Fatty Acid Amides: Scooting Mode-Based Discovery of Tight-Binding Competitive Inhibitors of Secreted Phospholipases A_2†

The 14-kDa secreted class of phospholipase A_2 (PLA2) may play a role in the inflammatory response.[1] For example, the enzyme isolated from human synovial fluid (also called the human nonpancreatic secreted PLA2) is secreted from hepatoma cells in response to acute phase inflammatory stimuli.[2] Levels of this enzyme are higher in the synovial fluid from patients suffering from rheumatoid arthritis compared to serum levels;[3,4] however, see Stoner et al.[5] Thus, inhibitors of this secreted PLA2 may have therapeutic value.

Many of the previously reported inhibitors of PLA2 function as nonspecific agents. Such compounds do not bind directly to the enzyme but cause a change in the physical properties of the membrane interface in a way that causes the enzyme to desorb from the surface under conditions in which the interfacial binding is weak.[6-8] This problem has been overcome by analyzing PLA2 in the scooting mode in which the enzyme binds irreversibly to the interface and undergoes processive catalysis.[7,9] This type of behavior has been observed in the action of several different secreted PLA2s on vesicles of anionic phospholipids such as 1,2-dimyristoyl-*sn*-glycerophosphomethanol (DMPM).[10]

Known inactivators of PLA2 are the nonspecific agents substituted phenacyl bromides[11] and manoalide and some of its analogues.[12-14] Phospholipid-based acylating agents have also been reported.[15]

Competitive inhibitors of PLA2 are mainly phospholipid analogues in which the enzyme-susceptible ester has been replaced with an amide,[16-18] a phosphonate,[19] or an α,α-difluoro ketone.[20] The structures of some of these enzyme-inhibitor complexes have been recently reported.[21,22] A series of second-generation PLA2 inhibitors are phospholipid analogues that lack the *sn*-2-phosphate but still contain a tetrahedral phosphorous species as a transition-state mimic.[23]

In this study, a new class of PLA2 inhibitors are described that lack the entire glycerol skeleton, including the attached phosphate group. This is significant since it is likely that ionic phospholipid analogues will not be cell permeable and therefore not useful in determining the role(s) of PLA2s in cellular processes. Using a screening protocol based on interfacial catalysis in the scooting mode, we have found that culture filtrates of an actinomycete collected from soil in Taiwan (deposited as SC0043 in the culture collection of Sterling Winthrop, Malvern, PA) display strong inhibitory effects on a number of secreted PLA2s. In light of the problems associated with analyzing inhibitors of interfacial catalysis, it is noteworthy that no false positive PLA2 inhibitors were found after screening several thousand compounds using the scooting-based assay. Four inhibitors (compounds 1-4) were isolated by liquid chromatography (see supplementary material).

These compounds were identified as primary amides of fatty acids on the basis of structural studies using ^1H and ^{13}C NMR, IR, mass spectrometry, capillary GC of the corresponding methyl ester, and total synthesis (Table I). The structures are as follows: 1, a mixture of *all-cis*-5,8,-11-tetradecatrienamide (major component) and an unidentified dodecadienamide (minor component); 2, *all-cis*-5,8-tetradecadienamide; 3, *all-cis*-9,12,15-octadecatrienamide (α-linolenoyl amide); and 4, *all-cis*-9,12-octadecadienamide (linoleoyl amide).

The PLA2 inhibition data for compounds 1-4 are summarized in Table II. Two protocols have been used to evaluate these inhibitors. First, the effect of the inhibitor on the initial velocity for the hydrolysis of DMPM vesicles in the scooting mode was analyzed according to eq 1 for competitive inhibition adopted for interfacial catalysis.[24] Here, $(v_0)^0$ and $(v_0)^I$ are the initial velocities

$$\frac{(v_0)^0}{(v_0)^I} = 1 + \frac{\left(1 + \frac{1}{K_I^*}\right)}{\left(1 + \frac{1}{K_M^*}\right)} \frac{X_I}{(1 - X_I)} \qquad (1)$$

