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Chem Rev. Author manuscript; available in PMC 2012 October 12.

Published in final edited form as:

Chem Rev. 2011 October 12; 111(10): 6130–6185. doi:10.1021/cr200085w.

Phospholipase A₂ Enzymes: Physical Structure, Biological Function, Disease Implication, Chemical Inhibition, and Therapeutic Intervention

Edward A. Dennis^{1,*}, Jian Cao¹, Yuan-Hao Hsu¹, Victoria Magrioti², and George Kokotos^{2,*}

¹Department of Chemistry and Biochemistry and Pharmacology, School of Medicine, University of California, San Diego, La Jolla, California 92093-0601

²Laboratory of Organic Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis, Athens 15771, Greece

1. Introduction

1.1. Discovery of the Phospholipase A₂ Superfamily

Phospholipases represent one of the earliest enzyme activities to be identified and studied and the phospholipase A₂ (PLA₂) superfamily (see defining specificity¹ in Figure 1) traces its roots to the identification of lytic actions of snake venom at the end of the 19th century. The enzyme was first purified and characterized from cobra venom and later from rattlesnake venom. As protein sequencing methodologies advanced in the 1970's, it became apparent that these enzymes had an unusually large number of cysteines (over 10% of the amino acids) and as secreted enzymes, that they were all in the form of disulfide bonds. It was further recognized that in the case of PLA₂, cobras and rattlesnakes had six disulfides in common, but one disulfide bond is located in distinctly different locations. This led to the designation of Type 1 and Type 2 for cobras (old world snakes) and rattlesnakes (new world snakes), respectively.² During that same period, studies on the porcine pancreatic digestive enzyme that hydrolyzes phospholipids led to the determination that this mammalian enzyme (and also the human pancreatic enzyme) had the same disulfide bonding pattern as cobras and hence the designation as IB with the cobra enzyme as IA.

A dramatic change in the phospholipase A₂ field that attracted the attention of the broader scientific community occurred in July, 1988 when at the first FASEB Summer Conference on Phospholipases, Jeffery J. Seilhamer and Lorin K. Johnson from California Biotechnology Inc.³ and Ruth M. Kramer from Biogen Research Corporation⁴ independently and with much fanfare and excitement reported the purification, sequencing and cloning of the first human non-pancreatic secreted PLA₂ which they each had isolated from the human synovial fluid of arthritic knee joints. Since the sequence revealed that the disulfide bond pattern was more like the rattlesnake than the human pancreatic enzyme, this new form of PLA₂ was designated IIA. All of these enzymes then became known as secreted or sPLA₂s.

It wasn't until the late 1980's that PLA₂-like activities were reported in mammalian cells in contrast to extracellular secreted activities from venom and pancreas. In July, 1992, at the second FASEB Summer Conference on Phospholipases, James D. Clark from the Genetics Institute⁵ and Ruth M. Kramer (who had moved to Lilly Research Laboratories)⁶

*Address correspondence to EAD: Phone, 858-534-3055; FAX, 858-534-7390; edennis@ucsd.edu. GK: Phone, 30-210-727-4462; FAX, 30-210-727-4761; gkokotos@chem.uoa.gr.

independently reported the purification, sequencing, and cloning of the first human cytosolic PLA₂ (cPLA₂) from the U937 macrophage cell line. The sequence was unrelated to those of the secreted enzymes. To track this new enzyme and potentially additional PLA₂s, a Group Numbering System⁷ was established utilizing the preexisting venom designation of I and II and expanding them to include subgroups IA, IB, and IIA (GIA, GIB, GIIA); adding Group III (GIII) for the clearly different PLA₂ which had been purified from bee venom; and establishing the Group IV (GIV) designation for the new cytosolic PLA₂ (cPLA₂).

This was fortuitous because soon thereafter a new form of secreted PLA₂ was discovered. It was produced by macrophages and it had the same six disulfide bonds as Group I and Group II, but lacked the seventh disulfide bond entirely. To make clear that this sPLA₂ was neither GI nor GII, this enzyme was designated as Group V (GV). At the Third FASEB Summer Conference on Phospholipases held in July, 1995, Edward A. Dennis from the University of California, San Diego⁸ reported on another cystosolic PLA₂ purified from macrophages that differed from Group IV cPLA₂ in that its activity was not dependent on Ca²⁺ and Simon S. Jones from the Genetics Institute⁹ reported that the cloned form from CHO cells had a very different sequence than cPLA₂. This new Ca²⁺-independent PLA₂ (iPLA₂) was designated as Group VI PLA₂ (GVI).¹⁰

Earlier, investigators from the University of Utah¹¹ had isolated an enzyme from human plasma which hydrolyzed platelet activating factor (PAF), a phosphatidylcholine containing an acetate at the *sn*-2 position, and in 1995 Larry W. Tjoelker from ICOS¹² reported its cloning. This enzyme and other related PAF acetyl hydrolases (PAF-AH) were later recognized more broadly as PLA₂s with a specificity for a short acyl chain on the *sn*-2 position and for the plasma one for oxidized lipids for which the same enzyme was independently named lipoproteinassociated phospholipase A₂ (Lp-PLA₂). These enzymes were designated Group VII and VIII (GVII and GVIII).¹³ As additional specific PLA₂s were discovered, they were either designated by letters as subgroups of the original Groups indicated above or as additional Groups. Especially noteworthy was the discovery of a number of additional sPLA₂s in which the sequence and/or disulfide bonding pattern varied significantly from the traditional Groups I, II, III, and V sPLA₂s. These new forms led to the additional Groups IX, X, XI XII,XIII, and XIV sPLA₂s representing new human forms (especially Group X, which may have important functions) as well unique enzymes from snail venom, rice shoots, parvovirus, and fungi/bacteria. The only new type of PLA₂ reported that did not naturally fit in the four types discussed above (secreted, cystosolic, Ca²⁺-independent, PAF acetylhydrolases) is the lysosomal PLA₂ (LPLA₂) which was designated as Group XV (GXV).¹⁴ Recently, a new PLA₂ was isolated from adipose tissue and designated as Group XVI (GXVI);¹⁵ it appears to be a new type of PLA₂ called adipose-PLA₂ (AdPLA). The current designations are summarized in Table 1.

In this review, we will discuss in turn each of the six types of PLA₂. For each, we will first discuss the various forms, in terms of groups, subgroups and mechanism of action, their structure and interaction with membranes, their biological activities and role in disease, and the development of selective inhibitors. Of course the commonly used type designation has little meaning today since as we have learned more about these enzymes, it has been recognized that secreted, cystosolic, Ca²⁺-independent, PAF-AH, and lysosomal make little sense since all four of the later categories are actually intracellular (cytosolic) enzymes, that the secreted ones may occur intracellularly in various vesicles, and that the PAF-AHs, lysosomal and some forms of cPLA₂ are also Ca²⁺-independent. Thus the Group Numbering System designation provides an unambiguous definition of each enzyme form. Over the years, numerous excellent reviews on either the broad family of PLA₂s¹⁶ or specific types including sPLA₂s,¹⁷ cPLA₂s,¹⁸ iPLA₂s,¹⁹ PAF-AHs²⁰ and LPLA₂²¹ have appeared as well as several review articles summarizing the classes of PLA₂ inhibitors and their potential role

for the treatment of inflammatory diseases.²² We have employed all of these prior reviews heavily in preparing this up-to-date and comprehensive single review covering all aspects of the entire phospholipase A₂ superfamily.

1.2 Accessing the *In Vitro* Activity of Phospholipase A₂

Studying phospholipases has posed significant challenges because unlike classical water-soluble enzymes acting on water soluble substrates, phospholipases act on phospholipids which aggregate in aqueous solution to form structures termed micelles, vesicles, liposomes, etc. While most of the PLA₂s studied in depth to date are water-soluble themselves, they catalyze hydrolysis of their water-insoluble substrates by catalytic action at the lipid-water interface. Since the substrate phospholipids are not monomeric, but rather are lined up in a two-dimensional interfaces, when the enzyme is associated with that interface, it binds its substrate phospholipid molecule in the interface where its substrate concentration can be best expressed in surface terms. Thus in kinetic experiments to determine activity, the observed activity depends on non-substrate lipids in the interface whether they be other non-substrate phospholipids, surface active detergents (surfactants), and even inhibitors that aggregate with the surface. The concept of “surface dilution kinetics”²³ has been useful in measuring PLA₂ activities, particularly in comparing the different enzyme forms and substrates as reviewed elsewhere²⁴ and illustrated in Figure 2. Thus as one compares the specific activity, specificity, and especially the inhibition²⁵ of each PLA₂ Group, subgroup, and species, one must note the particular assay conditions and aggregated form of substrate used including the kinetic ramifications.

2. Secreted Phospholipase A₂ (sPLA₂ Groups I, II, III, V, IX, X, XI, XII, XIII, XIV)

2.1 Groups, Subgroups, Specificity and Mechanism

The sPLA₂s are small secreted proteins of 14–18 kDa (except for Group III sPLA₂) that usually contain 6 to 8 disulfide bonds (Table 2).¹⁴ A schematic presentation of the sequences (Figure 3) provides an overview of this PLA₂ types. This group of enzymes uses an active site His/Asp dyad and requires mM Ca²⁺ for catalytic activity. Members of this type were first studied in phenomenological detail over 100 years ago using “poison” – venom from cobras.^{16c} As various snake venom PLA₂s were sequenced and disulfide bond patterns determined, those from old world snakes (cobras and kraits) were referred to as type I and those from new world snakes (rattlesnakes) were referred to as type II. The first non-venom PLA₂, named GIB, was isolated from the pancreatic juices of cows, and was also found in many other mammals (similar disulfide bond pattern to GI snake venom). Later, another mammalian sPLA₂, a non-pancreatic form (similar disulfide bond pattern to GII snake venom) named GIIA was found in the synovial fluid of patients with rheumatoid arthritis.^{3–4} To date, seventeen forms of sPLA₂ (Table 2) have been identified in mammals, insects, mollusks, reptiles, plants and bacteria. The sPLA₂s display a wide range of different tissue distribution patterns and distinct physiological functions.

Ten members of the sPLA₂ family (group IB, IIA, IIC, IID, IIE, IIF, III, V, X and XII) have been identified in mammals; these are numbered and grouped according to their pattern of disulfide bonds and in order of their discovery.¹⁴ The human genome contains 9 sPLA₂s and the mouse genome contains all ten, including GIIC sPLA₂, which exists in the human genome only as a pseudogene.

Mammalian GIII sPLA₂ is a multi-domain protein with a molecular weight of 55 kDa. It contains a central domain similar to that of bee venom GIII sPLA₂, including a 130-amino acid N-terminal domain extension and a 219-amino acid C-terminal domain extension. GXIIB sPLA₂ in humans and mice (a homologue of GXIIA sPLA₂), has a natural mutation in the active site (H48L) and totally lacks enzymatic activity.²⁶ GXIIB sPLA₂ is highly

expressed in the liver, small intestine and kidney, both in human and mouse, and the functions of GXIIB sPLA₂ seem not to rely on enzymatic catalytic activity and might be related to its non-catalytic role in which the protein forms supermolecular aggregates with phospholipid vesicles and/or acts as a ligand for specific cellular targets.²⁶ The prokaryotic sPLA₂s have only two or zero disulfide bonds (GXIII and GXIV), which is strikingly different from the eukaryotic enzymes, which normally contain 6–8 disulfide bonds.

All of the sPLA₂s display a characteristic increase in activity when the substrate concentration is changed from monomers to aggregates, which is referred as “interfacial activation”.²⁴ The structural basis for the interfacial activation mechanism will be discussed in section 2.2. In contrast with cytosolic PLA₂s (cPLA₂s), which have a marked specificity for arachidonic acid at the *sn*-2 position of its phospholipid substrates, sPLA₂s do not show distinct preference for the *sn*-2 position fatty acyl chains; there is, however, some specificity for certain head groups of the phospholipid substrates. In general, most of the sPLA₂s show a higher activity with anionic phospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylserine (PS). GIA and GXIV sPLA₂s are more active against zwitterionic phosphatidylcholine (PC) vesicles. Mammalian GV and GX sPLA₂s can hydrolyze both anionic phospholipid vesicles and zwitterionic PC-rich vesicles at comparable rates.²⁷

2.2 Structural Characteristics and Interactions with Membranes

Almost all sPLA₂s contain a highly conserved Ca²⁺ binding loop (*XCGXGG*) and a catalytic site (*DXCCXXHD*). Among the sPLA₂s, crystal structures available are the cobra venom GIA, human, porcine and bovine pancreatic GIB, human GIIA, human GX, plant GXIB and prokaryotic GXIV sPLA₂s. Although the amino acid identity level is low, all of these enzymes share a common protein fold (Figure 4 A) with slight differences and feature the same catalytic His/Asp dyad. GI, IIA and X sPLA₂s have very similar structures, which contain three long α -helices, two-stranded β -sheets referred to as β -wings, and a conserved Ca²⁺ binding loop. The plant GXIB sPLA₂ has been shown to lack β -wings.²⁸ The prokaryotic GXIV sPLA₂ shows a different, all α -helical, protein folding.²⁹

Although the structures of other sPLA₂ family members have not been reported to date, based on available structural data and sequence alignment, the GI, GII, GV and GX mammalian sPLA₂s are expected to share a common three-dimensional structure. The human GIII sPLA₂ would represent another class of fold. Its PLA₂ domain is expected to be similar to that of bee venom GIII sPLA₂, while its extra C- and N-terminal domains, as we mentioned above, are functionally unknown and structurally are not homologous to known proteins. GXIIA sPLA₂ comprises the third structural class, which has an unusual Ca²⁺ binding loop.

The cobra venom GIA sPLA₂ is one of the most extensively studied PLA₂ enzymes and it has been considered an important model for phospholipase A₂ enzymology. Therefore, we will use GIA sPLA₂ as an example to show the unique structural characteristics of sPLA₂ enzymes. GIA sPLA₂ contains six conserved disulfide bonds with an additional disulfide bond between residue 11 and residue 71 (Figure 4 A). Calcium ions bind with the conserved aspartic acid residue as well as the carbonyl oxygens of the tyrosine and glycines from the calcium binding loop. Calcium is absolutely required for hydrolysis. The secreted sPLA₂s shown do not form a classical acyl enzyme intermediate characteristic of serine proteases.^{7,30} Rather, they utilize the catalytic site His, assisted by an Asp, to polarize a bound H₂O, which then attacks the carbonyl group. The calcium ion stabilizes the transition state by coordinating the carbonyl group and the negative charge from the phosphate oxygen.

