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Communications to the Editor

Pyrrolidine Inhibitors of Human Cytosolic Phospholipase A_2

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Introduction. Phospholipase A_2 (PLA_2) 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. PLA_2s have been generally classified into secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), and Ca^{2+}-independent PLA_2 ($iPLA_2$) by their molecular weights, amino acid sequences, and calcium requirements.[1]

$cPLA_2$ comprises three distinct types of enzymes: α, *β*, and *γ*.[2] $cPLA_2\alpha$, an 85-kDa protein, contains a calcium-dependent lipid binding domain and a catalytic domain, requires micromolar levels of Ca^{2+} for membrane translocation, and has a specificity for arachidonic acid bound to the *sn*-2 position of phospholipids[3] in contrast with $sPLA_2$ and $iPLA_2$ which have broad substrate specificities, suggesting that $cPLA_2\alpha$ is involved in the production of eicosanoids. In fact, mice deficient in $cPLA_2\alpha$ showed a marked decrease in eicosanoid biosynthesis,[4] allergic symptoms,[4a] and postischemic brain injury,[4b] indicating a direct contribution of $cPLA_2\alpha$ to the production of eicosanoids and the importance of $cPLA_2\alpha$ in inflammation and reperfusion injury. Therefore, $cPLA_2\alpha$ inhibitors are very attractive targets as agents to treat inflammatory diseases and stroke. Although some compounds have been reported as $cPLA_2\alpha$ inhibitors,[5] none have been worthy of further pharmacological and clinical study in terms of potency, specificity, and material characteristics.[6] In searching for PLA_2 inhibitors, kinetic analysis alone is not sufficient to determine whether compounds can inhibit PLA_2 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 $sPLA_2$ 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 data[8] 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 $cPLA_2\alpha$ has recently been solved;[9] however, the complexity of the enzyme kinetics and the fact that X-ray cocrystallography of $cPLA_2\alpha$ and

Chart 1

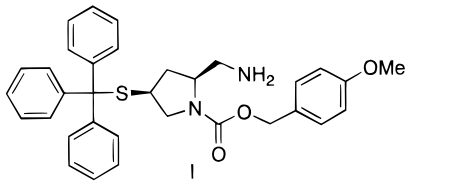

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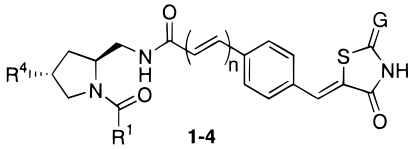
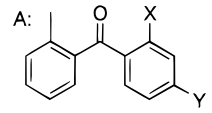
Chart 2. Synthesized Compounds

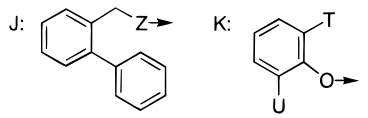


variation of R^1



B: 3-benzoylphenyl
E: 4-benzoylphenyl

variation of R^4



	R^1	X	Y	G	n	R^4	Z	T	U
1a	A	H	H	O	0	J	O		
1b	A	H	F	O	0	J	O		
1c	A	F	F	O	0	J	O		
1d	A	H	F	S	0	J	O		
1e	B	H	H	O	0	J	O		
1f	E	H	H	O	0	J	O		
2a	A	H	F	O	0	J	S		
2b	A	H	F	S	0	J	S		
3a	A	H	F	O	0	K		H	Ph
3b	A	H	F	S	0	K		H	Ph
3c	A	H	F	O	0	K		*i*-Bu	Br
3d	A	F	F	O	0	K		*i*-Bu	Br
3e	A	H	F	O	0	K		*i*-Bu	L
3f	A	F	F	O	0	K		*i*-Bu	L
4a	A	H	F	O	0	J	N(*i*-Bu)		
4b	A	F	F	O	0	J	N(*i*-Bu)		
4c	A	H	F	O	1	J	N(*i*-Bu)		
4d	A	F	F	O	1	J	N(*i*-Bu)		

L = 2-isopropylphenyl

one of its inhibitors has not been successful make it difficult to synthesize more potent and useful cPLA$_2\alpha$ inhibitors.

We searched for nonpeptide and low-molecular-weight inhibitors of cPLA$_2\alpha$ and identified two compounds (**I** and **II**) as lead compounds for cPLA$_2\alpha$ inhibitors from our compound library (Chart 1). These compounds showed cPLA$_2\alpha$ inhibitory activities with IC_{50} 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 cPLA$_2\alpha$ inhibitors having structures **1**−**4** (Chart 2), which possess very potent cPLA$_2\alpha$ 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-Aryloxypyrrolidine derivatives (**3**) and *N*,*N*-disubstituted 4-aminopyrrolidines (**4**) were also synthesized from the same *cis*-*N*-Boc-4-hydroxy-L-proline methyl ester.

