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Communications to the Editor

Pyrrolidine Inhibitors of Human Cytosolic Phospholipase A₂

Kaoru Seno,* Takayuki Okuno, Koichi Nishi, Yasushi Murakami, Fumihiko Watanabe, Takaharu Matsuura, Masaaki Wada, Yasuhiko Fujii, Masaaki Yamada, Tomoyuki Ogawa, Tetsuo Okada, Hiroshi Hashizume, Makoto Kii, Shin-ichiro Hara, Sanji Hagishita, Shozo Nakamoto, Katsutoshi Yamada, Yukiko Chikazawa, Masahiko Ueno, Isao Teshirogi, Takashi Ono,* and Mitsuaki Ohtani*

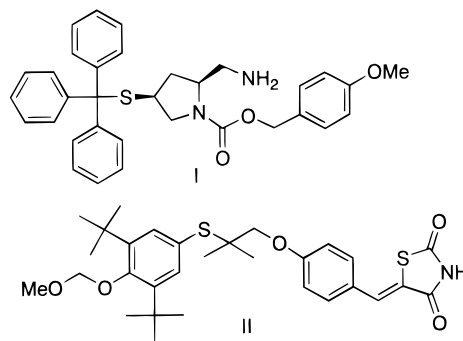
Shionogi Research Laboratories, Shionogi & Company, Ltd.,
12-4, Sagisu 5-chome, Fukushima-ku,
Osaka 553-0002, Japan

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Introduction. Phospholipase A₂ (PLA₂) is a group of lipolytic enzymes that catalyze the hydrolysis of fatty acid ester bonds at the *sn*-2 position of phospholipids. This enzyme is thought to play an important role in the biosynthesis of eicosanoids via the release of arachidonic acid from biomembranes. Another product from biomembranes, a lysophospholipid, is converted to platelet-activating factor (PAF) known as an inflammatory mediator. PLA₂s have been generally classified into secretory PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂), and Ca²⁺-independent PLA₂ (iPLA₂) by their molecular weights, amino acid sequences, and calcium requirements.¹

cPLA₂ comprises three distinct types of enzymes: α , β , and γ .² cPLA₂ α , an 85-kDa protein, contains a calcium-dependent lipid binding domain and a catalytic domain, requires micromolar levels of Ca²⁺ for membrane translocation, and has a specificity for arachidonic acid bound to the *sn*-2 position of phospholipids³ in contrast with sPLA₂ and iPLA₂ which have broad substrate specificities, suggesting that cPLA₂ α is involved in the production of eicosanoids. In fact, mice