Using the native and inhibitor-bound structure of GIA sPLA₂ and a space-filling model for the phospholipid substrate, a model of how the substrate interacts with the active site was created (Figure 4B).⁷ In the model only about 9–10 carbons of the *sn*-2 acyl chain interact with the enzyme and the rest of the chains are buried in the lipid-water interface. This explains why sPLA₂s fail to show specificity for the particular fatty acid in the *sn*-2 position. The hydrophobic residues, Leu-2, Phe-5, Trp-19, Tyr-52 and Tyr-69 wrap around the acyl chain of the lipid substrate.

The activity of most phospholipase A₂ family members depends critically on the interaction of the protein with large lipid aggregates. The interfacial activation mechanism of PLA₂ enzymes has long been an interesting topic in membrane protein enzymology.^{24,31} GIA sPLA₂ can hydrolyze zwitterionic phospholipids. This is most likely due to the aromatic residues present on the interfacial binding surface. Recently hydrogen deuterium exchange mass spectrometry (DXMS) was used to study GIA sPLA₂/phospholipid vesicles and calcium ion interaction in solution.³² The DXMS results show that the surface hydrophobic residues Tyr-3, Trp-61, Tyr-63 and Phe-64 penetrate into the lipid membrane phase to allow the enzyme to access the substrate from the lipid aggregates (Figure 5). A second calcium binding site was also indicated in the DXMS study, although there is only one calcium ion in the crystal structure. A second calcium binding site has also been found in other related sPLA₂ structures.³³ Among the aromatic amino acids, tryptophan was thought to be the most potent contributor to the interfacial binding. Replacing Try-31 causes human GV sPLA₂ to lose its ability to bind to zwitterionic PC vesicles.³⁴ A similar result was found in GX sPLA₂, where replacing the only surface Try-67 residue with an alanine caused an 8-fold reduction in binding affinity to PC vesicles and also significantly decreased the hydrolysis activity of PC-rich vesicles.^{27b} Human GIIA sPLA₂, which binds poorly to PC-rich vesicles, exhibits much higher activity on DOPC membranes when a surface valine residue is mutated to tryptophan.^{27b,35}

Both electrostatic and hydrophobic interactions contribute to the interfacial binding of sPLA₂ to anionic phospholipid membranes. The interaction between basic residues on the binding surface with anionic vesicles plays an important role in interfacial binding.³⁶ The large number of basic residues scattered over the GIIA sPLA₂ surface could be the reason that this enzyme has highly selective binding to anionic vesicles. The recently defined crystal structure of GIB sPLA₂ in a premicellar complex has shown that the enzyme uses the hydrophilic residues Arg-6 and Lys-10 to bind at the anionic interface.³⁷ However, studies on the interfacial binding of bee venom GIII sPLA₂ have shown that the interaction occurs predominantly through a nonelectrostatic mechanism.³⁸ When the five basic residues on the bee venom sPLA₂ binding surface are all changed to neutral glutamine residues, the resulting mutant does not show a significant decrease in binding to the anionic vesicles.³⁸ However if the basic residues are mutated to charge-reversed glutamate residues, the mutant binds to PS/PC vesicles 3000-fold more weakly than the wild-type protein.³⁸ This indicates that while the electrostatic interaction is not predominant between the enzyme and anionic phospholipids, the repulsion interaction will definitely destroy the binding. The interfacial binding surface of GX sPLA₂ is charge neutral, which may explain why this enzyme is active on both zwitterionic and anionic phospholipids.³⁹

The crystal structure of the trimeric form of human pancreatic pro-GIB sPLA₂ was recently reported.⁴⁰ The trimeric form shows a much more positively charged interfacial surface. The authors suggested that human GIB sPLA₂ may use a different activation mechanism in which GIB sPLA₂ switches from monomeric to trimeric form when it associates with the membrane using the highly positive charged trimer back side.⁴⁰ The crystal structure of the trimeric form has also been shown in studies on cobra venom⁴¹ and *Naja naja* GIA sPLA₂s.⁴² Anion-assisted dimer crystal structures were reported for other pancreatic

sPLA₂s.⁴³ While the existence of dimeric and trimeric forms may be a functionally important feature of these sPLA₂s, the higher oligomeric states of sPLA₂ structures are found under high concentration crystallization conditions, so they may not be representative of the real physiological state. Whether the trimeric or dimeric form is important for sPLA₂ catalysis or not requires further investigation.

2.3 Biological Functions and Disease Implications

sPLA₂s exhibit a large variety of cellular functions, though the specific function varies by group or subgroup. The major functions will be summarized below and include the ability to kill Gram-positive and Gram-negative bacteria thereby affecting host defense against bacterial infections.⁴⁴ sPLA₂s also show antiviral activity.⁴⁵ sPLA₂s are expressed and released by human inflammatory cells including macrophages, monocytes, T cells, mast cell and neutrophils and increased concentrations of different isoforms of sPLA₂s have been detected in the blood of patients with inflammatory and autoimmune diseases.⁴⁶ sPLA₂s also play a role in the hydrolysis of oxidized lipids in low- and high-density lipoproteins contributing to the development of atherosclerosis.⁴⁷ Many experiments carried out both *in vitro* and *in vivo*, especially transgenic and gene knockout mice studies, have expanded our knowledge of the sPLA₂ family. However, due to the large number of sPLA₂ family members and the redundant expression in tissues, there is still much we do not understand about the functions and physiological roles of each individual sPLA₂.

2.3.1 Antibacterial and Antiviral Functions of sPLA₂s (sPLA₂ Groups I, II, III, V, X)—There is a considerable body of evidence supporting the antibacterial functionality of sPLA₂. GIIA sPLA₂ has displayed antibacterial activity towards Gram-positive bacteria including *Staphylococcus aureus*⁴⁸, *Listeria monocytogenes*,⁴⁹ and others.^{17a} The enzyme has also demonstrated antibacterial activity against some Gram-negative bacteria, such as *E. coli* and *Salmonella typhimurium*.⁴⁴ High concentrations of GIIA sPLA₂ are found in tears, where the majority of bactericidal action is due to GIIA sPLA₂.⁵⁰ The concentration of GIIA sPLA₂ increases up to 500-fold in the serum samples of patients with severe acute diseases compared with healthy controls.^{49b} High concentrations of GIIA sPLA₂ have also been found in seminal plasma, inflammatory exudates, bronchoalveolar lavage and intestinal lumen.^{17e,51} Overexpression of GIIA sPLA₂ in transgenic mice has resulted in decreased mortality in experimental *Staphylococcus aureus* infections and has improved clearance of bacteria from organs and body fluids of experimental animals.⁵² This enzyme also plays a protective role *in vivo* against experimental anthrax. Transgenic mice expressing human GIIA sPLA₂ and mice that have had recombinant human GIIA sPLA₂ administered *in vivo* are resistant to *B. anthracis* infection.⁵³

The antibacterial activity of GIIA sPLA₂ is calcium-dependent and is negated in the presence of EGTA.^{44,54} The antibacterial activity also depends on PLA₂ activity to hydrolyze the cell membrane.⁵⁵ To kill the bacteria, GIIA sPLA₂ first penetrates the peptidoglycan envelope of Gram-positive bacteria, thus gaining access to the bacterial cell membrane phospholipids.⁵⁶ The highly positively charged surface of GIIA sPLA₂ also enables it to bind with the lipoteichionic acids of Gram-positive bacteria.⁵⁷ The bactericidal effect of GIIA sPLA₂ is highest against bacteria in the phase of logarithmic growth, which may be due to its ability to reach the bacterial plasma membrane through the dividing cell wall.⁵⁸

In addition to GIIA other sPLA₂s also have antibacterial activity.^{49a,56,59} The ranking of most to least potent sPLA₂s against Gram-positive bacteria is GIIA > GX > GV > GXII > GIIE > GIB, GIIF for human, and GIIA > GIID > GV > GIIE > GIIC, GX > GIB, GIIF for murine.⁵⁶ The antibacterial efficiency of a particular sPLA₂ depends significantly on the

highly positively charged protein surface. For example, the GIIA sPLA₂ shows more antibacterial function than other sPLA₂s—this may be related to its highly cationic nature (pI > 10.5). Antibacterial activity was also found in snake venom GIA sPLA₂ against both Gram-positive and Gram-negative bacteria,⁶⁰ however the mechanism is different. The catalytically inactive form (H49K), as well as a synthetic peptide containing residues 115–129, retained the bactericidal effect of the catalytically active intact protein,⁶⁰ which indicates that the bactericidal effect is independent of the enzymatic activity.

The antibacterial mechanism against Gram-negative bacteria was thought to be different from the action against Gram-positive bacteria. In addition to the anionic peptidoglycan cell wall, Gram-negative bacteria have an outer layer of lipopolysaccharide, which makes it difficult for the enzyme to access and hydrolyze the plasma membrane phospholipids. GIIA sPLA₂ has been shown to be effective against bactericidal/permeability-increasing protein (BPI)-treated *E. coli* which depends on the presence of a cluster of basic residues within the surface region near the N-terminus.⁶¹ By mutating Ser-7 to a lysine residue, the pig pancreas GIB sPLA₂ can be converted into an enzyme active against *E. coli* treated with BPI.⁶² GV sPLA₂ was also found to hydrolyze phospholipids from *E. coli* in the presence of serum, but not as efficiently as GIIA sPLA₂.⁵⁹ GV sPLA₂ has a lower pI (>7) and thus has lower intermediate activity on anionic cell wall-bound *E. coli* membranes.

In addition to their antibacterial functions, sPLA₂s also display antivirus activity. GIII, GV and GX sPLA₂s are capable of preventing host cells from being infected with adenovirus.⁶³ The antivirus activity of GV and GX sPLA₂s is due to the enzyme activity which converts PC to lysoPC in the host cell membranes. This hydrolysis of the cell membrane protects the host cells from adenovirus entry.^{63a} GIII sPLA₂ has a different antivirus mechanism, which requires the presence of both the catalytic domain and the N-terminal domain for the antiadenovirus effect.^{63b} By degrading the virus membrane and recognizing the virus envelop, human GX sPLA₂ is capable of neutralizing HIV-1.⁶⁴ Bee and snake venom sPLA₂s and a peptide derived from bee venom sPLA₂ can block HIV-1 entry into the host cells by steric inhibition of the chemokine receptor on the target cells without requiring enzyme catalytic activity.^{45,65}

2.3.2 sPLA₂ and Inflammation (sPLA₂ Groups I, II, V, X)—The sPLA₂s appear to play a role in several inflammatory diseases. The first evidence was from GIIA sPLA₂, which is present at high concentrations in the synovial fluid of patients with rheumatoid arthritis.³ GIIA sPLA₂ deficient mice have shown reduced signs of arthritis when compared with wild-type mice. Recently, GV sPLA₂ has also been found in rheumatoid arthritis synovial fluid, but the expression was notably lower than GIIA sPLA₂.⁶⁶ However, GV sPLA₂ may play an anti-inflammatory role rather than the normal pro-inflammatory role.⁶⁶ GV sPLA₂ deficient mice were protected from K/BxN arthritis when treated with exogenous recombinant GV sPLA₂.⁶⁶ Increased levels of sPLA₂s have also been detected in the plasma or serum of patients with acute pancreatitis, septic shock, Crohn's diseases and ulcerative colitis.⁴⁷ Furthermore, sPLA₂s seem to be involved in adult respiratory distress syndrome (ARDS) and inflammatory bowel disease.⁶⁷

sPLA₂s participate in inflammation through their enzymatic activity by releasing free fatty acids, including arachidonic acid (AA), thus initiating the biosynthesis of lipid mediators, including prostaglandins, thromboxanes and leukotrienes. In addition, the hydrolytic product, lysophospholipid, is also a proinflammatory lipid mediator. AA release by the PLA₂ catalytic reaction is the initial and rate limiting step for the biosynthesis of eicosanoids.⁶⁸ GIVA PLA₂ has for a long time been considered the major PLA₂ enzyme to release AA for eicosanoid production.⁶⁸ However, more recent results have shown that sPLA₂s may also be involved in AA release and eicosanoid biosynthesis. GIIA sPLA₂s are

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript capable of mediating cytokine-induced delayed AA release and ionophore-induced immediate AA release.⁶⁹ Exogenously added human GV sPLA₂ could induce AA and leukotriene C4 (LTC4) release from unprimed human neutrophils.³⁴ Peritoneal macrophages from GV sPLA₂ knockout mice show reduced production of LTC4 upon stimulation with the yeast cell wall particle zymosan which amplifies the action of GIV cPLA₂ in regulating eicosanoid biosynthesis.⁷⁰ In mouse peritoneal macrophages stimulated with zymosan, GV sPLA₂ can translocate to the phagosome and regulate phagocytosis through regulation of eicosanoid generation.⁷¹ The exogenously added human GV sPLA₂ could also induce leukotriene B₄ (LTB4) synthesis in human neutrophils by the activation of GIVA PLA₂.⁷² A putative mechanism has been proposed whereby human GV sPLA₂ first hydrolyzes the outer plasma membrane of neutrophils to release lysoPCs and fatty acids. The lysoPC and fatty acid then cause an increase in the calcium concentration and in turn activate membrane translocation of 5-lipoxygenase and cPLA₂.⁷² In addition, exogenous GV sPLA₂ could induce arachidonic acid release and LTC4 synthesis in isolated human peripheral blood eosinophils in a manner independent of activating cPLA₂.⁷³ Recombinant GX sPLA₂ has been shown to induce a substantial release of AA independent of the action of GIVA PLA₂, when added to human myeloid leukemia cells or adherent mammalian cells.⁷⁴ A GIVA PLA₂ knockout mice study further supports the results of the *in vitro* study which showed that the amount of AA released by GX sPLA₂ from spleen cells was not significantly altered by GIVA PLA₂ deficiency.⁷⁵ GX sPLA₂ knockout mice have shown a significant reduction in eicosanoid generation and this provides additional strong evidence for GX sPLA₂ involvement in eicosanoid-mediated inflammation.⁷⁶

sPLA₂s can release AA by two mechanisms: an external plasma membrane pathway and a heparan sulfate proteoglycan (HSPG)-shuttling pathway.⁷⁷ Among sPLA₂s, GV and GX show high binding affinity and catalytic activity towards PCs. This would allow GV and GX sPLA₂s to directly act on the outer leaflet of plasma membranes, to hydrolyze PCs to release fatty acids and lysophospholipids.⁷⁷ Heparin-binding sPLA₂s GIIA, GIID and GV may bind to the heparan sulfate chains of glypcan, allowing the accumulation of the enzyme on the cell surface and promoting enzyme trafficking to intracellular compartments to release AA.⁷⁷

