Communications to the Editor

N-(Carboxymethyl)-N-[3,5-bis(decyloxy)-phenyl]glycine (Ro 23-9358): A Potent Inhibitor of Secretory Phospholipases A_2 with Antiinflammatory Activity

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Phospholipases A_2 (PLA_2's) are a class of enzymes which catalyze the hydrolysis of membrane phospholipids at the *sn*-2 position to release fatty acids and lysophospholipids. When the fatty acid is arachidonic acid, further metabolism leads to proinflammatory mediators such as prostaglandins, leukotrienes and platelet activating factor (PAF). The low molecular weight (14 kDa), Ca^{2+}-dependent, extracellular PLA_2's found in mammalian pancreas, several snake venoms, human platelets, human placenta, and rheumatoid synovial fluid have been widely investigated.[1] Although the pancreatic enzymes are involved in hydrolysis of dietary phospholipids, the PLA_2's secreted by other mammalian tissues may be involved in various inflammatory conditions.[2] The human nonpancreatic secretory PLA_2's (hnps-PLA_2) found in platelets, synovial fluid, and placenta have been shown to be identical.[3-5] Structures determined by X-ray crystallography of hnps-PLA_2,[6] porcine pancreatic PLA_2 (pp-PLA_2),[7] and bovine pancreatic PLA_2[8] show that the structural features of the active site are similar. The catalytic mechanism is most likely identical for these enzymes. Much less studied is a high molecular weight (85 kDa) cytosolic PLA_2 (cPLA_2), such as that isolated from the human monocytic cell line U937,[9] which differs from the secretory PLA_2's by its preference for 2-arachidonyl phospholipids and by activation in response to the low Ca^{2+} levels found in stimulated cells. There is evidence to support the contention that both the secretory and the cytosolic PLA_2's are involved in various inflammatory conditions[10,11] and therefore inhibitors of either of these enzymes might become useful therapeutics. Despite considerable effort to evaluate inhibitors of secretory PLA_2's in recent years, no inhibitor with clinical potential has emerged.[12]

Earlier workers in this field have described the use of X-ray structural data and molecular modeling to design PLA_2 inhibitors.[13-15] We began our PLA_2 inhibitor program by docking a representative phospholipid substrate into the active site of bovine pancreatic PLA_2 based on the published mechanism and crystallographic structure of the uncomplexed enzyme.[8] The Roche Interactive Molecular Graphics program[16] was used for visual docking and subsequent optimization based on target atoms, intermolecular steric interactions, and intramolecular strain energy. The docked substrate had the carbonyl oxygen of the 2-acyl chain and a phosphate oxygen as ligands of the essential Ca^{2+} ion (displacing two water molecules in the crystallographic structure), and had the two fatty acyl chains coiled into the large hydrophobic cavity of the active site. One of our strategies for inhibitor design was to replace the glycerol backbone of the substrate with an aromatic ring which had substituents to mimic the three substrate chains. Docking optimizations with a variety of benzene derivatives indicated that the 1,3,5-substitution pattern best fit the active site. One such compound, 3,5-bis(decyloxy)benzoic acid (**3**), prepared early in our program, was found to inhibit human synovial fluid PLA_2 (HSF-PLA_2) with an IC_{50} of 3 μM. To increase potency, we performed further docking optimizations with putative inhibitors which had superior Ca^{2+}-binding ligands replacing the carboxylate group of **3**. Selection of the potentially tridentate Ca^{2+}-binding ligand, the iminodiacetic acid group, led to *N*-(carboxymethyl)-*N*-[3,5-bis(decyloxy)phenyl]glycine (**1**, Ro 23-9358), which is among the most potent inhibitors of secretory PLA_2's reported to date. Figure 1 shows **1** modeled into the active site of bovine PLA_2.[8] Each carboxylate of the inhibitor has one oxygen atom as a calcium ligand. The benzene ring and the alkyl chains occupy the large hydrophobic substrate binding cavity. The chain on the lower left is coiled in the cavity, while the upper chain occupies a narrow crevice and then projects out from the enzyme.