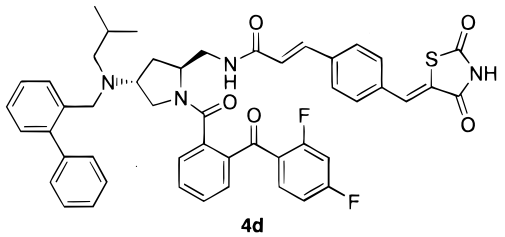
Table 1. Inhibitory Activity of Pyrrolidine Derivatives against Human cPLA$_2$ and Effect on Production of Arachidonic Acid from THP-1 Cells Stimulated with A23187[a]

	IC_{50} (μM)	
compd	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 PLA$_2$ activity was measured using the liposome containing 2.5 μM 1-palmitoyl-2-[^{14}C]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 pretreated 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 cPLA$_2\alpha$ has recently been solved,[9] there were no available data on the three-dimensional structure of cPLA$_2\alpha$ when we started this structure−activity relationship study. Since it was reported at that time that Ser_{228} was the most important amino acid residue in the active site of cPLA$_2\alpha$ and both Arg_{200} and Asp_{549} were essential for the activity from the result of site-directed mutagenesis,[12,13] we expected that Arg_{200} could be trapped by the thiazolidinedione part with the acid−base interaction and Asp_{549} might exist near this active center of the enzyme. Furthermore, we supposed that Ser_{228} 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 Ser_{228} 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 cPLA$_2\alpha$ 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

Chart 3



4d

Table 2. Effect of Pyrrolidine Derivatives on Production of Eicosanoids by Cultured THP-1 Cells Stimulated with A23187[a]

	THP-1 cellular assay IC_{50} (μM)		
compd	AA[b]	PGE_2[c]	LTC_4[c]
1a	0.67 ± 0.07	0.41 ± 0.07	0.37 ± 0.07
1b	0.32 ± 0.03	0.26 ± 0.10	0.26 ± 0.06
3a	0.31 ± 0.04	0.21 ± 0.11	0.14 ± 0.06
4d	0.022 ± 0.001	0.031 ± 0.043	0.013 ± 0.001
indomethacin	>10	0.0036 ± 0.0043	>10

[a] Data are expressed as the mean ± SD of three independent determinations. [b] See footnote c of Table 1. [c] After incubation with A23187 at 37 °C for 20 min as shown in footnote c of Table 1, the reaction was stopped by cooling on ice, and LTC_4 and PGE_2 levels in the supernatant were measured by radio immunoassay and enzyme immunoassay.

rhodanine (**1d, 2b, 3b**) led to a slight decrease of the inhibition (Table 1). The inhibitory activity was not affected by the substituents at the 4-position being substituted benzyl groups (**1b,d**) or substituted aryl groups (**3a,b**). In addition to the hydrophobic interaction between the lipophilic substituent at the 4-position of pyrrolidine and the lipophilic part of the enzyme, the sterically crowded environment also seemed to be needed (**3c**−**f**). Introduction of a voluminous tertiary amino group to the 4-position offered a new way of crowding the environment at this position without loss of lipophilicity to increase the activity (**4a,b**). Also, elongation of the substituent at the 2-position with insertion of a double bond between the amide carbonyl group and the phenyl group led to a great increase in the activity (**4c,d**). The inhibition of arachidonic acid release in human monocytic leukemia cells (THP-1 cells) stimulated with A23187 was enhanced in parallel with the increase of inhibitory activity against $cPLA_2\alpha$ by the chemical modification as shown in Table 1. The most active compound **4d** (Chart 3) among those listed in Table 1 inhibited the $cPLA_2\alpha$ enzyme with an IC_{50} value of 1.8 nM and arachidonic acid release in A23187-stimulated THP-1 cells with an IC_{50} value of 22 nM and was more active by about 230-fold in the enzyme assay and about 3900-fold in the cellular assay than arachidonyl trifluoromethyl ketone ($AACOCF_3$), which is generally known as a potent $cPLA_2\alpha$ inhibitor.[5a,15] Compound **4d** at 1 μM almost completely inhibited arachidonic acid release from THP-1 cells.