In addition to eicosanoid production, sPLA₂s also are able to activate inflammatory cells to induce the production of other proinflammatory mediators from macrophages, neutrophils, eosinophils, monocytes and endothelial cells⁷⁸ and the function may not require enzymatic activity. GIB, GIIA, GV and GX sPLA₂s induce the production of proinflammatory cytokines and chemokines independent of their hydrolytic activity.⁷⁹ GIIA and GIII sPLA₂s are capable of upregulating the surface molecules on a variety of inflammatory cells.⁸⁰

Adult respiratory distress syndrome is characterized by lung surfactant disorders that lead to increased surface tension, alveolar collapse, loss of liquid balance in the lung and severe disturbance of pulmonary gas exchange.^{67a} Increased levels of sPLA₂s have been detected in bronchoalveolar lavage fluids of ARDS patients at levels that correlate positively with the severity of ARDS.⁸¹ The involvement of sPLA₂s in ARDS seems to depend on the enzymatic hydrolysis of surfactant phospholipids, since increased amounts of lyso-PC and decreased PC concentrations were found in bronchoalveolar lavage fluids of ARDS patients.⁸² In an animal model of acute lung injury inhibiting GIIA sPLA₂ activity ameliorated lung dysfunction by protecting against surfactant degradation.⁸³ Transgenic mice expressing human GV sPLA₂, but not GIIA or GX sPLA₂, show a significant reduction in the lung surfactant phospholipids phosphatidylcholine and phosphatidylglycerol, a condition that leads to neonatal lethality due to lung dysfunction.⁸⁴ The overexpression of human GX sPLA₂ enzyme in transgenic mouse macrophages leads to massive degradation of surfactant phospholipids causing lethal lung inflammation.⁸⁵

sPLA₂ may be involved in the pathogenesis of inflammatory bowel disease including Crohn's disease and ulcerative colitis.⁸⁶ GIIA sPLA₂ protein and mRNA were detected in Paneth cells of the small intestinal mucosa in the intestine in Crohn's disease patients.^{86b} GIIA sPLA₂ enzymatic activity was found to be significantly increased in actively inflamed colonic mucosa of Crohn's disease patients and severely inflamed mucosa of ulcerative colitis patients compared with non-inflamed mucosa.^{86a} GIIA sPLA₂ was found to be localized to the Paneth's cells at the site of active inflammation in the Crohn's disease patients.^{86b}

2.3.3 sPLA₂s in Atherosclerosis (sPLA₂ Groups II, III, V, X)—There is considerable evidence to show that sPLA₂s play an important role in atherosclerosis. GIIA, GV and GX sPLA₂s have been detected in human and/or mouse atherosclerotic lesions and are believed to enhance lipid accumulation in arterial intima.^{17c} A study in patients with coronary artery disease shows that increased levels of plasma GIIA sPLA₂ may be a significant risk indicator for coronary artery disease (CAD) and a predictor for clinical coronary events.⁸⁷ Young adults with metabolic syndrome (MetS) were also found to have increased serum levels of sPLA₂.⁸⁸

Transgenic mice expressing human GIIA sPLA₂ have exhibited a dramatic increase in atherosclerotic lesions compared with nontransgenic littermates, regardless of whether they were fed a high-fat, high-cholesterol diet or a low-fat chow diet.⁸⁹ The transgenic mice also exhibit decreased levels of HDL-cholesterol and increased levels of LDL/VLDL-cholesterol.⁸⁹ An animal model that transplanted bone marrow cells from transgenic mice to lethally irradiated LDL receptor knockout (LDLR^{-/-}) mice indicates that the macrophage cells expressing human GIIA sPLA₂ promote atherosclerotic lesion formation without plasma lipoprotein concentration changes.⁹⁰ Additionally, the same mouse model shows 2.3 fold larger lesions compared with control mice when fed a high-fat diet and also shows enhanced collagen deposition independent of lesion size.⁹¹ This indicates that GIIA sPLA₂ is a proatherogenic factor and it may regulate the collagen production in the plaque. The macrophage-specific expression of GIIA sPLA₂ in transgenic mice induces increased mouse 12/15-lipoxygenase (12/15-LO), causing the generation of oxidative stress, thought to be a major contributing factor to atherogenesis.⁹² Studies of transgenic mice have found that the expression of recombinant apolipoprotein B100 (apoB100) and GIIA sPLA₂ induces the formation of slightly smaller LDL particles, enriched with lysoPC.⁹³ This is because GIIA sPLA₂ modifies the LDL in circulation so that site A (residues 3148–3158) in apoB100 is exposed, increasing LDL/proteoglycan binding and making the LDL more proatherogenic.⁹³

One possible mechanism for the role of sPLA₂s in atherogenesis is the ability of sPLA₂ to hydrolyze the phospholipids on LDL particles. The modified LDL could then promote lipid accumulation and lead to enhanced macrophage uptake. Among mammalian sPLA₂s, GV and GX sPLA₂ have shown higher activity in hydrolyzing LDL and HDL than other sPLA₂s due to their high affinity binding to PCs.⁹⁴ Thus, they may contribute more to atherosclerosis. GV sPLA₂ was detected in both human and mouse atherosclerosis lesions.⁹⁵ LDL particles modified by GV sPLA₂ are significantly smaller than native LDL particles and promote foam cell formation through a mechanism that is independent of scavenger receptors SR-A and CD36.^{95–96} Using gain-of-function and loss-of-function approaches, Bostrom and coworkers have shown that macrophages are the major source of GV sPLA₂ in mouse lesions and that overexpression of GV sPLA₂ in bone marrow cells significantly increases collagen deposition in atherosclerotic lesions, providing the first *in vivo* data showing that GV sPLA₂ promotes atherosclerosis.⁹⁷

GX sPLA₂ shows the highest activity towards PC, which is the major lipid component of both LDL and HDL. Elevated expression of GX sPLA₂ was found in atherogenic lesions in

the arterial intima of both human and apoE-deficient mice fed a high fat diet.⁹⁸ LDL modified by GX sPLA₂ enhances accumulation of cholesterol ester in macrophages,⁹⁸ where it may promote the atherosclerotic process by hydrolyzing lipoproteins. Unlike GIIA and GV sPLA₂, GX sPLA₂ does not promote LDL particle aggregation and does not bind to proteoglycans.⁹⁹ Recent research has shown GX sPLA₂ may suppress macrophage expression of two transport proteins, ABCA1 and ABCG1, which efflux excess cholesterol to extracellular acceptors.¹⁰⁰ This indicates that GX sPLA₂ may negatively regulate the genes critical for cellular cholesterol efflux to affect atherosclerotic lipid accumulation.

In addition to GIIA, GV and GX, GIID, GIIE and GIII sPLA₂s were also expressed in macrophage and smooth muscle cells (SMCs), and their expression increased with atherosclerosis development.¹⁰¹ Intimal SMCs are thought to contribute to the progression of atherosclerosis due to their ability to produce an extracellular matrix which binds to low-density lipoprotein (LDL) and leads to further intimal cholesterol deposition.¹⁰²

Human GIII sPLA₂, which is distinctive among mammalian sPLA₂s due to its additional N- and C-terminal domains, may also play a role in atherosclerosis. Transgenic mice overexpressing human GIII sPLA₂ have marked alterations in the levels of plasma lipoproteins and GIII sPLA₂ modified LDL could promote the formation of foam cells.¹⁰³

sPLA₂ may also contribute to the atherosclerotic process by generating proinflammatory lipid mediators such as prostaglandins, thromboxanes and lysophospholipids. As we discussed in section 2.3.2, GIIA, GV and GX sPLA₂ may be involved in AA release and thus promote eicosanoid biosynthesis. Moreover, as part of the release of fatty acids, another proinflammatory lipid mediator – lysophospholipid – is produced. Lysophosphatidylcholine, a highly atherogenic lipid, can induce multiple deleterious processes in the atherosclerotic plaque.¹⁰⁴ In addition, GX sPLA₂ can hydrolyze both platelet activating factor (PAF) in free form and PAF that has been partitioned into either large unilamellar PC vesicles or lipoproteins,¹⁰⁵ which indicates that GX sPLA₂ may function like GVIIA lipoprotein-associated PLA₂ (Lp-PLA₂), which regulates PAF in the inflammatory process.

sPLA₂ may also exacerbate atherosclerosis through induction of proinflammatory cytokines. NFκB is considered to be a key regulator of inflammation in the atherosclerosis process.¹⁰⁶ LDL containing GV sPLA₂ incubated with J-774 macrophage-like cells showed a significant increased TNF-α and IL-6 mRNA expression and the induced cytokine secretion was promoted by activation of NFκB.¹⁰⁷ The activation of NFκB was promoted by the lipid products generated by GV sPLA₂ hydrolysis of LDL.¹⁰⁷

2.3.4 Other Functions—Many of the biological functions of sPLA₂s appear to be independent of their catalytic activity. A variety of sPLA₂s exhibit potent anticoagulant activity.^{67b} The mammalian GIIA, GIID and GV sPLA₂s, in addition to several venomous sPLA₂s, contain many basic residues on the protein surface which inhibit prothrombinase activity by binding to factor Xa. This effect is independent of phospholipid hydrolysis.¹⁰⁸ However, at limiting concentrations of phosphatidylserine, sPLA₂ enzymes inhibit the prothrombinase complex in a phospholipid-dependent manner either by hydrolyzing or binding to phospholipids and therefore inhibiting formation of coagulation complexes.¹⁰⁸

Two mammalian cell surface sPLA₂ receptors, N-type and M-type receptors, have been identified and found to bind to both sPLA₂s from venom and mammalian.¹⁰⁹ The N-type receptor is highly expressed in mammalian brain membranes. The M-type receptor is a 180 kDa protein, membrane-bound and soluble secreted receptor.^{17d} The M-type receptor was cloned in various mammalian species and is expressed in various tissues. Several lines of

evidence indicate that sPLA₂s may exert physiological roles by acting in a cytokine-like fashion and independent of their catalytic function.^{79a,79c,110}

GIB sPLA₂, which is present at high levels in pancreatic juice, is the major enzyme responsible for the dietary phospholipid phosphatidylcholine.¹¹¹ GIB knockout mice fed on a chow diet show no difference in the absorption of dietary lipids compared with wild type mice.^{111a} However, GIB knockout mice fed a high-fat diet are resistant to diet-induced obesity and obesity-related insulin resistance.¹¹²

Higher levels of sPLA₂ activity were found in the bronchoalveolar lavage fluid of asthmatic patients than that of control groups.¹¹³ GIIA and GX sPLA₂s were found in the airways of both asthmatic patients and controls. These two sPLA₂s should be responsible for the majority of PLA₂ enzymatic activity detected in the bronchoalveolar lavage fluid.¹¹⁴ GX sPLA₂ is more highly expressed than GIIA sPLA₂ in the airway epithelium of asthmatic patients.¹¹⁴ In a mouse asthma model, GX sPLA₂ deficiency was shown to potentially reduce allergen-induced airway inflammation and remodeling.⁷⁶ When transgenics expressing human GX sPLA₂ in GX sPLA₂ knockout mice were used in a recent study in a Th2 cytokine driven mouse asthma model, the mouse shown an induced airway inflammation and hyperresponsiveness which was not found in the GX sPLA₂ knockout mouse asthma model.¹¹⁵ Altogether these studies suggest that GV sPLA₂ may play an important role in asthma.

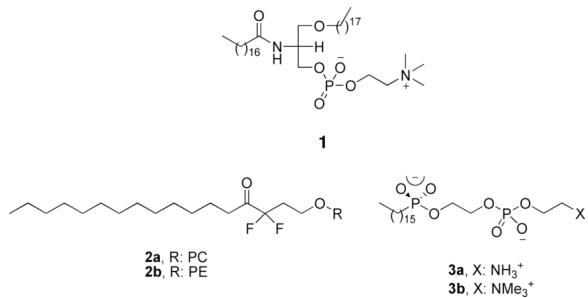
sPLA₂s may also play a role in tumorigenesis.^{16e} GIIA sPLA₂ is overexpressed in almost all human prostate cancer specimens and increased levels correlate with advanced tumor grades.¹¹⁶ GIIA sPLA₂ may stimulate tumor cell growth¹¹⁷ and be involved with the progression of prostate cancer, and could therefore potentially serve as a biomarker for prostate cancer.¹¹⁸ Increased expression of GIIA sPLA₂ was also detected in colorectal adenomas of familial adenomatous polyposis patients, and the enzyme may also be involved in human colorectal tumor development and progression¹¹⁹. However the protective role of GIIA sPLA₂ in colorectal cancer was also found in a mouse model. Transgenic Min (multiple intestinal neoplasia) mice carrying the functional PLA2G2A^{AKR} allele have shown resistance to tumor development, including both reduced tumor multiplicity and size.¹²⁰ In addition to GIIA, GIB, GX and GIII sPLA₂s are expressed in various type of cancers and may also play a role in tumorigenesis.^{16e} However the function of sPLA₂s in cancer is a controversial issue, as it is not clear whether the enzyme is a tumor suppressor or tumor promoter.¹²¹

2.4 Chemical Inhibitors and Therapeutic Intervention

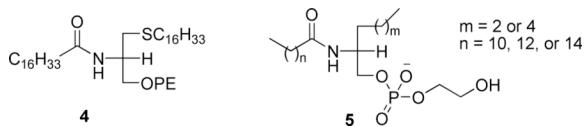
Numerous assays to evaluate the activity of inhibitors toward each PLA₂ type exist,¹²² ranging from simple procedures to methods involving expensive instrumentation and from low sensitivity to highly sensitive procedures. As mentioned in section 1.2, it has to be emphasized that it makes no sense to compare the activity of various inhibitors (for example, the IC₅₀ values) if they are measured in different assay systems, especially if assayed at different substrate concentrations. The IC₅₀ is the inhibitor concentration that is required to reduce the activity of the enzyme in half. A review article in 2005 reported the various inhibitors of sPLA₂s.^{22b} The most recent review article on PLA₂ inhibitors highlights the recent patent literature.^{22f}