1 was synthesized as shown in Scheme I. Alkylation of methyl 3,5-dihydroxybenzoate with 1-bromodecane followed by basic hydroylsis provided **3**. The benzoic acid **3** was smoothly converted via the acid azide and the benzyl carbamate to the corresponding aniline **4** in 77% overall yield. Alkylation of **4** with excess benzyl bromoacetate in the presence of 1,8-bis(dimethylamino)naphthalene (Proton Sponge) in refluxing acetonitrile gave the benzyl ester precursor of **1** in 45% yield. Finally, catalytic hydrogenolysis readily furnished **1** in 75% yield as a colorless solid, mp 110–114 °C.

Compound **1** was found to be a potent inhibitor of crude HSF-PLA_2 exhibiting an IC_{50} of 0.23 μM.[17] **1** also inhibited purified recombinant human placental PLA_2 (r-hpPLA_2) with an IC_{50} of 0.087 μM.[18] Both assays utilized [^{14}C]oleate-labeled *Escherichia coli* as the substrate in the presence of 2 mM Ca^{2+}.

In contrast, **1** either stimulated or weakly inhibited the cPLA_2 from the human monocytic tumor cell line U937, depending on the concentration of free Ca^{2+} in the assay.[19] At high Ca^{2+} (2 mM), **1** (50 μM) caused a 52% stimulation of cPLA_2 activity, while at low free Ca^{2+} (0.8 μM) **1** gave a weak inhibition (IC_{50} = 48 μM) of this activity. This suggests that, at low Ca^{2+}, **1** may inhibit cPLA_2 by acting as a chelator to deprive the enzyme of the Ca^{2+} required to maintain activity. In the absence of inhibitor, cPLA_2 activity was stimulated by 0.1–1 μM Ca^{2+} and further stimulated by 1–20 mM Ca^{2+}.

Many of the previously reported inhibitors of secretory PLA_2's do not bind to the enzyme active site but act by disrupting the membrane interface. Analysis of PLA_2 inhibitors in the "scooting mode" avoids apparent inhi-

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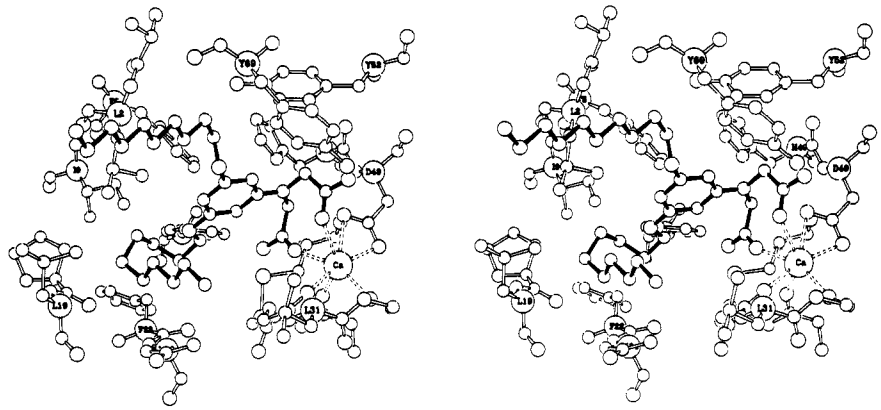
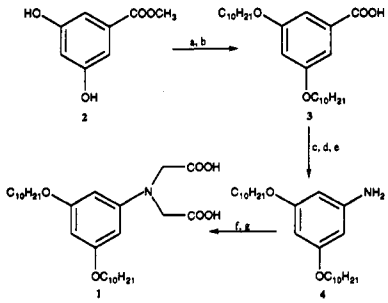


Figure 1. Stereoview of 1 modeled into the active site of bovine PLA_2.[8] Protein residues within 5 Å of the inhibitor are shown. The inhibitor is shown with filled bonds, the protein with open bonds, and calcium ligands with dashed bonds. The calcium ion and selected protein residues are labeled.