per enzyme in the absence and presence of the inhibitor in the interface at mole fraction X_I, respectively. K_M^* is the interfacial Michaelis constant for the DMPM substrate and K_I^* is the dissociation equilibrium constant for interfacially bound enzyme-inhibitor complex. The mole fraction of inhibitor necessary to cause a 50% reduction in the initial velocity is designated as $X_I(50)$, and values of this parameter for amides 1-4 acting against the PLA2s from porcine pancreas, bee venom (*Apis mellifera*), cobra venom (*Naja naja atra*), and human synovial fluid are listed in Table II. It can be seen that the fatty acid amides are potent PLA2 inhibitors ($X_I(50)$ as low as 0.0003 mol fraction) and the profile of inhibition with these isomers depends on the source of the enzyme. In addition, the $X_I(50)$s for the naturally-obtained amides 3 and 4 are the same, within experimental error, as the values for the

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Table I. Structural Characterization of the PLA2 Inhibitors[a]

property	1[b]	2	3	4
HPLC, retention time (min)	51.1	54.5	60.5	62.2
equivalent chain length (Me esters)	11.868	13.706	17.787	7.719
CI-MS (*m/e*)	221	223	277	279
IR (KBr) (cm^{-1})	1660.5, 1631.1	1656.7, 1631.1	1657.5, 1628.0	1657.9, 1626.2
^1H NMR ($CDCl_3$)	0.96 t; 1.71, quin; 2.11, m; 2.21, t; 5.36, m	0.85 t, 1.26, m; 1.69, quin; 2.03, quar; 2.13, quar; 2.20 t; 2.75, t; 5.35, m	0.96, t; 1.30, m; 1.62, m; 2.06, m; 2.20, t; 2.79, t; 5.34, m	0.87, t; 1.29, m; 1.62, m; 2.03, quar; 2.20, t; 2.75, t; 5.34, m
^{13}C NMR ($CDCl_3$)	nd	14.06, 22.55, 25.27, 25.63, 26.54, 27.22, 29.30, 31.50, 35.14, 127.58, 128.69, 129.29, 130.46, 175.25	14.24, 20.52, 25.48, 25.50, 25.59, 27.17, 29.08, 29.17, 29.20, 29.56, 35.91, 127.09, 127.71, 128.22, 128.26, 130.22, 131.94, 175.64	14.06, 22.56, 25.50, 25.63, 27.18, 29.11, 29.20, 29.23, 29.34, 29.59, 31.52, 35.89, 127.90, 128.06, 130.03, 130.23, 175.33

[a] Additional details are described in the Supplementary Material. [b] Data are for the major component.

Table II. $X_I(50)$ Values (Mole Fraction) for Fatty Acid Amide Inhibitors of PLA2s[a]

inhibitor	porcine pancreatic	bee venom	cobra venom	human synovial fluid
1	0.004	0.02	>0.1	0.02
2	0.002 (0.0007)[b]	0.002 (0.0007)	0.04 (0.01)	0.01 (0.003)
3	0.001	0.002	0.003	nd
4	0.001	0.001	0.02	0.004
synthetic oleoyl amide	0.0005	nd	nd	0.03
synthetic elaidoyl amide	0.001	nd	nd	0.005
synthetic petroselaidoyl amide	0.01	nd	nd	nd
synthetic γ-linolenoyl amide	0.0008	nd	nd	0.002
synthetic arachidonyl amide	0.0003	0.002	0.015	0.004
N-methyl synthetic 3	0.05	no inhibn at $X_I = 0.04$	0.05	0.2

[a] Additional details are given in the supplementary material. The moles of DMPM used to calculate $X_I(50)$ is 65% of the moles of DMPM in the assay since this is the fraction that is in the outer layer of the vesicles. If the amides distribute on both sides of the bilayer, then the true $X_I(50)$s will be 2-fold smaller. All $X_I(50)$ values are estimated from at least three independent determinations, and the estimated errors for all numbers is ≤30%. [b] The numbers in parentheses are the $X_I(50)$s corrected for the fact that 34% of amide 2 is partitioned into the vesicles.