2.4.1 Early Attempts with Phospholipid Analogues—The early attempts to develop PLA₂ inhibitors were focused on phospholipid analogues and started in earnest in the 1980's. 1-Stearyl-2-stearoylaminodeoxy phosphatidylcholine (**1**) was studied and found to be a reversible inhibitor of PLA₂ from cobra venom (*Naja naja naja*).¹²³ At the same time, A series of long chain difluoro ketones was studied.¹²⁴ Derivative **2a** based on

phosphatidylethanolamine was the most active in this series against cobra venom PLA₂. Phospholipid analogues (**3**) containing a phosphonate group in place of the ester at the *sn*-2 position of the glycerol backbone were found to be tight-binding inhibitors of the same enzyme.¹²⁵

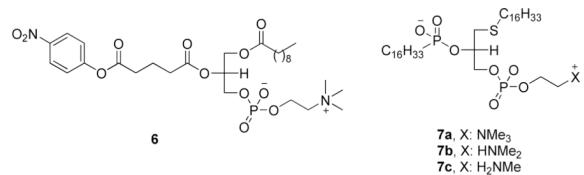


A series of structurally modified phospholipids were used to delineate the structural features involved in the interaction between cobra venom PLA₂ and its substrate.¹²⁶ A very potent inhibitor (thioether amide PE, **4**) was identified among them. At the same time, a class of acylamino analogues of phospholipids (**5**) was developed and studied as inhibitors of porcine pancreatic PLA₂.¹²⁷

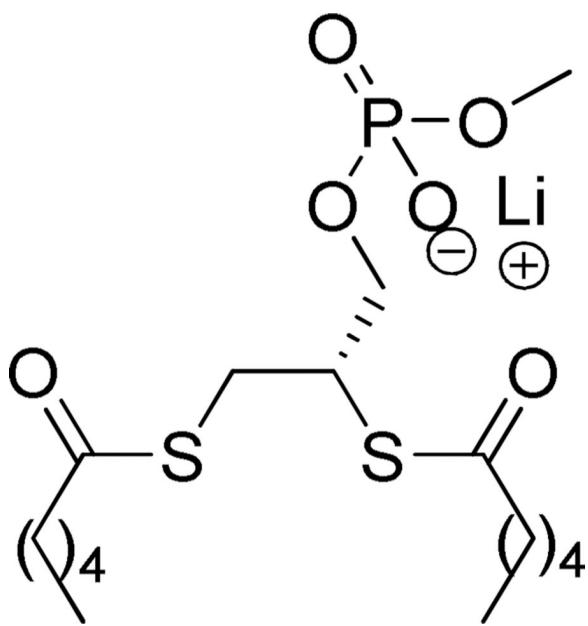


Some of the above mentioned inhibitors, an acylamino and a phosphonate phospholipid analogue, were useful tools to resolve the structure of various sPLA₂s by X-ray crystallography.^{30a,33,33,128} Thus, the interfacial catalytic mechanism of sPLA₂ was proposed.³³ The first structure of recombinant human synovial fluid PLA₂ was reported in 1991¹²⁸ and it was expected to be applied in structure based design.

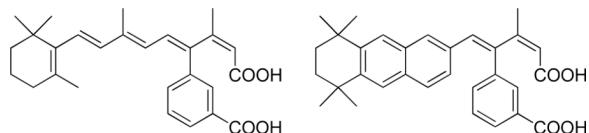
A class of “suicide-inhibitory bifunctional linked substrates” (SIBLINKS, for example compound **6**) has been reported.¹²⁹ In addition, these investigators also studied the effect of polar head groups on the interaction of cobra venom PLA₂ with phosphonate transition-state analogues **7**.¹³⁰



The substrate specificity at the active site of recombinant human synovial fluid PLA₂ was investigated by using a series of short-chain phospholipid analogues such as **8**.¹³¹

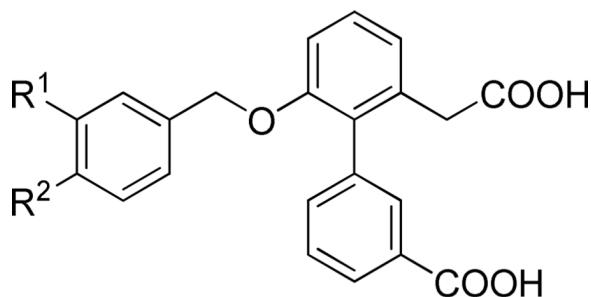


2.4.2 Dicarboxylic Acids—In 1992, Bristol-Myers Squibb presented the dicarboxylic acid **9a** (BMS-181162) as the first specific inhibitor of a 14 kDa PLA₂ (specifically compared to other types of phospholipases, PLC, PLD and PLA₁).¹³² BMS-181162 blocked the arachidonic acid release (IC_{50} 10 μ M) and the biosynthesis of LTB4 and PAF in calcium ionophore (A23187) stimulated human PMN's.^{132b} A similar derivative, BMS-188184, presented better stability and activity as a backup agent for BMS-181162 in clinical studies. BMS-181162 reached Phase II clinical trials as a cream for topical application for the treatment of psoriasis, but the results were disappointing as the drug could not penetrate beyond the outer layer of the skin. After the evaluation of these results, this inhibitor series was discontinued.

**9a, BMS-181162****9b, BMS-188184**

The mechanism of inhibition of GIIA sPLA₂ (referred to by the authors as human nonpancreatic sPLA₂) by the anti-inflammatory agent BMS-181162 was studied.^{132c} BMS-181162 inhibited human platelet PLA₂ with an IC_{50} 40 μ M and it was able to reduce mouse ear edema with an ED_{50} 160 μ g/ear in a phorbol-ester induced acute inflammation assay, while BMS-188184 inhibited human platelet PLA₂ with an IC_{50} 17 μ M and reduced mouse ear edema with an ED_{50} 9.37 μ g/ear.^{132d} It is unclear whether the inhibition observed in the mouse ear edema model reflected inhibition of just GIIA sPLA₂ or other PLA₂s as well.

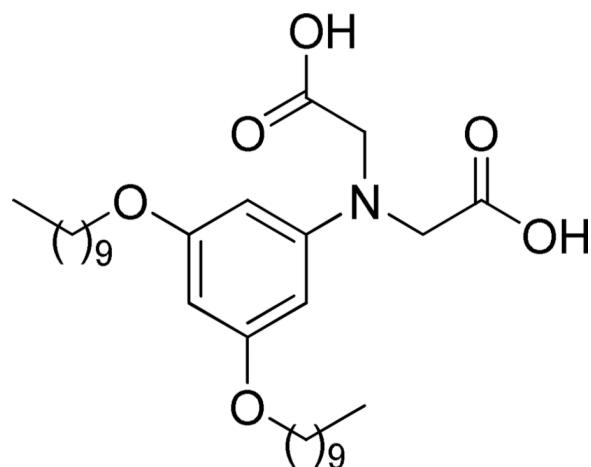
Among a series of biaryl diacid inhibitors of human sPLA₂, biarylacetic acid derivatives were found to be more active than biaryl acids or biarylpropanoic acids.¹³³ Compounds with larger hydrophobic groups were usually more potent inhibitors of the enzyme. Compounds **10a** and **10b** were found to possess significant anti-inflammatory activity in a phorbol ester induced mouse ear edema model of chronic inflammation (Table 3).



10a, $R^1=R^2=OC_5H_{11}$

10b, $R^1=H$, $R^2=OC_{10}H_{21}$

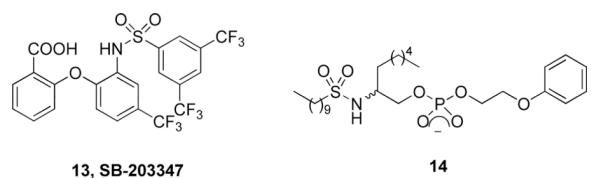
Through computer-assisted methods, Roche developed the very potent inhibitor **11** containing the iminodiacetic acid group (IC_{50} 0.23 μM for human synovial fluid PLA₂).¹³⁴ Inhibitor **11** exhibited anti-inflammatory activity in two separate animal models of inflammation.



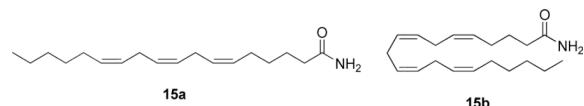
11

2.4.3 Sulfonamides—A novel series of benzenesulfonamides were prepared and evaluated as membrane-bound PLA₂ inhibitors.¹³⁵ Several compounds (**12a,b**), which proved to be potent inhibitors in vitro, significantly reduced the size of myocardial infarction in coronary occluded rats by iv administrations prior to the ligation. Compound **12a** (ER-3826), which showed the protective in vivo effects at doses higher than 0.3 mg/kg iv (Table 4), was finally chosen as a leading candidate.¹³⁵

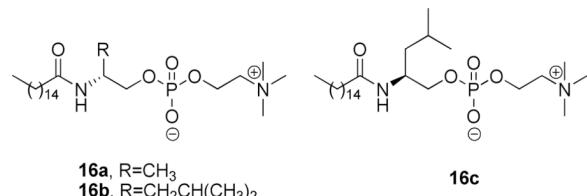
Sulfonamide **13** (SB-203347) was used to understand the contribution of GII sPLA₂ to prostaglandin formation.¹³⁶ Computer-assisted methods contributed to the rational design of the sulfonamide inhibitor **14**.¹³⁷



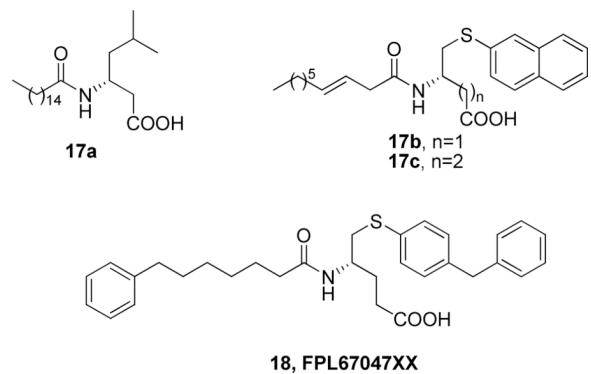
2.4.4 Amides—Taking into consideration that ionic phospholipid analogues would not be cell permeable and therefore not useful in determining the roles of PLA₂s in cellular processes, primary amides of long unsaturated acids were synthesized and studied. Two of them (**15a,b**) presented interesting inhibition of porcine pancreatic and human synovial fluid PLA₂ (Table 5).¹³⁸



Acylamino phospholipid analogues were synthesized and evaluated as pancreatic PLA₂ inhibitors (Table 6).¹³⁹ The mode of binding of these inhibitors to the active site of the enzyme was determined using two-dimensional NMR and molecular modeling techniques.



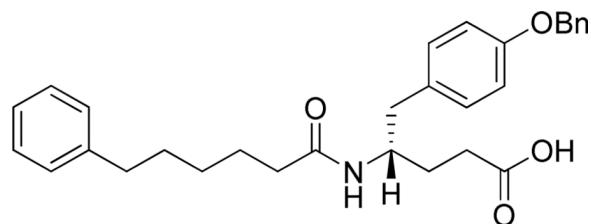
A continuation of this work reported non-phospholipid inhibitors of sPLA₂, where a simple carboxylic group replaced the phosphocholine moiety.¹⁴⁰ Structure-activity relationship studies led to interesting results (Table 7). The most potent inhibitor in this series (FPL67047XX) presented an IC₅₀ value against the human platelet sPLA₂ of 21 ± 4 nM. The precise binding interactions of this inhibitor with the human nonpancreatic sPLA₂ were determined by high-resolution X-ray crystallography.¹⁴¹



Most recently, new GIIA sPLA₂ inhibitors were designed based on docking calculations by modifying the pharmacophore segments of the FPL67047XX inhibitor.¹⁴²

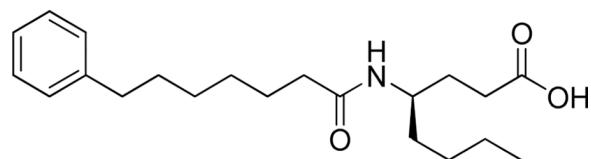
Another similar series of potent inhibitors was created by derivatization of D-tyrosine.¹⁴³ The activities of various derivatives are summarized in Table 8. Inhibitor **19b** (R=benzyl) was co-crystallized with hGIIA PLA₂ and the crystal structure revealed a chelation to a Ca²⁺

ion through carboxylate and amide oxygen atoms, H-bonding through an amide NH group to His48, multiple hydrophobic contacts and a T-shaped aromatic group – His6 interaction.¹⁴³

**19a** IC_{50} 0.662 μM

Inhibitor **19b** (R=benzyl) was found to protect the rat small intestine from I/R injury after oral or intravenous administration.¹⁴⁴ In addition, this inhibitor of GIIA sPLA₂ protected rats from TNBS-induced colitis,¹⁴⁵ exhibited antifibrotic activity in young spontaneously hypertensive rats,¹⁴⁶ and preserved bone architecture following ovariectomy in adult rats.¹⁴⁷

A recent study on natural and non-natural amino acid-based amide and 2-oxoamide inhibitors of human PLA₂ enzymes showed that amide **20**, based on (*R*)- γ -norleucine, is a selective inhibitor of GV sPLA₂ ($X_I(50)$ 0.003 \pm 0.0004) not affecting the activities of intracellular GIVA PLA₂ and GVIA PLA₂.¹⁴⁸

**20**

2.4.5 Indoles—In 1995, researchers at Lilly reported a highly potent sPLA₂ inhibitor having a novel indole structure by using computer-aided drug design and chemical modification of a lead compound, which was discovered in the course of high-volume screening. Inhibitor **21a** was co-crystallized with human recombinant GIIA PLA₂ and the three dimensional structure showed that the inhibitor was in fact located in the active site.¹⁴⁹ The replacement of the carboxylic acid functionality with an amide one and the methyl group by an ethyl group led to considerable increase of activity (Table 9).¹⁵⁰

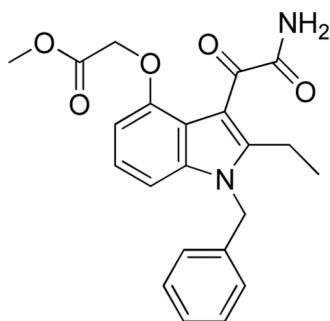
Further implementation of this structure-based design strategy and continued SAR development led to indole-3-acetamides with additional functionalities which provide increased interaction with important residues within the enzyme active site. These inhibitors **22** presented substantially enhanced potency and selectivity (Table 10).¹⁵¹

Structure-activity relationship studies were extended to include a series of indole-3-glyoxamide derivatives. Functionalized indole-3-glyoxamides with an acidic substituent appended to the 4- or 5-position of the indole ring were prepared and studied. Indole-3-glyoxamides with a 4-oxyacetic acid substituent had optimal inhibitory activity. These inhibitors exhibited an improvement in potency over the best of the indole-3-acetamides

(Table 11), and LY315920 or Varespladib (**24**) was selected for evaluation clinically as a hGIIA PLA₂ inhibitor.¹⁵²

LY315920 was 40-fold less active against human GIB pancreatic PLA₂ and was inactive against cPLA₂ and the constitutive and inducible forms of cyclooxygenase.¹⁵³ LY315920Na showed prophylactic effects on the high mortality, severe pancreas tissue damage, and blood biochemical changes in a lipolytic enzyme-related severe pancreatitis model.¹⁵⁴ Varespladib was advanced in clinical trials as an intravenously-administered therapy for sepsis-induced systemic inflammatory response syndrome.¹⁵⁵ At the end of the Phase I study, Varespladib was found to have an acceptable safety profile in patients with severe sepsis.¹⁵⁶ However, the development of Varespladib for the treatment of severe sepsis was terminated because the Phase II study showed poorer than expected efficacy.