Scheme I[a]



[a] Reagents: (a) $C_{10}H_{21}Br$, K_2CO_3; (b) NaOH, CH_3OH; (c) DPPA; (d) BnOH, heat; (e) H_2, Pd; (f) $BrCH_2COOBn$, Proton Sponge; (g) H_2, Pd.

bition by these nonspecific effects at the interface.[20] The scooting assay monitors the kinetics of interfacial hydrolysis of phospholipids by s-PLA_2 under conditions where the enzyme does not leave the strongly anionic phospholipid vesicle interface. Inhibition of ppPLA_2 and r-hpPLA_2 by 1 in vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol (DMPM) in the scooting assay[21] gave $X_I(50)$ (mole fraction of 1 in the vesicle which gives 50% inhibition) values of 0.00033 and 0.003 mole fraction, respectively.[22] One of the most potent competitive inhibitors of which we are aware, 1-octyl-2-(heptylphosphonyl)phosphatidylethanolamine (MG14)[23] has been reported to exhibit $X_I(50)$ values of 0.0034 mole fraction against ppPLA_2[24] and 0.052 mole fraction against recombinant hnps-PLA_2.[25] Our data on 1 in the scooting assay suggests that it acts as a competitive inhibitor of these two 14 kDa PLA_2's.

The rat established adjuvant arthritis model,[26] developed to detect nonsteroidal antiinflammatory drugs which inhibit cyclooxygenase, is thought to involve activation of a PLA_2 and subsequent eicosanoid production. When rats with adjuvant-induced arthritis were treated once daily for 7 days with a 30 mg/kg intraperitoneal dose of 1, paw swelling decreased relative to control animals.[27] The paw volume of vehicle-treated animals increased by 0.96 ± 0.14 mL while the paw volume of animals treated with 1 decreased by 1.00 ± 0.24 mL during the treatment period. The effect of 1 in this model is probably due to PLA_2 inhibition since it did not inhibit 5-lipoxygenase from RBL-1 cells or ram seminal vesicle cyclooxygenase when tested *in vitro* at 50 μM.

Paw edema models which use PLA_2 to initiate an inflammatory response have been reported using mice[28] and rats.[29] Compound 1 when tested intraperitoneally at 30 mg/kg inhibited the paw swelling in rats induced by *Naja naja* venom PLA_2 by 41%.[30]

In conclusion, we have reported a potent, structurally novel, selective inhibitor of secretory PLA_2's which also exhibits inhibitory activity in two animal models of inflammation. Forthcoming publications will describe this new series in greater detail.

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Supplementary Material Available: Detailed experimental procedures for the preparation of 1 (3 pages). Ordering information is given on any current masthead page.