In addition to the arachidonic acid release, we determined whether the pyrrolidine derivatives could inhibit eicosanoid production in A23187-stimulated THP-1 cells (Table 2). Compounds **1a,b, 3a,** and **4d** inhibited the formation of PGE_2 and LTC_4 with IC_{50} values comparable to those of the arachidonic acid release. Because none of these compounds induced the release of lactate dehydrogenase as an indicator of cell viability even at the concentration of 100 μM, the observed inhibitions were not due to a cytotoxic effect.

To determine whether the observed inhibition of the pyrrolidine derivatives for eicosanoid production involves action against a step-down arachidonic acid release, the effect of compound **4d** on the production of PGE_2 and LTC_4 by direct addition of arachidonic acid to THP-1 cells was examined (Table 3). On addition of arachidonic acid to the cells, the standard cyclooxygenase inhibitor indomethacin and the standard 5-lipoxygenase inhibitor 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA-861)[16] inhibited the production of PGE_2 and LTC_4, respectively, as in A23187 stimulation, whereas compound **4d** did not inhibit either production. Therefore, the inhibition of PGE_2 and LTC_4 biosynthesis by compound **4d** was apparently due to inhibition of only the step of arachidonic acid release. In contrast to the pyrrolidine derivatives, $AACOCF_3$ inhibited not only the step of arachidonic acid release but also the thromboxane biosynthetic step located further downstream, probably because its fundamental structure is arachidonic acid.[15a]

Recently, $cPLA_2\beta$ and $cPLA_2\gamma$ have been identified as new members of $cPLA_2$ and characterized.[2] The molecular weights of $cPLA_2\beta$ and $cPLA_2\gamma$ were about 114 and 61 kDa, respectively, and the sequences of both PLA_2s revealed about 30% identity with that of $cPLA_2\alpha$, but the physiological roles of $cPLA_2\beta$ and $cPLA_2\gamma$ have not yet been clarified. Although the inhibitory activity of pyrrolidine derivatives for $cPLA_2\beta$ and $cPLA_2\gamma$ have not been examined, it is possible that these compounds also inhibit $cPLA_2\beta$ and $cPLA_2\gamma$, because amino acids essential for catalysis were strictly conserved in $cPLA_2\alpha$, $cPLA_2\beta$, and $cPLA_2\gamma$.[2]

Taken together, these results indicate that the pyr-

Table 3. Effect of Various Inhibitors on Production of PGE_2 and LTC_4 by Cultured THP-1 Cells Stimulated with Arachidonic Acid[a]

	THP-1 cellular assay IC_{50} (μM)				
	A23187			AA	
compd	AA[b]	PGE_2[c]	LTC_4[c]	PGE_2[d]	LTC_4[e]
4d	0.022 ± 0.001	0.031 ± 0.043	0.013 ± 0.001	>10	>10
indomethacin	>10	0.0036 ± 0.0043	>10	0.013 ± 0.007	>10
AA861	>10	nd[f]	0.0047 ± 0.0010	nd[f]	0.068 ± 0.026

[a] Data are expressed as the mean ± SD of three independent determinations. [b] See footnote c of Table 1. [c] See footnote c of Table 2. [d] DMSO differentiated THP-1 cells, suspended in Hanks' balanced salt saline (pH 7.6) containing 0.1% bovine serum albumin, were preincubated with the inhibitor at 37 °C for 15 min, 50 μM arachidonic acid was added, and the reaction mixture was incubated at 37 °C for 20 min. The reaction was stopped by cooling on ice, and PGE_2 levels in the supernatant were measured by radio immunoassay. [e] DMSO differentiated THP-1 cells, suspended in Hanks' balanced salt saline (pH 7.6) containing 0.1% bovine serum albumin, were preincubated with 200 μM aspirin at 37 °C for 60 min; the cells were washed with PBS. Following preincubation with the inhibitors at 37 °C for 15 min, 250 μM arachidonic acid was added and the reaction mixture was incubated at 37 °C for 20 min. The reaction was stopped by cooling on ice, and LTC_4 levels in the supernatant were measured by enzyme immunoassay. [f] nd, not done.

rolidine derivatives inhibit PGE_2 and LTC_4 production by inhibiting $cPLA_2\alpha$ 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 PGE_2 and cysteinyl leukotrienes were significantly decreased in stimulated peritoneal macrophages from $cPLA_2\alpha$-deficient mice,[4] demonstrating that $cPLA_2\alpha$ is important for eicosanoid biosynthesis and that the pyrrolidine derivatives are of great value for elucidating the physiological role of $cPLA_2\alpha$ as well as the role of $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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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