Lilly also synthesized methyl varespladib **25**, LY333013, which functions as a prodrug and is rapidly converted in vivo to Varespladib. Using inhibitor **25** the role of GIIA PLA₂ in rat colitis induced by dextran sulfate sodium was studied.¹⁵⁷ A randomized, double-blinded, placebo-controlled clinical trial of LY333013 showed that the treatment for 12 weeks was well tolerated, but ineffective as an adjunct to disease modifying antirheumatic drugs.¹⁵⁸ LY333013 was also used to study the possible role of GII sPLA₂ in asthma, however it had no impact on the primary outcome variables of the areas under the FEV1 response curve early (0–3 hours) (AUCEarly) and late (3 –8 hours) (AUCLate) following inhaled allergen challenge.¹⁵⁹



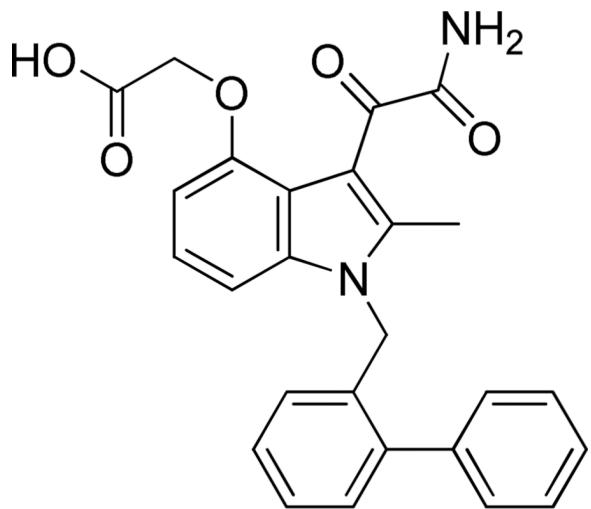
25, LY333013 or Methyl Varespladib

In 1996, Shionogi reported the synthesis, structure-activity relationship, and inhibitory activities of indolizine and indene derivatives. 1-(Carbamoylmethyl) indolizine derivatives were potent inhibitors, but were not stable to air oxidation. Introduction of an oxamoyl group to the C-1 position made the derivative stable and highly potent. By chemical modification at the C-3 position with various hydrophobic substituents and at the C-1 or C-8 position with hydrophilic substituents, some compounds approached the stoichiometric limit of the chromogenic assay (Table 12).¹⁶⁰

The effect of indoxam on murine endotoxic shock was studied and the results suggested that indoxam blocks the production of proinflammatory cytokines during endotoxemia through PLA₂-IIA independent mechanisms, possibly via blockade of the PLA₂R function.¹⁶¹

In 2002, the expression of the full set of human and mouse groups I, II, V, X, and XII sPLA₂s in *Escherichia coli* and insect cells provided pure recombinant enzymes for detailed comparative interfacial kinetic and binding studies. Analysis of the inhibition by a set of 12 active site-directed, competitive inhibitors revealed a large variation in the potency among

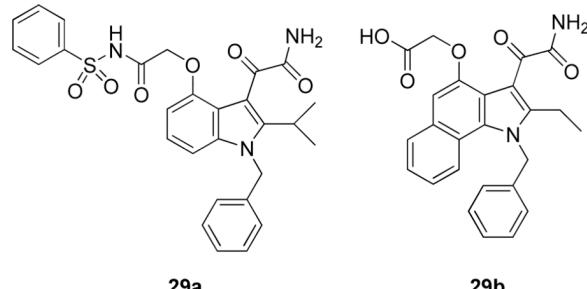
the mammalian sPLA₂s, with Me-Indoxam being the most generally potent sPLA₂ inhibitor.^{27a}



28, Me-Indoxam

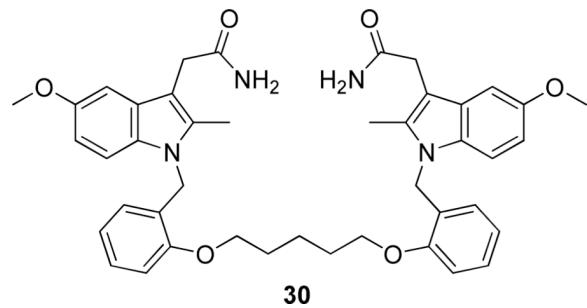
A structure-guided design was employed in a search for potent and selective inhibitors of mammalian sPLA₂s. Although no compounds were found to be highly specific for a single human or mouse sPLA₂, combinations of Me-Indoxam analogues were discovered that could be used to distinguish the action of various sPLA₂s in cellular events.¹⁶²

Using the X-ray structure of human GX sPLA₂, the first potent inhibitor of this enzyme was developed.¹⁶³ In addition, a series of inhibitors of sPLA₂s based on substituted indoles, 6,7-benzoindoles, and indolizines derived from LY315920 were reported.¹⁶⁴ Compound **29a** was found to be selectively potent against hGX over all other human and mouse sPLA₂ enzymes, while the substituted 6,7-benzoindole **29b** inhibited nearly all human and mouse sPLA₂s in the low nanomolar range.



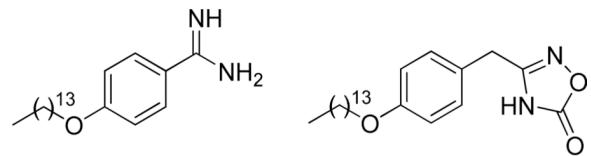
Most recently, molecular docking and 3D Quantitative Structure-Activity Relationship Comparative Molecular Field Analysis (3D-QSAR CoMFA) studies on indole inhibitors of GIIA sPLA₂ led to a model which was used for the design and evaluation of new compounds.¹⁶⁵

A series of bis-indole compounds were designed and synthesized on the basis of the enzyme structure of human nonpancreatic sPLA₂. Their inhibition activities were improved compared to that of the monofunctional protocompound. The potent compound **30** (IC₅₀ 24 nM) revealed that cooperative binding interactions between the two enzyme molecules also contributed to the stability of the ternary complex.¹⁶⁶



Recently, Anthera Pharmaceuticals disclosed the sPLA₂ inhibitors Varespladib (A-001, previously known as LY315920) and Varespladib methyl (A-002, previously known as LY333013) for the treatment of cardiovascular diseases.¹⁶⁷ A-002 was shown to lower levels of GIIAs PLA₂ by >90%, LDL-C by 12% to 18% and high-sensitivity CRP by 20% to 40% in stable CHD patients.¹⁶⁸ A-002 acts synergistically with pravastatin to decrease atherosclerosis in the heart and proximal aorta of apoE^{-/-} mice, possibly through decreased levels of systemic inflammation or decreased lipid levels.¹⁶⁹ The FRANCIS (Fewer Recurrent Acute Coronary Events With Near-Term Cardiovascular Inflammation Suppression, <http://clinicaltrials.gov/>, Identifier: NCT00743925) study demonstrated that treatment with Varespladib methyl reduced concentrations of LDL-C, hsCRP and sPLA₂ in ACS patients treated with evidence-based therapies inclusive of high-dose atorvastatin.¹⁷⁰ Enrollment has commenced in the Phase 3 clinical study named VISTA-16 (Vascular Inflammation Suppression to Treat Acute Coronary Syndrome for 16 Weeks, <http://clinicaltrials.gov/>, Identifier: NCT01130246). The primary objective of this study is to determine whether 16 weeks of treatment with A-002 plus atorvastatin and standard of care is superior to placebo plus atorvastatin and standard of care for reducing the hazard of the first occurrence of the combined endpoint of cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, or documented unstable angina with objective evidence of ischemia requiring hospitalization.

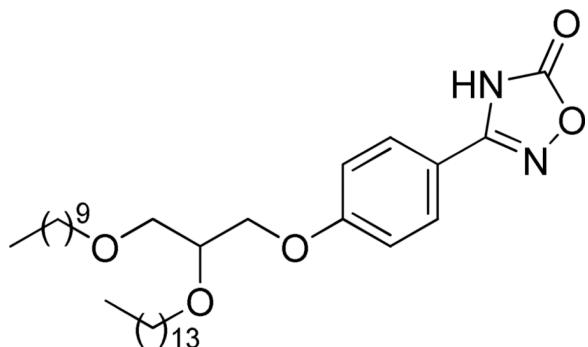
2.4.6 Oxadiazolones—A series of 4-alkoxybenzamidines was synthesized and their inhibitory potency against sPLA₂ was evaluated.¹⁷¹ 4-Tetradecyloxybenzamidine (**31a**, **PMS815**) was shown to exert an anti-inflammatory effect *in vivo* on the carrageenan-induced rat paw edema. Starting from **PMS815**, a series of oxadiazolones was synthesized and studied.¹⁷² The leading compound **31b** (**PMS1062**) exhibited a micromolar IC₅₀ towards three GII PLA₂s, while inactive towards four GI and one GIII enzymes in two *in vitro* enzymatic assay conditions.



31a, PMS815

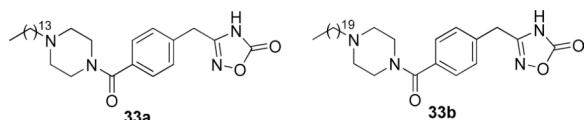
31b, PMS1062

In a continuation, glycerol-containing derivatives of PMS1062 were synthesized.¹⁷³ Among them, compound **32** was as potent as Me-Indoxam for hGIIA PLA₂.

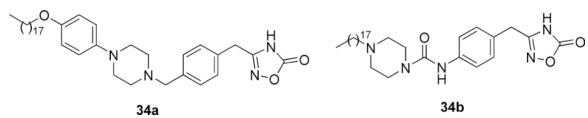


32

Replacement of the long chains by substituted piperazine derivatives led to inhibitors **33**.¹⁷⁴ Compound **33a** inhibited hGIIA sPLA₂ and in a carrageenan-induced edema test in rats showed to be as potent as indomethacin (Table 13).



Most recently, further SAR towards the change of the rigidity of the piperazine region produced the more potent inhibitors **34** (Table 13).¹⁷⁵



2.4.7 In Silico-Guided Identification of Inhibitors—A collection of 2150 druggable active sites from the Protein Data Bank was screened by high-throughput docking to identify putative targets for five representative molecules of a combinatorial library sharing a 1,3,5-triazepan-2,6-dione scaffold.¹⁷⁶ Out of the five proposed proteins, sPLA₂ was shown to be a true target for a panel of 1,3,5-triazepan-2,6-diones which exhibited micromolar affinities toward two human sPLA₂ members (Table 14).

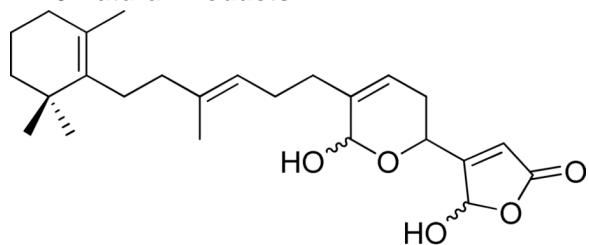
2.4.8 Aptamers and Peptides—A family of sequence-related 2'-aminopyrimidine, 2'-hydroxylpurine aptamers, developed by oligonucleotide-based combinatorial chemistry, SELEX (systematic evolution of ligand by exponential enrichment) technology, was found to bind human nonpancreatic sPLA₂ with nanomolar affinities and inhibit enzymatic activity.¹⁷⁷

A number of synthetic peptides were designed and screened for sPLA₂ inhibition.¹⁷⁸ The linear peptide **36** (PIP-18) inhibited the recombinant human synovial sPLA₂ activity with an IC₅₀ of 1.19 μM. The peptide interfered with the function of sPLA₂, but it also appeared to inhibit mRNA expression of sPLA₂ and various MMPs in IL-1β-stimulated RA synovial fibroblast (RASF) cultures and thereby the production of the corresponding proteins (>80% inhibition).