References

(1) Dennis, E. A.; Rhee, S. G.; Billah, M. M.; Hannun, Y. A. Role of Phospholipases in Generating Lipid Second Messengers in Signal Transduction. *FASEB J.* **1991**, *5*, 2068–2077.
(2) Chang, J.; Musser, J. H.; McGregor, J. M. Phospholipase A_2: Function and Pharmacological Regulation. *Biochem. Pharmacol.* **1987**, *36*, 2429–2436.
(3) Kramer, R. M.; Hession, C.; Johansen, B.; Hayes, G.; McGray, P.; Chow, E. P.; Tizard, R.; Pepinsky, R. B. Structure and Properties of a Human Non-pancreatic Phospholipase A_2. *J. Biol. Chem.* **1989**, *264*, 5768–5775.
(4) Crowl, R.; Stoner, C.; Stoller, T.; Pan, Y.-C.; Conroy, R. Isolation and Characterization of cDNA Clones from Human Placenta Coding for Phospholipase A_2. *Adv. Exp. Med. Biol.* **1990**, *279*, 173–184.
(5) Levin, W.; Daniel, R. F.; Stoner, C. R.; Stoller, T. J.; Swanson, J. A. W.; Angelillo, Y. M.; Familetti, P. C.; Crowl, R. M. Purification of Recombinant Human Secretory Phospholipase A_2 (Group II) Produced in Long-Term Immobilized Cell Culture. *Protein Expression Purification* **1992**, 3, 27–35.
(6) Scott, D. L.; White, S. P.; Browning, J. L.; Rosa, J. J.; Gelb, M. H.; Sigler, P. B. Structures of Free and Inhibited Human Secretory Phospholipase A_2 from Inflammatory Exudate. *Science* **1991**, *254*, 1007–1010.
(7) Thunnissen, M. M. G. M.; Ab, E.; Kalk, K. H.; Drenth, J.; Dijkstra, B. W.; Kuipers, O. P.; Dijkman, R.; de Haas, G. H.; Verheij, H. M. X-ray Structure of Phospholipase A_2 Complexed with a Substrate-Derived Inhibitor. *Nature* **1990**, *347*, 689–691.
(8) Slotboom, A. J.; Verheij, H. M.; De Haas, G. H. On the Mechanism of Phospholipase A_2. In *Phospholipids*; Hawthorne, J. N.; Ansell, G. B., Eds.; Elsevier Biomedical Press: Amsterdam, 1982; pp 359–434.
(9) Clark, J. D.; Milona, N.; Knopf, J. L. Purification of a 110-Kilodalton Cytosolic Phospholipase A_2 from the Human Monocytic Cell Line U937. *Proc. Natl. Acad. Sci., U.S.A.* **1990**, *87*, 7708–7712.
(10) Hulkower, K. I.; Hope, W. C.; Chen, T.; Anderson, C. M.; Coffey, J. W.; Morgan, D. W. Interleukin-1β Stimulates Cytosolic Phospholipase A_2 in Rheumatoid Synovial Fibroblasts. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 712-718.