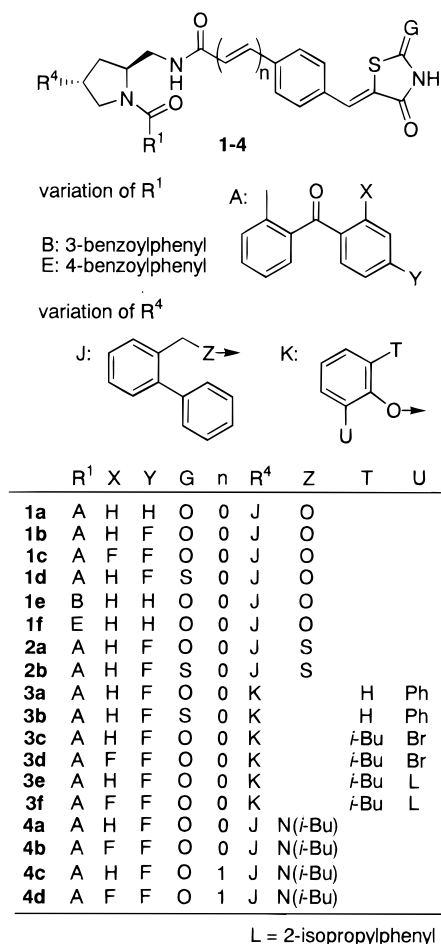
Chart 1



deficient in cPLA₂ α showed a marked decrease in eicosanoid biosynthesis,⁴ allergic symptoms,^{4a} and postischemic brain injury,^{4b} indicating a direct contribution of cPLA₂ α to the production of eicosanoids and the importance of cPLA₂ α in inflammation and reperfusion injury. Therefore, cPLA₂ α inhibitors are very attractive targets as agents to treat inflammatory diseases and stroke. Although some compounds have been reported as cPLA₂ α inhibitors,⁵ none have been worthy of further pharmacological and clinical study in terms of potency, specificity, and material characteristics.⁶ In searching for PLA₂ inhibitors, kinetic analysis alone is not sufficient to determine whether compounds can inhibit PLA₂ activity by affecting the interfacial quality of the substrate or by directly inhibiting the interaction between the substrate and the active site of the enzyme. To screen sPLA₂ inhibitors identified as clinical candidates,⁷ in addition to enzyme assays, a tissue assay system was introduced as a secondary in vitro assay to eliminate the false positive compounds such as those inhibiting the interaction between the substrate interface and enzyme, but not its catalytic site. The most useful were the X-ray cocrystallographic data⁸ of the enzyme and its lead compound which were valuable for improving the true inhibitory activity at the active site. The X-ray crystal structure of cPLA₂ α has recently been solved;⁹ however, the complexity of the enzyme kinetics and the fact that X-ray cocrystallography of cPLA₂ α and

* To whom correspondence should be addressed. Phone: +81-6-6458-5861. Fax: +81-6-6458-0987. E-mail: kaoru.seno@shionogi.co.jp, takashi.ono@shionogi.co.jp, mitsuaki.ohtani@shionogi.co.jp.

Chart 2. Synthesized Compounds



one of its inhibitors has not been successful make it difficult to synthesize more potent and useful cPLA₂α inhibitors.

We searched for nonpeptide and low-molecular-weight inhibitors of cPLA₂α and identified two compounds (**I** and **II**) as lead compounds for cPLA₂α inhibitors from our compound library (Chart 1). These compounds showed cPLA₂α inhibitory activities with IC₅₀ values of 1.5 μM (**I**) and 1.7 μM (**II**) with the enzyme assay. By combining these two compounds and conducting structure–activity relationship studies using both enzyme assay and secondary-cell-based assay established to select the inhibitor specific for arachidonic acid release and penetrating the cell membrane, we found a compound having inhibitory activity about 900-fold more than the lead compounds.

In this communication, we report on these pyrrolidine cPLA₂α inhibitors having structures **1–4** (Chart 2), which possess very potent cPLA₂α inhibitory activity.

Chemistry. We synthesized four types of pyrrolidine derivatives (**1–4**) using known synthetic methods. Compounds (**1**) having substituted benzyloxy groups at the 4-position of pyrrolidine were synthesized from *trans*-N-Boc-4-hydroxy-L-proline methyl ester.¹⁰ Pyrrolidine derivatives (**2**) having substituted benzylthio groups were synthesized from *cis*-N-Boc-4-hydroxy-L-proline methyl ester.¹¹ 4-Aryloxy-pyrrolidine derivatives (**3**) and *N,N*-disubstituted 4-aminopyrrolidines (**4**) were also synthesized from the same *cis*-N-Boc-4-hydroxy-L-proline methyl ester.

Table 1. Inhibitory Activity of Pyrrolidine Derivatives against Human cPLA₂ and Effect on Production of Arachidonic Acid from THP-1 Cells Stimulated with A23187^a

compd	IC ₅₀ (μM)	
	PC/DOG ^b	THP-1 ^c
1a	0.165 ± 0.020	0.67 ± 0.07
1b	0.078 ± 0.009	0.32 ± 0.03
1c	0.049 ± 0.018	0.23 ± 0.03
1d	0.090 ± 0.004	0.70 ± 0.23
1e	>10	5.9 ± 0.5
1f	>10	5.5 ± 3.2
2a	0.034 ± 0.007	0.25 ± 0.01
2b	0.072 ± 0.033	0.40 ± 0.02
3a	0.069 ± 0.016	0.31 ± 0.04
3b	0.083 ± 0.026	0.66 ± 0.24
3c	0.038 ± 0.002	0.30 ± 0.03
3d	0.022 ± 0.003	0.22 ± 0.02
3e	0.0053 ± 0.0018	0.092 ± 0.011
3f	0.0021 ± 0.0004	0.055 ± 0.011
4a	0.021 ± 0.001	0.18 ± 0.06
4b	0.0062 ± 0.0026	0.047 ± 0.008
4c	0.0031 ± 0.0006	0.052 ± 0.009
4d	0.0018 ± 0.0005	0.022 ± 0.001
AACOCF ₃	0.42 ± 0.28	86 ± 15