VDIHVWDGV-VDIHVWDGV

36, PIP-18

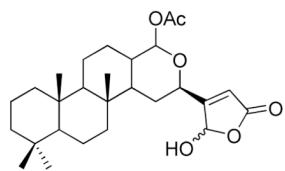
2.4.9 Natural Products—



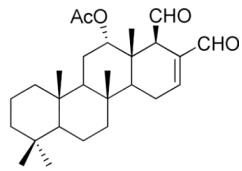
37, Manoalide

In the mid 1980s, the natural product manoalide **37** was reported to be the first inhibitor of cobra venom PLA₂.¹⁷⁹ Manoalide is a sesterpene, which was isolated in the early 1980s from the sponge *Luffariella variabilis* and it was found to have anti-inflammatory activity *in vivo*. Manoalide inhibits also bee venom PLA₂¹⁸⁰ and human synovial fluid PLA₂.¹⁸¹ Mechanistic studies on manoalide and analogues¹⁸² revealed that two specific lysine residues are responsible for the inhibition of the enzyme.^{183,184} Manoalide was licensed to Allergan Pharmaceuticals and reached Phase II clinical trials as a topical antipsoriatic, its development was, however, discontinued due to formulation problems.¹⁸⁵

A number of sesquiterpenes of marine origin that contain the γ -hydroxybutenolide moiety have been studied for their anti-inflammatory activity and inhibition of PLA₂. Petrosaspongiolide M (**38**) displayed a potent inhibitory activity toward GII and GIII sPLA₂ and the molecular basis of the inhibition of this product as well as petrosaspongiolides M-R was studied.¹⁸⁶ More recently, the binding mode of petrosaspongiolide to human GIIA PLA₂ was analyzed in detail.¹⁸⁷ Scalaradial (**39**) is another marine metabolite, which inhibits GII and GIII PLA₂ and presents *in vivo* anti-inflammatory activity.¹⁸⁸

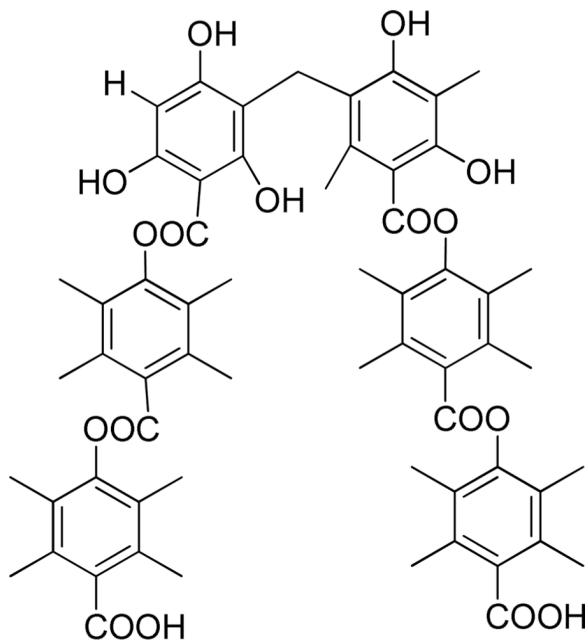


38, Petrosaspongiolide M



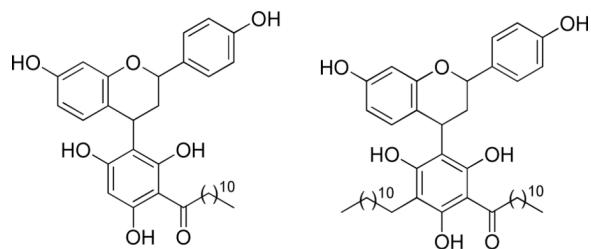
39, Scalaradial

Thielocin B3 is a very potent naturally occurring inhibitor of human nonpancreatic sPLA₂ (GII). Structure-activity relationship studies led to a number of analogues with potency comparable to the parent natural product.^{189,190}



40, Thielocin B3

YM-26567 (**41**), a natural product isolated from the fruit of *Horsfieldia amygdalina*, is a micromolar inhibitor of rabbit platelet sPLA₂.¹⁹¹ Further studies led to YM-26734 (**42**) which has increased potency against the enzyme (IC_{50} 85 nM)¹⁹² and to simplified analogues.¹⁹³



41, YM-26567

42, YM-26734

Flavonoids are widely produced polyphenolic plant secondary metabolites. Some flavonoids have demonstrated inhibition of PLA₂¹⁹⁴ and the mechanism of inhibition of human sPLA₂ has been investigated.¹⁹⁵

2.4.10 Summary Status of sPLA₂ Inhibitors—As presented above, a wide variety of structurally different inhibitors has been studied for their inhibition of several sPLA₂s. Starting in the mid 1980s, before crystal structures were widely available, the first studied inhibitors of sPLA₂ were substrate analogues and marine natural products. The first potent and specific (when compared to PLC, PLD and PLA₁) dicarboxylic acid inhibitor of sPLA₂ was presented by Bristol-Myers Squibb in 1992. It reached Phase II clinical trials for the topical treatment of psoriasis, but since it could not reach the inner layer of the skin, the studies were discontinued. Sulfonamide and various amide inhibitors developed over the

years proved useful tools for mechanistic studies. Indole inhibitors have attracted a great deal of attention as drug candidates and they constitute the most comprehensively studied class of sPLA₂ inhibitors. Lilly Research Laboratory presented a highly potent sPLA₂ inhibitor (LY315920) which was developed via molecular modeling techniques combined with chemical modification of a lead compound. This inhibitor, which is selective for GIIA sPLA₂, reached Phase II studies as a treatment for severe sepsis, but the trials were discontinued when results did not meet expectations. One of the reasons that indole inhibitors may not have shown the expected efficacy in clinical studies may be that they are cell impermeable and therefore incapable of blocking intercellular effects. Even though sPLA₂ is a secreted enzyme, inhibitors that are both potent and cell permeable may show greater effects clinically. Furthermore, similar properties in inhibitors may affect tissue distribution and permeability as well but this has not been extensively investigated.

A prodrug of the Lilly inhibitor was also used to study the possible role of GII sPLA₂ in asthma. At the same time, a structurally similar inhibitor was introduced by Shionogi, namely Me-Indoxam. A few years later, when the full set of human sPLA₂s was expressed, indole-type inhibitors were developed that could somewhat distinguish the action of various sPLA₂s in cellular events, even though no compound was highly specific for a single sPLA₂. Currently, the previously mentioned LY315920, also called Varespladib or A-001, together with Varespladib methyl or A-002 are in Phase III trials by Anthera Pharmaceuticals to treat cardiovascular diseases. In the quest for sPLA₂ inhibitors, one should keep in mind that while selective sPLA₂ inhibitors can help us study the biological activity of specific sPLA₂s *in vitro* and *in vivo*, they are also essential for helping us to distinguish the activities of the different groups of sPLA₂.

3. Cytosolic Phospholipase A₂ [Group IV cPLA₂]

3.1 Groups, Subgroups, Specificity and Mechanism

The first cytosolic PLA₂, now attributed to GIVA PLA₂ (or cPLA₂ α), was reported as an activity by Christina Leslie and Ruth Kramer in human neutrophils¹⁹⁶ and platelets¹⁹⁷ respectively in 1986. The enzyme was purified, sequenced and cloned by James Clark at the Genetics Institute and Ruth Kramer at Lilly Research Laboratories in 1991.^{5–6} GIVB PLA₂ (cPLA₂ β)¹⁹⁸ and GIVC PLA₂ (cPLA₂ γ)^{198a,199} were subsequently reported in 1998–99 by Lilly Research Laboratories and Genetics Institute investigators. GIVD (cPLA₂ δ), GIVE (cPLA₂ ε) and GIVF (cPLA₂ ζ) PLA₂ were identified in mice by the Shimizu's group in Japan in 2004–2005.²⁰⁰ Currently, the group IV PLA₂ is comprised of these six known phospholipases.¹⁴ A summary of the characteristics of each member of the GIV PLA₂ family (Table 15) and a schematic presentation of the sequences (Figure 6) provide an overview of this PLA₂ group.

3.1.1 Group IVA Phospholipase A₂ (cPLA₂ α)—After the identification of cytosolic PLA₂ in neutrophils and platelets by cell-free assay,^{196–197} the human GIVA PLA₂ was cloned and sequenced in 1991 from U937 cells.^{5–6} The GIVA PLA₂ gene has been mapped to human chromosome 1q25 and is ubiquitously expressed in most human tissues (Table 15).²⁰¹ This enzyme contains 749 amino acids and was shown to be a 85 kDa protein using SDS-PAGE.^{5–6} GIVA PLA₂ consists of an N-terminal C2 domain and a C-terminal catalytic domain (Figure 6),^{5,202} and the structural details of this enzyme can be seen in the X-ray crystal structure produced by Dessen et al in 1999.^{202a} GIVA PLA₂ is regulated by intracellular calcium, and calcium binding to the C2 domain of GIVA PLA₂ can activate the enzyme resulting in the localization of the enzyme to the phospholipid membrane.^{5,203} After localization to the membrane, the catalytic domain of GIVA PLA₂ utilizes an active site dyad Ser-228/Asp-549, located in the α/β hydrolase domain, to catalyze the hydrolysis.^{202a,204} In addition, MAP kinase phosphorylation²⁰⁵ and the lipid mediators

ceramide-1-phosphate (C1P)²⁰⁶ and phosphatidylinositol 4,5-bis phosphate (PIP₂)²⁰⁷ were shown to further increase GIVA PLA₂ activity.

Most phospholipids, such as phosphatidylcholine (PC), phosphatidylethanoamine (PE) and phosphatidylinositol (PI), are substrates of GIVA PLA₂.²⁰⁸ Depending on the experimental setup, the preference for these phospholipids may vary slightly. But, PC is a relatively good substrate and is commonly used to determine the enzymatic activity of GIVA PLA₂ since its discovery.^{5–6} GIVA PLA₂ hydrolysis of phospholipid substrates has high substrate specificity for arachidonic acid at the *sn*-2 position.^{5,18a} In addition to phospholipase activity, GIVA PLA₂ also possesses lysophospholipase activity and transacylase activity.^{204b} In contrast to the calcium dependent phospholipase activity, the lysophospholipase activity toward micelle substrates was found to be calcium-independent.^{204b} Recently, a comprehensive interfacial kinetic study compared the activities of all six GIV PLA₂ isoforms and showed that they all exhibit phospholipase A₁/A₂ and lysophospholipase activities.²⁰⁹

3.1.2 Group IVB Phospholipase A₂ (cPLA₂β)—The full length human GIVB PLA₂ was initially cloned in 1999 and the recombinant protein was then expressed in Sf-9 insect cells.¹⁹⁸ The GIVB PLA₂ gene is encoded by chromosome 15q11.2–q21.3 and the mRNA is expressed ubiquitously, but found at especially high levels in the pancreas and cerebellum (Table 15).¹⁹⁸ The enzyme contains 1012 amino acids and its size is reported at 100–114 kDa using SDS-PAGE (Figure 6).¹⁹⁸ GIVB PLA₂ contains a C2 domain and a catalytic domain that are similar to those in GIVA PLA₂, with the primary sequences sharing 30% identical residues.^{18a} The major difference is that the GIVB PLA₂ has two insertions (before and after the C2 domain). The first is a 242-residue insertion at the N-terminus and the second is a 120-residue insertion between the C2 domain and the catalytic domain (Figure 6). The 242-residue insertion is part of a JmjC domain, which is often found to regulate chromatin stability in nuclear proteins.²¹⁰ Interestingly, the JmjC domain is in the gene upstream from that of GIVB PLA₂. The α/β hydrolase domain is highly homologous to GIVA PLA₂ and the active site dyad Ser-538 and Asp-566 is conserved. When we ran a multiple sequence alignment of all six GIV PLA₂s, the alignment shows that GIVB PLA₂ does not have the phosphorylation sites, C1P binding sites, or PIP₂ binding sites, and has a significantly different cap and lid region (Figure 6). Three GIVB PLA₂ splice variants, b1, b2 and b3, have been observed.²¹¹ The alternative splicing on the catalytic domain results in the splice variants b2 and b3, which are shorter than the original b1 variant. GIVB PLA₂ preserves the conserved calcium binding sites on the C2 domain in the sequence alignment and thus demonstrates calcium-dependent PLA₂ activity. Compared to GIVA PLA₂, its phospholipase activity is low and it has low substrate specificity for *sn*-2 fatty acids.^{14,212} It also has low lysophospholipase A₁/A₂ and transacylase activities.^{198b,204b,213}

3.1.3 GIVC PLA₂ (cPLA₂γ)—The human GIVC PLA₂ gene, located at chromosome 19q13.3, was cloned in 1998 (Table 15).^{198a,199} Its transcript is expressed highly in the heart and skeletal muscle.^{198a,199} The GIVC PLA₂ is a 61 kDa protein and has 541 amino acid residues, showing 30% homology to the catalytic domain of GIVA PLA₂ (Figure 6). The active site dyad in the catalytic domain is conserved. Because this enzyme does not contain a C2 domain, GIVC PLA₂ has no calcium-dependent phospholipase activities.^{198a,199} Neither the C1P nor the PIP₂ binding sites nor the phosphorylation sites are observed. Cys-538 has shown to be farnesylated in insect cells.²¹⁴ GIVC PLA₂ is also able to hydrolyze both *sn*-1 and *sn*-2 acyl chains from a phosphatidylcholine substrate with PLA₂ and PLA₁ activity.^{198b,199} It has low *sn*-2 specificity for arachidonic acid, less than the GIVA PLA₂.¹⁹⁹

3.1.4 GIVD (cPLA₂δ), GIVE (cPLA₂ε) and GIVF (cPLA₂ζ)—The human GIVD PLA₂ gene was found to be differentially expressed between normal and psoriatic skin, and was then cloned in 2004.^{200b} The Shimizu laboratory later performed the cloning of murine GIVD, GIVE and GIVF in 2005.^{200a} All three genes form a cluster with GIVB PLA₂ at human chromosome 15q15.1 (Table 15). The murine transcripts of all three genes are tissue specific. GIVD PLA₂ was detected in the placenta, GIVE PLA₂ was detected in the thyroid, heart and skeletal muscles, and GIVF PLA₂ was detected in the thyroid.^{200a} The sequence lengths of GIVD, E and F PLA₂ are 818 amino acids/91 kDa, 838 amino acids/95 kDa and 849 amino acids/95 kDa respectively (Figure 6). All three proteins contain a conserved C2 domain and a phospholipase domain. The calcium binding sites are conserved in the C2 domain and the active site dyad is also conserved in the catalytic domain, but no C1P, PIP2 or phosphorylation sites are observed. All GIVD, E and F PLA₂s exhibit calcium-dependent PLA₂ activities.^{200a} GIVF PLA₂ has higher activity toward phosphatidylethanolamine (PE) than phosphatidylcholine (PC), which is the opposite of GIVA PLA₂, suggesting preference for charged substrates. A comparison of the three enzymes shows GIVF PLA₂ has higher PLA₂ activity than GIVD PLA₂ and GIVE PLA₂. GIVF PLA₂ shows high phospholipase A₂ and lysophospholipase activities.²¹⁵ In response to ionomycin, GIVF PLA₂ translocates to ruffles and dynamic vesicular structures, which is different from how GIVA PLA₂ translocates to the Golgi and endoplasmic reticulum.²¹⁵ The biological functions of these enzymes have not been well studied to date.