(11) Kramer, R. M.; Johansen, B.; Hession, C.; Pepinsky, R. B. Structure and Properties of a Secretable Phospholipase A_2 from Human Platelets. In *Advances in Experimental Medicine and Biology*; Wong, P. Y-K.; Dennis, E. A., Eds.; Plenum Press: New York, 1990; pp 35–53.
(12) Mobilio, D.; Marshall, L. A. Recent Advances in the Design and Evaluation of Inhibitors of Phospholipase A_2. In *Annual Reports in Medicinal Chemistry*; Allen, R. C. Ed.; Academic Press, Inc.: San Diego; 1989; *24*; pp 157–166.
(13) Davis, P. D.; Nixon, J. S.; Wilkinson S. E.; Russell, M. G. N. Inhibition of Phospholipase A_2 by Some Long Chain Alkylamines. *Biochem. Soc. Trans.* 1988, *16*, 816–817.
(14) Campbell, M. M.; Long-Fox, J.; Osguthorpe, D. J.; Sainsbury, M.; Sessions. R. B. Inhibition of Phospholipase A_2: A Molecular Recognition Study. *J. Chem. Soc. Chem. Commun.* 1988, 1560–1562.
(15) Bennion, C.; Connolly, S.; Gensmantel, N. P.; Hallam, C.; Jackson, C. G.; Primrose, W. U.; Roberts, G. C. K.; Robinson, D. H.; Slaich, P. K. Design and Synthesis of Some Substrate Analogue Inhibitors of Phospholipase A_2 and Investigations by NMR and Molecular Modeling into the Binding Interactions in the Enzyme-Inhibitor Complex. *J. Med. Chem.* 1992, *35*, 2939–2951.
(16) Mueller, K.; Ammann, H. J.; Doran, D. M.; Gerber, P.; Schrepfer, G. Aspects and Applications of Computer-assisted Molecular Modeling to Drug Design (CAMMDD). In *Innovative Approaches in Drug Research*; Harms A. F., Ed.; Elsevier: Amsterdam, 1986; pp 125–134.
(17) The assay for HSF-PLA_2 monitored the liberation of [1-^{14}C]oleic acid from the *sn*-2 position of membrane-associated phospholipids of [1-^{14}C]oleate-labeled, autoclaved *E. coli* (specific activity of 0.484 μCi/μmol of phospholipid phosphorus) as described: Hope, W. C.; Patel, B. J.; Fiedler-Nagy, C.; Wittreich, B. H. Retinoids Inhibit Phospholipase A_2 in Human Synovial Fluid and Arachidonic Acid Release from Rat Peritoneal Macrophages. *Inflammation* 1990, *14*, 543–559. The substrate was present in excess at a concentration of 20 000 dpm/mL, which was equivalent to 18.2 μM of cell membrane phospholipid phosphorous and 2×10^9 *E. coli*/mL. The enzyme source was cell-depleted human rheumatoid arthritic synovial fluid (HSF). The final concentration in the assay mixture was 0.15% HSF (v/v), which was equivalent to 66 μg protein/mL. The IC_{50} for 1 (0.23 μM) was the mean of three experiments (0.22, 0.23, and 0.24 μM) and was determined from five to six concentration points (two determinations of percent inhibition per point) at half-log intervals. IC_{50} represents the concentration of 1 which gives 50% inhibition of PLA_2 activity. Under the assay conditions selected, the hydrolysis rate was linear with respect to protein concentration and reaction time.
(18) The assay for r-hpPLA_2 was the same as that for HSF-PLA_2 with the following modifications. The final concentration of r-hpPLA_2 was 5 ng/mL (0.36 nM) and the reaction time was reduced from 30 to 15 min to maintain linearity. In order to optimize activity, the sodium ion concentration was increased from 150 to 250 mM. The purified r-hpPLA_2 used was prepared as described.[5] The IC_{50} for 1 (0.087 μM) was the mean of two experiments (0.084 and 0.090 μM).
(19) The assay for cPLA_2 activity in a 100000*g* supernatant of U937 cells lysed in the presence of 2 mM EGTA by nitrogen cavitation was a modification of the method of Clark et al.[9] It measures the release of [1-^{14}C]-arachidonic acid ([^{14}C]AA) from L-α-1-palmitoyl-2-([1-^{14}C]arachidonyl)phosphatidylcholine ([^{14}C]PC, adjusted to a specific activity of 10 μCi/μmol with nonradioactive PC). The assay was conducted using 50 μM [^{14}C]PC substrate (120 000 dpm = 5 nmol PC in a final assay volume of 100 μL) in mixed micelles with 100 μM Triton X-100. The mixture also contained 50 mM sodium HEPES buffer (pH 7.3), 2 mM net free Ca^{2+}, 125 mM NaCl, and 1% (v/v) DMSO used to dissolve the inhibitor. After adding the inhibitor, the reaction was initiated by the addition of enzyme (final concentration of 0.5 mg protein/mL of 100000*g* supernatant of lysed cells) and duplicate samples of the mixture were incubated with shaking for 30 min at 37 °C. Total lipids were extracted from the reaction mixture and 10 μg of nonradioactive AA was added to the extract as a carrier. Enzymatically-released [^{14}C]AA was separated from unreacted [^{14}C]PC by thin layer chromatography and the radioactivities of the AA and PC zones were quantitated by liquid scintillation counting.[17]
