipid mediator research.

A new class of cPLA$_2\alpha$ inhibitors has been recently developed, the pyrrolidine inhibitors comprising pyrrolidine-1 and pyrrophenone. These were shown to inhibit cPLA$_2\alpha$ *in vitro* with an important gain of potency compared to $AACOCF_3$ and MAFP (Ono *et al.,* 2002). Moreover, the IC_{50} of the pyrrolidine inhibitors for cPLA$_2\alpha$ was at least 2 orders of magnitude lower than for several other members of the PLA_2 family, namely group IB, IIA, IVC, V, VIB and X PLA_2 (Seno *et al.,* 2000, 2001; Ghomashchi *et al.,* 2001; Ono *et al.,* 2002), indicating a good level of specificity of pyrrolidine inhibitors for cPLA$_2\alpha$.

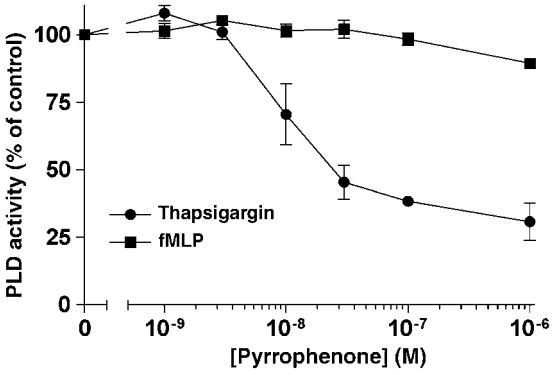


Figure 6 Effect of pyrrophenone on PLD activity in activated PMN. PMN suspensions (37°C, 10^7 cells ml^{-1}) were labeled with 1-*O*-[^{3}H]alkyl-2-lyso-phosphatidylcholine. Cells were then stimulated for 10 min with either 1 nM fMLP or 100 nM thapsigargin in the presence of 1% EtOH (final concentration). Incubations were stopped by the addition of 3.6 volumes of a cold (4°C) stop solution (CH_3Cl/MeOH/HCl, 50/100/1, v/v/v) containing unlabeled 1-*O*-alkyl-2-lyso-phosphatidylethanol as internal standard. 1-*O*-[^{3}H]alkyl-2-lyso-phosphatidylcholine and phosphatidylethanol were separated by TLC and analyzed for radioactivity content as described in Methods. Data represent the mean (±s.e.m.) of three separate experiments.

In the present study, we characterized the effect of pyrrophenone on LT, PG and PAF biosynthesis in activated PMN, a major source of bioactive lipid mediators in man and animals. As expected, the biosynthesis of eicosanoids and PAF was inhibited by pyrrophenone at low nanomolar concentrations, in agreement with a central role of cPLA$_2\alpha$ in the biosynthesis of these lipid mediators in activated-PMN (Syrbu *et al.,* 1999; Degousee *et al.,* 2002). Our results show that pyrrophenone potently inhibits three different classes of lipid mediators of inflammation in PMN, activated by their natural ligands fMLP and PAF as well as by the commonly used pharmacological agents A23187, ionomycin and thapsigargin and are in agreement with the reported inhibitory effects of pyrrolidine-1 on fMLP- and zymosan-induced AA release and PAF biosynthesis in human PMN (Rubin *et al.,* 2005). The IC$_{50}$s obtained for inhibition of LT and PGE$_2$ biosynthesis are very consistent with those also obtained with pyrrophenone for inhibition of the A23187-induced LTC$_4$ and PGE$_2$ biosynthesis in THP-1 (Seno *et al.,* 2001; Ono *et al.,* 2002). It is very likely that the much increased IC$_{50}$ observed for inhibition of 5-LO product biosynthesis in whole blood as opposed to isolated PMN in a protein-free buffer is the consequence of binding of the lipophilic drug to plasma proteins, albumin in particular. The complete reversal of LT biosynthesis inhibition by exogenous AA in pyrrophenone-treated, thapsigargin-activated PMN is in agreement with the effect of exogenous lyso-PAF on PAF biosynthesis described earlier using the same experimental conditions (Flamand *et al.,* 2006a), suggesting that a submicromolar concentration of pyrrophenone results in the specific inhibition of both AA and Lyso-PAF release in PMN.