3.2 Structural Characteristics and Activation Mechanisms

The six members of GIV PLA₂ show some structural similarities in the C2 and the α/β hydrolase domains, but the differences among them are significant. Because our understanding of GIVB, C, D, E and F PLA₂ are still limited, we utilize the well-studied GIVA PLA₂ enzyme to represent the structural characteristics and activation mechanisms of the entire GIV PLA₂ type in this section. The early work showed that GIVA PLA₂ is activated by calcium, and determined that the C2 domain is responsible for the calcium binding and GIVA PLA₂ translocation.^{202b,203} The crystal structure and the NMR structure of the C2 domains were reported in 1998, and showed calcium binding to the C2 domain.^{202b,216} Dessen et al. (1999) solved the crystal structure of the whole GIVA PLA₂^{202a}, including the C2 domain which is linked to a catalytic domain (Figure 7). The C2 domain structure of the intact enzyme is similar to the C2 domain structure alone, showing two calcium ions binding to the anion hole at the tip of the C2 domain.

The catalytic domain is tethered to the C2 domain by a single peptide strand consisting of residues 139–143. The catalytic domain is composed of an α/β hydrolase core and a cap region. The α/β hydrolase core folds similarly to many lipases, but the sequence similarity is low.^{202a} The active site is located on the top of the α/β hydrolase core, which is also similar to other lipases. The novel cap region (residues 370–548) is found only in GIVA PLA₂, and is not conserved in other members of the GIV PLA₂ family. The homologies among GIVA, B and C PLA₂ in the cap region are very low. The homologies in the cap region among GIVD, E and F PLA₂ are higher than the rest of GIV PLA₂s. The cap region of GIVA PLA₂ sits on top of the α/β hydrolase core and so the active site dyad is buried within a tunnel. The cap region seems to be very flexible, so that a wide range of residues cannot provide enough electron density for crystallization.

Within the cap region, a segment of peptide (415–432) has been crystallized, characteristically surrounded by low-electron density residues. This segment is right on top of the active site and is defined as a lid. The presence of a lid region blocks the phospholipid substrate from approaching the active site. The region of the lid that faces away from the enzymes contains three Glu residues, Glu-418, Glu-419, and Glu-420. In addition, one of the

hinges that hold the lid in place also contains four negatively charged groups, i.e., Asp-436, Asp-438, Asp-39, and Asp-440. It appears that they interact with the lipid interface and that the presence or absence of negatively-charged lipids in the surface affects binding due to their interactions with these groups. At the same time the negative interactions may facilitate the movement of the lid away from the catalytic site.

This crystal structure defined the catalysis pocket, which is composed of Ser-228 in the consensus sequence GXSXS and Asp-549. These two residues act as an active site dyad (Figure 7) for both PLA₂ and lysophospholipase activities. Arg-200 was shown to be critical for phospholipase activity^{204a} and is in the proximity of the pocket. It may bind to the charged headgroups of the phospholipid substrate and may therefore be responsible for substrate affinity and the release of lysophospholipid.^{202a,217} Although the determination of the crystal structure has led to an understanding of the GIVA PLA₂ catalytic activity, due to the lack of electron density in some regions of the crystal structure, the C2 domain and the catalytic domain do not show any contact or interaction. This disparity with the deuterium exchange results suggests that the unstructured linker region is the only region that could bring the two domains together. The hydrogen/deuterium exchange experiments on the intact GIVA PLA₂ enzyme allowed the determination of the rates of exchange in regions that lack electron density. Additionally, the exchange results of the intact enzyme were compared with the C2 domain deletion mutant and the catalytic domain alone. The hydrogen/deuterium exchange experiments indicated that there are extensive intradomain interactions between the C2 domain and the catalytic domain.²¹⁸ The cap region shows a significant increase in deuterium exchange in the C2 domain deletion mutant, suggesting the intradomain interaction may play a role in the enzymatic activity.

The activation mechanism of this enzyme includes many steps. The enzyme is first recruited to the membrane by a calcium-dependent translocation of the C2 domain. The lipid second messengers ceramide-1-phosphate²¹⁹ and phosphatidylinositol (4, 5) bisphosphate²²⁰ can further activate the enzyme, at least in vitro. The phosphorylation status has also been shown to regulate the enzymatic activities.^{205,221}

3.2.1 Calcium Activation—In response to extracellular stimuli, the intracellular Ca²⁺ concentration increases, and the GIVA PLA₂ then translocates from the cytosol to the perinuclear membrane region.²⁰³ Calcium binding to the C2 domain is crucial for membrane localization, but is not directly involved in the catalytic activity of the GIVA PLA₂.^{202a} The GIVA PLA₂ C2 domain has three calcium binding loops, CBL1, CBL2, and CBL3, which form an anion hole on the tip of the C2 domain. Two calcium ions coordinate with the calcium-binding loops, CBL1, CBL2, and CBL3, in the C2 domain, as illustrated in Figure 7.^{202,216} These two Ca²⁺ ions neutralize the negative charge in the anion hole to facilitate the C2 domain's hydrophobic interaction with phospholipid membranes.^{202,216} We utilized hydrogen/deuterium (H/D) experiments to show that calcium binds to a low H/D exchange region, indicating that the anion hole is a stable region before calcium binding.²¹⁸ Calcium binding also causes a conformational change in the C2 domain to stabilize the C2 domain structure, as shown by a decreased rate of H/D exchange.²¹⁸ This rigidified conformation of the C2 domain is crucial for proper orientation of the stabilized C2 domain binding to the phospholipid membrane, leading to enzyme interfacial activation.

The calcium-bound C2 domain is also important for phospholipid hydrolysis by GIVA PLA₂. The catalytic domain of GIVA PLA₂ alone does not have phospholipase activity.^{218,220} We have shown that the intradomain interactions between the C2 domain and the catalytic domain stabilize both domains²¹⁸. This intradomain interaction changed our understanding of the relative position between the C2 domain and the catalytic domain as illustrated in the crystal structure.²¹⁸ Membrane binding to the calcium-activated GIVA

PLA₂ has shown an increased H/D exchange on the cap region, including the part of the region involved in the intradomain interaction.²²² Active site targeting inhibitors also shows changes in the H/D exchange rate in the same cap region.²¹⁷ While the calcium binding effects are mainly seen in the C2 domain, calcium binding may extend its effects and have implications for the catalytic domain as well through the contact residues in the cap region.

3.2.2 PIP₂/C1P Activation—GIVA PLA₂ is also activated by binding to the lipid second messenger phosphatidylinositol-4,5-bis phosphate (PIP₂) in a Ca²⁺ independent manner.^{207b,220} An increase of intracellular PIP₂ levels caused the increase of arachidonic acid release by the calcium-independent activation of GIVA PLA₂.²²³ GIVA PLA₂ has a high binding affinity and specificity toward PIP₂.^{207b} The lysine cluster, Lys-488, Lys-541, Lys-543, and Lys-544, have been identified as PIP₂ binding sites and critical for PIP₂-mediated GIVA PLA₂ activation^{220,224} and translocation of the enzyme to phagosomes.²²⁵ The lysine cluster is located in the intradomain contact region (Figure 7).²¹⁸ PIP₂ activation requires the presence of the C2 domain, indicating that the orientation of the C2 domain and the catalytic domain is critical for phospholipase activity.²²⁰

Ceramide 1-phosphate (C1P) is a phosphorylated bioactive sphingolipid involved in inflammation.²²⁶ C1P is also a lipid second messenger involved in cell signaling.²²⁷ A C1P analog was shown to inhibit GIVA PLA₂ and increase cell toxicity.²²⁸ Ceramide kinase, which phosphorylates ceramide to synthesize C1P, was also found to be an activator of GIVA PLA₂.^{226,229} Ceramide kinase is also involved in the biogenesis of lipid droplets through GIVA PLA₂ activation.²³⁰ C1P binds directly to the C2 domain of GIVA PLA₂ at Arg-57, Lys-58, and Arg-59 (Figure 7).²¹⁹ It is also a required bioactive lipid for GIVA PLA₂ to translocate to intracellular membranes in response to inflammatory agonists.²³¹ Unlike PIP₂ activation, the C1P activation mechanism is Ca²⁺ dependent. Interestingly, recent research showed that C1P activates GIVA PLA₂ by decreasing the dissociation constant to increase the residence time on membranes, while PIP₂ activates GIVA PLA₂ by increasing membrane penetration for higher catalytic efficiency.²³² More recently, GIVA PLA₂ was also shown to be inhibited by sphingomyelin at the biomembrane interface.²³³

3.2.3 Phosphorylation—GIVA PLA₂ phosphorylation regulates the enzymatic activity at the phospholipid membrane and its cellular functions. GIVA PLA₂ was initially found to be phosphorylated at Ser-505 and activated by p42-MAP kinase and PKC in 1993.^{205,234} Ser-515, and Ser-727 were also found to be phosphorylated by mitogen activated protein kinases (MAPKs), calmodulin kinase II (CamKII), and mitogen activated protein kinase interacting kinase (MNK1).²²¹ The phosphorylation sites were later reported at Ser-505, Ser-437, Ser-454, Ser-515, and Ser-727 in Sf9 cells.²³⁵ Since then, the level of phosphorylation, especially the level of Ser-505 phosphorylation by MAP kinase, has been implicated in the activation of GIVA PLA₂ in response to various cellular stimuli.^{203b,205,235–236}

The phosphorylation sites are all located in the low electron density areas in the crystal structure.^{202a} Among them, Ser-437, Ser-454, Ser-505 and Ser-515 are in the cap region, indicating the critical role of phosphorylation in affecting the conformation of the cap or the flexibility around the phosphorylated region. DXMS studies have revealed these regions are flexible and significantly increase the deuteration level after activation.^{218,222} When GIVA PLA₂ is already activated by calcium, Ser-505 phosphorylation increases activity by 30–60%.²³⁷ In low (2.5 μM) Ca²⁺ concentration, membrane binding showed a 60-fold increase in membrane affinity.²³⁸ Interestingly, this effect was also observed in the PIP₂ activation mechanism.²²⁰ Both effects indicate a conformational change in the cap region to facilitate interfacial activation. The heterotetramer (A2t) of p11 and annexin A2 has been shown to bind to GIVA PLA₂ and inhibit the membrane binding. Phosphorylation of Ser-727 has been

shown to disrupt the binding of GIVA PLA₂ to heterotetramer (A2t) to activate GIVA PLA₂.²³⁹ To date, Ser-505, Ser-515 and Ser-727 are considered as the three phosphorylation sites involved in cellular functions.

3.2.4 Membrane Interaction—Interfacial activation, showing higher activity against large phospholipid aggregates (or a cellular phospholipid membrane), is required for GIVA PLA₂ activity.²⁴⁰ For the enzyme to be active, it must be sequestered to a phospholipid interface. As we have described, the binding of the GIVA PLA₂ to the membrane is mediated through three mechanisms: Ca²⁺-mediated translocation, binding of secondary lipid messengers, and phosphorylation. Each of these mechanisms increases GIVA PLA₂ catalytic efficiency in a different way. Calcium binding assists in membrane penetration of the C2 domain. We found that the presence of the phospholipid membrane decreases the H/D exchange rate on CBL1 and CBL3 in the C2 domain, which are composed of amino acids 35–39, and 96–98 (Figure 8).²²²

The C2 domain anchors GIVA PLA₂ to the membrane and causes the catalytic domain to approach the membrane.^{202b,203} The catalytic sites in GIVA PLA₂ must maintain the correct orientation toward the membrane, although calcium activated, C1P/PIP₂ bound, or phosphorylated GIVA PLA₂ may have slightly different phospholipid interactions. The intradomain interaction may also be affected by the conformational change in the C2 domain, phosphorylation, or C1P/PIP₂ binding, and further changes the membrane interaction of the catalytic domain. Two helices in the regions 268–279 and 466–470 in the cap region have been shown to interact with the phospholipid membrane, in addition to the C2 domain (Figure 8).²²²

Once GIVA PLA₂ has localized to the membrane, the active site is in the correct orientation to allow substrate molecules to enter the active site. We have mentioned that within the cap region, there is a lid region that prevents the phospholipid substrate from binding to the active site.^{202a} A substrate mimicking the inhibitor MAFP was used to examine the substrate binding effects. Recent work using a lipid substrate and a covalent inhibitor bound to the active site has indeed shown a conformational change of the lid region in the presence of the substrate.²²² Further experiments using the 2-oxoamide inhibitor (see Section 3.4.7) show that the head group is binding to the Arg-200.²¹⁷ Based on the model developed, the phospholipid molecule must interact with the residues near the cap region and be extracted from the membrane interface.^{217–218,222} The molecule will then go into the tunnel or push away the lid and bind to the active sites.