(20) Gelb, M. H.; Jain, M. K.; Berg, O. Interfacial Enzymology of Phospholipase A_2. *Bioorg. Med. Chem. Let.* 1992, *2*, 1335–1342.
(21) Jain, M. K.; Yuan, W.; Gelb, M. H. Competitive Inhibition of Phospholipase A_2 in Vesicles. *Biochemistry* 1989, *28*, 4135–4139.
(22) The scooting mode kinetic assay described[21] was utilized. Measurements were conducted using the pH-stat titration method at 21 °C, pH 8.0, 1 mM NaCl, 2.6 mM $CaCl_2$, 5 μg/mL polymyxin B sulfate, and 0.6 mg of DMPM. The PLA_2 inhibitory potencies are expressed as $X_I(50)$'s, which represent the mole fraction concentration of inhibitor that gives 50% inhibition of enzymatic activity. Mole fraction was calculated as total moles of inhibitor divided by total moles of lipid in the vesicle. The reported $X_I(50)$ for MG14[24] was calculated on the basis of total moles of lipid in the outer leaflet of the vesicle (this value would be less, perhaps by a factor of 2/3, if calculated in the former manner).
(23) Yuan, W.; Quinn, D. M.; Sigler, P. B.; Gelb, M. H. Kinetics and Inhibition Studies of Phospholipase A_2 with Short-Chain Substrates and Inhibitors. *Biochemistry* 1990, *29*, 6082–6094.
(24) Jain, M. K.; Tao, W.; Rogers, J.; Arenson, C.; Eibl, H.; Yu, B-Z. Active-Site Directed Specific Competitive Inhibitors of Phospholipase A_2: Novel Transition-State Analogues. *Biochemistry* 1991, *30*, 10256–10268.
(25) Bayburt, Y.; Yu, B-Z.; Lin, H-K.; Browning, J.; Jain, M. K.; Gelb, M. H. Human Nonpancreatic Secreted Phospholipase A_2: Interfacial Parameters, Substrate Specificities and Competitive Inhibitors. *Biochemistry* 1993, *32*, 573–582.
(26) Ward, J. R.; Cloud, R. S. Comparative Effect of Antirheumatic Drugs on Adjuvant-Induced Polyarthritis in Rats. *J. Pharmacol. Exp. Ther.* 1966, *152*, 116–121.
(27) Adjuvant (0.1 mg *Mycobacterium butyricum* in 0.1 mL of mineral oil) was injected into the base of the tail of male Lewis rats and the resulting inflammation was allowed to develop untreated for 21 days. Vehicle (DMSO) or 1 in DMSO was then administered daily for 7 days by the intraperitoneal route. The change in paw volume was the volume on day 28 minus the volume on day 21. Mean values ± SE for 1-treated rats are given for six animals per group and are significantly different from those for vehicle-treated animals ($p < 0.01$, Student's *t*-test).
(28) Cirino, G.; Peers, S. H.; Wallace, J. L.; Flower, R. J. A Study of Phospholipase A_2-Induced Edema in Rat Paw. *Eur. J. Pharmacol.* 1989, *166*, 505–510.
(29) Calhoun, W.; Yu, J.; Sung, A.; Chau, T. T.; Marshall, L. A.; Weichman, B. M.; Carlson, R. P. Pharmacologic Modulation of D-49 Phospholipase A_2-Induced Paw Edema in the Mouse. *Agents Actions* 1989, *27*, 418–421.
(30) 1 was administered at 30 mg/kg to Lewis rats 1 h prior to the subplantar injection (right hind paw) of 5 μg (10 units) of PLA_2 from *Naja naja* venom (Sigma) in 0.1 mL of pyrogen-free saline. The volume of the right hind paw was measured in an aqueous plethysomometer immediately prior to injection of the PLA_2 and at 0.5, 2, and 4 h after injection. Paw edema was calculated by subtracting the paw volume at zero time from the readings taken after injection of PLA_2. Percent change from control was calculated to determine the activity of 1 and is expressed as the mean of the three time points (41, 45, and 38% inhibition, respectively). Each time point measurement utilized a group of six animals. The statistical significance ($p < 0.01$) of the inhibitory effect of 1 was calculated using Student's *t*-test.