^a Data are expressed as the mean ± SD of three independent determinations. ^b Enzyme assay (PC/DOG assay): the PLA₂ activity was measured using the liposome containing 2.5 μM 1-palmitoyl-2-[¹⁴C]arachidonoyl-*sn*-glycero-3-phosphocholine (50 mCi/mmol) and 1.25 μM *sn*-1,2-dioleoylglycerol according to the method described previously.^{3c} ^c Cellular assay: human THP-1 cells were grown in RPMI 1640 containing 10% fetal calf serum and pre-treated with 1.3% dimethyl sulfoxide for 2 days. Following washing with phosphate-buffered saline (PBS), the cells were suspended in Hanks' balanced salt saline (pH 7.6) containing 0.1% bovine serum albumin. The cell suspension was preincubated with inhibitor at 37 °C for 15 min and then incubated with 3 μM A23187 (calcium ionophore) at 37 °C for 20 min. The reaction was terminated using Dole's solution (isopropyl alcohol:heptane:2 N sulfuric acid = 40:10:1). Arachidonic acid was labeled with 9-anthryldiazomethane and quantified by high-performance liquid chromatography.¹⁴

Biological Results and Discussion. Although the X-ray crystal structure of human cPLA₂α has recently been solved,⁹ there were no available data on the three-dimensional structure of cPLA₂α when we started this structure–activity relationship study. Since it was reported at that time that Ser₂₂₈ was the most important amino acid residue in the active site of cPLA₂α and both Arg₂₀₀ and Asp₅₄₉ were essential for the activity from the result of site-directed mutagenesis,^{12,13} we expected that Arg₂₀₀ could be trapped by the thiazolidinedione part with the acid–base interaction and Asp₅₄₉ might exist near this active center of the enzyme. Furthermore, we supposed that Ser₂₂₈ would be trapped by a functional group of the inhibitor which could interact with the hydroxyl group of the serine residue. We therefore focused our synthetic efforts on searching for groups which might interact with the hydroxyl group of Ser₂₂₈ and found the carbonyl group of the *o*-(benzoyl)-benzoyl group of compound **1a** to be suitable for this function. To confirm this, we synthesized *meta*- and *para*-substituted isomers of compound **1a** (see compounds **1e,f** of Table 1). The inhibitory activity of cPLA₂α was lost in both of these isomers. Introduction of fluorine atom(s) at the *para* and/or *ortho* position of the terminal benzoyl group of **1a** led to increased inhibitory activity (**1b–d** in Table 1). Replacement of the oxygen atom of the ether part at the 4-position of pyrrolidine to a sulfur atom (**2a,b**) led to increased inhibition, and substitution of thiazolidinedione to

rolidine derivatives inhibit PGE₂ and LTC₄ production by inhibiting cPLA₂α without any effect on enzymes located downstream of the arachidonic acid cascade, i.e., cyclooxygenase and 5-lipoxygenase. The effect of pyrrolidine derivatives on eicosanoid synthesis observed in this study was quite consistent with the findings that the syntheses of PGE₂ and cysteinyl leukotrienes were significantly decreased in stimulated peritoneal macrophages from cPLA₂α-deficient mice,⁴ demonstrating that cPLA₂α is important for eicosanoid biosynthesis and that the pyrrolidine derivatives are of great value for elucidating the physiological role of cPLA₂α as well as the role of cPLA₂α in the process of eicosanoid biosynthesis. We are now examining the pharmacological effect of the pyrrolidine derivatives.

We have described here the discovery of potent inhibitors of cPLA₂α having 1,2,4-trisubstituted pyrrolidine frameworks. Their structural features include the *ortho*-substituted (benzoyl)benzoyl group and 2,4-dioxo-(or 2-oxo-4-thioxo)thiazolidin-5-ylidenemethylphenyl group. Our newly synthesized pyrrolidine compounds are extremely potent cPLA₂α inhibitors compared to others reported to date. Detailed data from the syntheses and biological evaluations will be published in full papers soon.

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Supporting Information Available: Synthetic schemes and physical data of final compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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