corresponding synthetic compounds. Some additional fatty acid amides were also synthesized and are listed in Table II. Data in Table II also show that the presence of a methyl group on the amide nitrogen drastically reduces the potency of the inhibition. Analysis of the partitioning of the amides between the aqueous and vesicle phases reveals that the 18-carbon amide γ-linolenoyl amide is completely in the vesicles but approximately 35% of amide 2 is bound to DMPM (see supplementary material).

The $X_I(50)$ for the inhibition of the human synovial fluid PLA2 by oleoyl amide increased by a factor of 2 when the pH of the reaction mixture was changed from 8.0 to 7.3. Although a more thorough study will be required to fully interpret this result,[25] the data are consistent with the notion that the amide binds to the form of the enzyme in which the active-site histidine is in the depotonated state, as is the case for the binding of amide phospholipid analogues.[26] It is possible that the fatty acid amides interact with the active site of the enzyme in the same way as the amide-containing phospholipid analogues.[22] The contribution of the phospholipid polar head group to inhibitor binding may not be important if there is no differential interaction free energy for the head group–enzyme versus the head group–vesicle interactions.

An independent method for the evaluation of PLA2 inhibitors involves monitoring the inhibitor-afforded protection of the enzyme from inactivation by the active-site directed alkylating agent *p*-nitrophenacyl bromide.[8] In these studies, the enzyme is bound to the surface of a neutral diluent. Such a compound is defined as an amphiphile that forms an aggregate to which PLA2 binds via its interfacial recognition surface, but this agent offers no protection of the enzyme toward alkylation because it has no detectable affinity for the enzyme's active site. This approach yields the K_I^* for the inhibitor, and the data are analyzed according to equation given in the supplementary material and in Jain et al.[8] A value of $K_I^* = 0.04$ was determined for the interaction of oleoyl amide with the human synovial fluid PLA2 at pH 7.3, which is close to the $X_I(50)$ value of 0.008 observed at the same pH.[27] The 5-fold descrepancy in these values is most likely due to the fact that *p*-nitrophenacyl bromide was found to react with the amide present in the surface of the neutral diluent (under the experimental conditions employed, the half-time for reaction is approximately 1–2 h). Full protection of the enzyme at saturating levels of amide was measured which establishes that the active-site histidine residue is fully blocked by the binding of the amide.

The fatty acid amides do not inhibit the 85-kDa phospholipase A_2 found in the cytosol of mammalian cells.[28] In addition, the fatty acid amides are expected to be cell permeable and should be useful tools in establishing the role(s) of secreted PLA2s in biological processes as well as in diseases in man. Studies to determine the mode of

(25) The pH dependence of $X_I(50)$ may reflect a change in K_I^*, K_M^*, or both. However, the K_M^* for the human synovial fluid PLA2 acting on DMPM is ≫1 in the range pH 7–8 (Jain, M. K.; Gelb, M. H., unpublished observations), and thus the K_M^* term in eq 1 is close to unity and the change in $X_I(50)$ with pH reflects a change in K_I^* by the same factor.

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(27) Since K_M^* is ≫1 in this case,[25] $X_I(50) \approx K_I^*$.

(28) No detectable inhibition of the 85-kDa phospholipase A_2 (from U937 cells)[29] acting vesicles of 1-palmitoyl-2-arachidonylglycero-*sn*-3-phosphocholine containing 33 mol % dioleoylglycerol was seen when arachidonamide was present in the vesicles at 5 mol %.

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binding of these compounds to PLA2s as well as additional structure/function experiments are underway.

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Supplementary Material Available: Details of the kinetic analysis and isolation of the PLA2 inhibitors, structural characterization of 1–4, studies of protection from alkylation, and partitioning of the inhibitors between the vesicle and aqueous phases (8 pages). Ordering information is given on any current masthead page.