An important result of the present study is the observation that in contrast to pyrrophenone, MAFP and AACOCF$_3$ inhibited both the A23187- and the AA-stimulated LT biosynthesis with similar IC$_{50}$. Considering that the cPLA$_2\alpha$ is not involved in LT biosynthesis induced by exogenous AA, these data clearly demonstrate that in addition to their ability to inhibit the cPLA$_2\alpha$, MAFP and AACOCF$_3$ exert nonspecific effects on the 5-LO pathway resulting in inhibition of LT biosynthesis in intact PMN. This is in agreement with a recent study that showed an inhibitory effect of AACOCF$_3$ on 5-LO activity in a broken cell assay (Fonteh, 2002) and with another study which demonstrated that both MAFP and AACOCF$_3$ bound to a binding site for AA on the 5-LO-activating protein with an affinity similar to that of AA itself (Charleson *et al.,* 1994). It is noteworthy that pyrrophenone also appeared to exert an effect distinct from PLA$_2$ inhibition as it also blocked AA-induced LT biosynthesis; however, this 'non-PLA$_2$' inhibition by pyrrophenone occurred at a concentration 100-fold greater than that required for inhibition of the A23187-induced LT biosynthesis (IC$_{50}$ ~5 nM). Taken together, these comparative data clearly emphasize that pyrrophenone is a more potent and selective inhibitor of cPLA$_2\alpha$ than MAFP and AACOCF$_3$ and is therefore a more reliable pharmacological tool for investigation of eicosanoid and PAF biosynthesis and action in intact cells and tissues. Our recent report that AA regulates 5-LO translocation to nuclear structures in activated PMN (Flamand *et al.*, 2006b) provides an example of the usefulness of pyrrophenone in eicosanoid research. Indeed, pyrrophenone efficiently inhibited 5-LO translocation induced by all PMN stimuli tested and its effects were prevented by exogenous AA; in these experiments pyrrophenone turned out to be the key pharmacological tool in defining this novel regulatory mechanism of 5-LO activation.

The present study also demonstrated that the inhibitory effect of pyrrophenone on cPLA$_2\alpha$ was reversible in intact PMN. Indeed, lipid mediator biosynthesis was recovered when pyrrophenone-treated human PMN were washed with autologous plasma before stimulation, in agreement with the initial report of the reversibility of the pyrrophenone-mediated inhibition of AA release in a dilution system using the phosphatidylcholine/Triton X-100 micelle cPLA$_2\alpha$ assay (Ono *et al.,* 2002). Our data, however, emphasized that pyrrophenone is not easily washed out from whole cells, previously exposed to the drug, with buffered salt solutions and that recovery of cPLA$_2\alpha$ activity requires washing in the presence of plasma, enabling the trapping of the lipophilic drug by plasma proteins (albumin in particular).

Finally, the effect of pyrrophenone on PLD activity was also investigated in thapsigargin- and fMLP-activated PMN in order to assess the activity of the drug on this particular PL. PLD is an important enzyme in the regulation of PMN functions implicating vesicular transport such as phagocytosis and degranulation. PLD activity was not inhibited by pyrrophenone in fMLP-activated PMN. In contrast, the data presented herein showed that pyrrophenone inhibited PLD activity in thapsigargin-activated PMN, an effect that could be attributed to inhibition of cPLA$_2\alpha$ and biosynthesis of LTB$_4$, which acts as the direct activator of PLD in these experimental conditions (Grenier *et al.,* 2003). These results demonstrate that pyrrophenone has no direct inhibitory effect on PLD.

In conclusion, the results presented herein demonstrate that pyrrophenone is a very potent and specific inhibitor of cPLA$_2\alpha$ and consequently of the biosynthesis of lipid mediators in human PMN (and whole blood). This com-

pound provides a useful pharmacological tool to investigate the biosynthesis of lipid mediators in whole cells and tissues, and to assess their roles in biological processes.

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Conflict of interest

The authors state no conflict of interest.

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