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Book Reviews

Annual Review of Pharmacology and Toxicology. Volume 32, 1992. Edited by Arthur K. Cho, Terrence F. Blaschke, Horace H. Loh, and James L. Way. Annual Reviews, Inc., Palto Alto, CA. 1992. viii + 698 pp. 15.5 × 23 cm. ISBN 0-8243-0432-2. $44.00.

This volume presents up-to-date reviews on subjects of topical interest to pharmacologists, toxicologists, medicinal chemists, and other scientists in the health and allied sciences. The first review is one contributed by Nobel Laureate George H. Hitchings entitled "Antagonists of Nucleic Acid Derivatives as Medicinal Agents". Other reviews written by leaders in their fields are "Review of Revues", "Glucuronidation and Its Role in Regulation of Biological Activity of Drugs", "Concepts in Chronopharmacology", "Inhaled Toxicants and Airway Hyperresponsiveness", "Catalytic Sites of Hemoprotein Peroxidases", "Hydrogen Sulphide and Its Toxicologic Implications", "Pharmacology of Nonpeptide Angiotensin II Receptor Antagonists", "Mutagenesis of the Beta-2 Adrenergic Receptor: How Structure Elucidates Function", "Population Pharmacokinetics/Dynamics", "Mitochondrial Benzodiazepine Receptors and the Regulation of Steroid Biosynthesis", "Selective Naltrexone-Derived Opioid Receptor Antagonists", "Geriatric Pharmacology. Basic and Clinical Considerations", "Theoretical Basis for a Pharmacology of Nerve Growth Factor Biosynthesis", "Therapeutic Applications of Oligonucleotides", "Pharmacology of Protein Kinase Inhibitors", "The Central Role of Voltage-Activated and Receptor-Operated Calcium Channels in Neuronal Cells", Pharmacodynamic Modeling of Anesthetic EEG Drug Effects", "Calcium-Mediated Mechanisms in Chemically Induced Cell Death", "Role of Covalent and Noncovalent Interactions in Cell Toxicity: Effects on Proteins", "Biochemical and Molecular Pharmacology of Kinin Receptors", "The Chemistry of Avermectins", "Tandem Mass Spectrometry: The Competitive Edge for Pharmacology", "Cytotoxic Conjugates Containing Translational Inhibitory Proteins", and "Determinants of Metabolite Dispositions". Each review is followed by a comprehensive list of references. The book includes an excellent subject index followed by cumulative indexes for contributing authors and chapter titles for volumes 28–32.

Researchers in the fields reviewed will benefit from individual desk copies of this volume. Others will want access to the volume from institutional libraries.

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The Organic Chemistry of Drug Design and Drug Action. By Richard B. Silverman. Academic Press, Inc., San Diego, CA. 1992. xiv + 422 pp. 16 × 24 cm. ISBN 0-12-643730-0. $55.00.

Unlike traditional medicinal chemistry texts that are generally organized by classes of drugs and a description of their pharmacological effects, this book emphasizes the organic chemical aspects of medicinal chemistry. As such, it concentrates on the organic chemistry of drug design, drug development, and drug action. Organic chemical principles and reactions important to the design of drugs and an understanding of their action are emphasized. Clinically important therapeutic agents are used as examples. In this manner, the author has presented the concepts of medicinal chemistry in terms of rational physical organic chemistry. The principles that are clearly described in this book should provide the foundation for future elucidation of drug action and the rational discovery of new drugs based on organic chemical phenomena. The organization of this book and the clarity of presentation are outstanding. Thus, following an introductory chapter are ones directed toward drug discovery, design and development, receptors, enzymes, DNA, drug metabolism, and prodrugs and drug delivery systems. Each chapter is followed by specific references as well as general references to significant reviews. An excellent subject index is also included.

This book very clearly presents medicinal chemistry as a unified discipline based on sound principles of organic chemistry. It is highly recommended to medicinal chemists as well as to all others entering into the field or concerned with the science of medicinal chemistry.

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