3.3 Biological Functions and Disease Implications

3.3.1 Biological Functions

3.3.1.1 Phospholipid Hydrolysis: GIVA PLA₂ specifically favors hydrolyzing the phospholipid membrane at the *sn*-2 position of unsaturated arachidonic acid in response to cellular stimuli.^{18a,241} Free arachidonic acid may be metabolized along the cyclooxygenase (COX) pathway or lipoxygenase pathway (LOX). COX-1, COX-2 and other terminal synthases convert arachidonic acid and generate prostaglandins and thromboxanes.²⁴² On the other hand, arachidonic acid can be oxidized by 5-lipoxygenase or 12/15-lipoxygenase and other downstream enzymes leukotriene A4 hydrolase and LTC4 synthase to produce leukotrienes and lipoxins.^{242a,243} The eicosanoids are important in intracellular immunity²⁴⁴ and implicated in several diseases, including thrombosis, cancer, atherosclerosis, asthma, arthritis and rhinitis.^{242a,245}

GIVA PLA₂ deficiency in patients and knockout mouse models have shown decreased eicosanoid production and an easing in the effects of inflammatory diseases.^{241,246} Because

arachidonic acid is the precursor of these eicosanoids, it has been proposed that GIVA PLA₂ plays a major role in inflammatory diseases. GIVA PLA₂ is now considered a central enzyme for mediating eicosanoid production. GIVA PLA₂ has also been implicated in apoptosis triggered by the hydrolysis product arachidonic acid and its metabolites.²⁴⁷ The inhibition of arachidonoyl-CoA transferase results in accumulation of arachidonic acid, which triggers apoptosis.²⁴⁸ Arachidonic acid metabolites, including 12-EET and 19-HETE, have been shown to trigger apoptosis.^{245a,249}

Lysophospholipids represent another product of phospholipid hydrolysis by GIVA PLA₂. This biological function is not specific to GIVA PLA₂, since the lysophospholipid can be potentially generated by all PLA₂ members. But it has been suggested that lysophospholipids act as second messengers for GPCR signaling and are strongly associated with cancer.²⁵⁰

3.3.1.2 Golgi Function Regulation: The Golgi complex and the trans-Golgi network are cellular organelles involved in the trafficking of cell lipids and proteins as part of the endocytic and biosynthetic pathways.²⁵¹ Lipids are not only the main component of the cell membrane, but are also implicated in the regulation of membrane-protein trafficking, vesicular fusion, and signaling.^{242a,251} When GIVA PLA₂ is localized in the Golgi complex in epithelial cells, it functions as a Golgi regulatory enzyme instead of inducing cell proliferation.²⁵² GIVA PLA₂ also regulates the junction protein transports from the Golgi complex to endothelial cell contacts.²⁵³ It was recently shown that GIVA PLA₂ associates with the Golgi complex and is involved in regulation of the Golgi complex structure, tubule formation and intra-Golgi transport.^{18c,254}

3.3.1.3 Regulation of NADPH Oxidase: GIVA PLA₂ regulates the stimulation of NADPH oxidase to produce superoxides.²⁵⁵ Correlations have been demonstrated between PGE₂ production and GIVA PLA₂ translocation to the nuclear membranes and between superoxide production and GIVA PLA₂ translocation to the plasma membranes.^{255–256} While GIVA PLA₂ binds to PC-enriched nuclear membranes in a calcium-dependent process using its C2 domain, which is correlated with its phospholipid-binding specificity, it is anchored to the plasma membranes by the assembled NADPH oxidase which serves as an adaptor protein for GIVA PLA₂.^{255–257} Under oxidative stress, down-regulation of GIVA PLA₂ can suppress the NADPH oxidase-mediated reactive oxygen species production in astrocytes.²⁵⁸ GIVA PLA₂ is targeted to the p47phox-PX domain of the assembled NADPH oxidase via a novel binding site in its C2 domain.²⁵⁹

3.3.2 Disease Implications

3.3.2.1 Human Mutation and Knockout Mice: A patient with two heterozygous single base pair mutations (Ser111Pro; Arg485His) in the coding regions of his GIVA PLA₂ gene has been identified.^{246b,260} The Arg485His mutation is located in a critical region, and is involved in intradomain interaction and membrane interaction.^{218,222} Because PLA₂ activity in platelets was not observed in the patient, the mutation sites must be critical for GIVA PLA₂ activity. The patient showed small intestinal ulcerations without COX deficiency. Inherited human GIVA PLA₂ deficiency is associated with impaired eicosanoid biosynthesis.

Genetic knockout mice were developed in 1997 by two different groups.^{246a,261} The normal phenotype of GIVA PLA₂-null mice suggested that this enzyme is not crucial for development.^{246a,261} However, when the mice were tested for various disease models, especially those involving inflammation, the signs of illness were much milder than wild-type mice and the mice demonstrated a great reduction in lipid mediator production. In particular, GIVA PLA₂-null mice have been shown to be resistant to ischemia-reperfusion

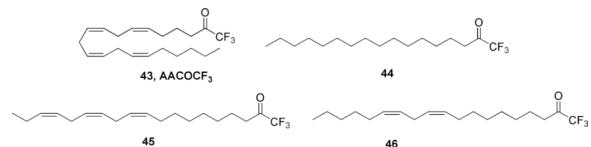
injury,²⁶² anaphylactic responses,²⁶³ acute respiratory distress syndrome caused by acid or endotoxin,²⁶⁴ bleomycin-induced pulmonary fibrosis,²⁶⁵ collagen-induced autoimmune arthritis,²⁶⁶ experimental allergic encephalomyelitis,²⁶⁷ fatty liver damage,²⁶⁸ and autoimmune diabetes.²⁶⁹ Concerning normal physiology, the loss-of- GIVA PLA₂ causes some defects to the renal concentrating function.²⁷⁰ The most notable defects caused by the loss-of- GIVA PLA₂ are found in the female reproduction system.²⁶¹ These female knockout mice showed a decreased level of eicosanoids and platelet-activating factor in peritoneal macrophages, gained less weight and had a smaller litter size. Ultimately, they failed to carry to term and deliver offspring. However, these effects could be prevented by injection of a progesterone-receptor antagonist during pregnancy.

3.3.2.2 Cancer: Alterations in the levels and functional activity of GIVA PLA₂ have also been associated with cancer pathogenesis.^{245a} GIVA PLA₂ activation was shown to mediate estrogen-dependent breast cancer cell growth.²⁷¹ Deletion of GIVA PLA₂ in the APC716 mouse of the human FAP model showed a decrease in the number of and repressed the growth of polyps in the small intestine.²⁷² GIVA PLA₂ plays an important organ-specific role in modulating intestinal tumorigenesis. Administering azoxymethane (AOM) triggered the formation of colon tumors in studies of GIVA PLA₂-null mice, showing significant increases in the number of tumors over wild type mice.²⁷³ GIVA PLA₂ null mice also developed 43% less tumors than wild type mice after exposure to the lung carcinogen urethane.²⁷⁴ In analysis of adenocarcinoma tumors, GIVA PLA₂ expression was detected in 18% of Barrett's oesophagus patients and is inversely associated with depth of tumor infiltration.²⁷⁵ Additionally, the GIVA PLA₂ null mice also show reduction of tumor progression in the glioblastoma (GL261) and Lewis lung carcinoma tumor models.²⁷⁶

3.4 Chemical Inhibitors and Therapeutic Intervention

The most recent review on inhibitors of the four major PLA₂ types focused on the recent patent literature.^{22f} The GIVA PLA₂ has been considered to be the PLA2 enzyme that plays the most central role in inflammatory diseases, thus numerous inhibitors have been reported.^{22c,d}

3.4.1 Fatty Acid Trifluoromethyl Ketones and Tricarbonyls—The first inhibitor of GIVA PLA₂ was the activated ketone, arachidonoyl trifluoromethyl ketone (**43**).²⁷⁷ AACOCF₃ was shown to be a slow tight binding inhibitor of GIVA PLA₂, 4 orders of magnitude more potent for this enzyme than for sPLA₂. ¹⁹F and ¹³C-NMR experiments elucidated the structure of the GIVA PLA₂•AACOCF₃ complex and that the mole ratio of AACOCF₃ with respect to the enzyme was 1:1.²⁷⁸ The concentration-dependent inhibition of the enzyme by **43** and the palmitoyl trifluoromethyl ketone (**44**) was also studied.²⁷⁹ The trifluoromethyl ketone analogues of γ -linolenic (**45**) and linoleic acid (**46**) were also found to be GIVA PLA₂ inhibitors.²⁸⁰



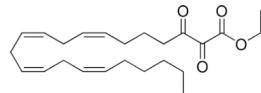
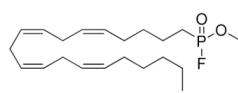
A series of fatty acid trifluoromethyl ketones were analyzed with phospholipid vesicle-, detergent-phospholipid mixed micelle-, and natural membrane-based assays.²⁸¹ With few exceptions, the relative inhibitor potencies measured with the three assays were similar (Table 16). Note that some trifluoromethylketones may also inhibit GVIA iPLA₂ (see, Section 4.4.1).

AACOCF₃ was also able to inhibit the lysophospholipase activity of GIVA PLA₂ at a similar level to PLA₂ activity.^{213b} Fatty acid tricarbonyl derivatives, like compound **47**, were found to be inhibitors of GIVA PLA₂.²⁷⁹ Their potency was approximately the same as that of the corresponding trifluoromethyl ketones.

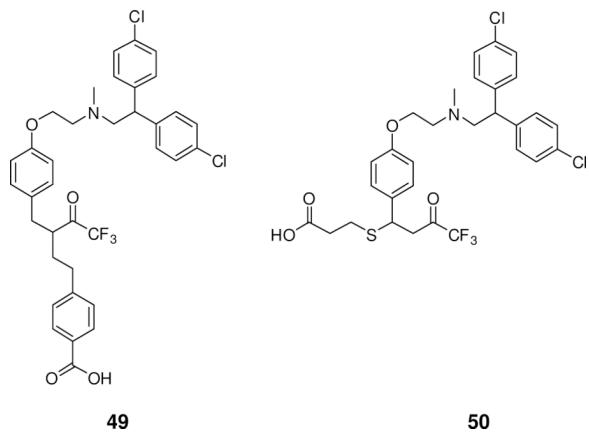
AACOCF₃ has been used in various experiments in cells and in vivo; however, the results obtained with this inhibitor have to be viewed with some caution, because it is not a selective GIVA PLA₂ inhibitor and it may inhibit other enzymes, for example cyclooxygenases.²⁸² AACOCF₃ blocked the production of arachidonic acid and 12-hydroxyeicosatetraenoic acid (12-HETE) in platelets²⁸³ and in human monocytes causing a dose dependent inhibition of GIVA PLA₂ activity and LDL lipid oxidation.²⁸⁴ Daily treatment of prion-infected cell lines with AACOCF₃ for seven days prevented the accumulation of protease-resistant PrP (PrP^{res}) indicating a pivotal role for GIVA PLA₂ in prion disease.²⁸⁵

The role of GIVA PLA₂ in allodynia has been studied. Intracerebroventricular injection of AACOCF₃ significantly reduced responses to von Frey hair stimulation at 8 h and 1 day after facial carrageenan injection.²⁸⁶ Intrathecal administration of AACOCF₃ dose-dependently prevented thermal hyperalgesia induced by intrapolar carrageenan as well as formalin-induced flinching.²⁸⁷ Using AACOCF₃, Kalyvas and David disclosed that cPLA₂ plays an important role in the pathogenesis of experimental autoimmune encephalomyelitis (EA), the animal model of multiple sclerosis.²⁸⁸ More recently, AACOCF₃ has been used in a study reporting that GIVA PLA₂ reduction ameliorates cognitive deficits in a mouse model of Alzheimer's disease.²⁸⁹

3.4.2 Methyl Arachidonyl Fluorophosphonate—Methyl arachidonyl fluorophosphonate (**48**, MAFP) was reported to be a potent time-dependent irreversible inhibitor of GIVA PLA₂ but not of the human sPLA₂. The inactivation of the enzyme by some additional alkyl methyl fluorophosphonates was also studied.²⁸¹ Like AACOCF₃, intrathecal administration of MAFP dose-dependently prevented thermal hyperalgesia induced by intraplantar carrageenan as well as formalin-induced flinching.²⁹⁰

**47****48**, MAFP

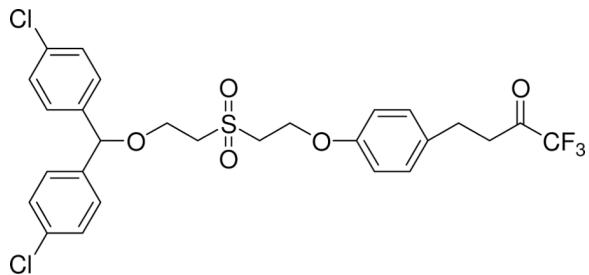
3.4.3 Trifluoromethyl Ketones—Bristol-Myers Squibb reported in several US patents a series of α - and β -substituted trifluoromethyl ketones,²⁹¹ such as compounds **49** and **50**, as GIVA PLA₂ inhibitors for the treatment of inflammatory diseases. These inhibitors presented IC₅₀ values in the 1–50 μ M range.



49

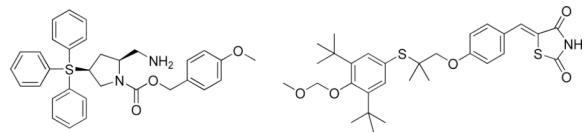
50

BMS-229724 is a tight-binding inhibitor of GIVA PLA₂ that acts at the lipid/water interface and possesses anti-inflammatory activity in skin inflammation models.²⁹² In a UVB-induced skin erythema model in hairless guinea pigs, BMS-229724 was orally active.



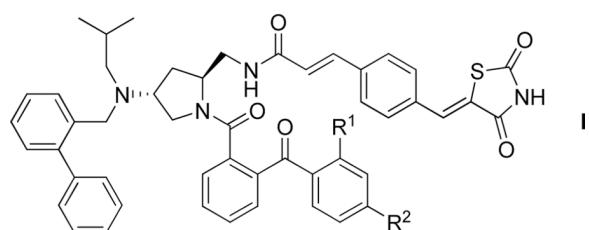
51, BMS-229724

3.4.4 Pyrrolidines—In the search for low molecular weight inhibitors of GIVA PLA₂, Shionogi identified compounds **52** (IC_{50} 1.5 μM) and **53** (IC_{50} 1.7 μM) as lead candidates. When these two structures were combined and studied in SAR experiments, a series of even more potent inhibitors were identified.²⁹³ Compounds **54** and **55** were the most promising (Table 17). Compound **55** inhibited the arachidonic acid release in A23187 stimulated THP-1- cells with an IC_{50} value of 22 nM.²⁹³ Compound **54** inhibited GIVA PLA₂ by 50%, when present at approximately 0.002 mole fraction in the interface in a number of in vitro assays.²⁹⁴

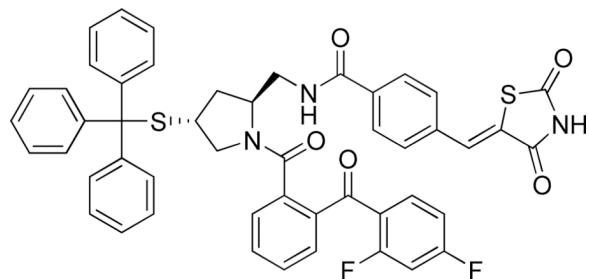
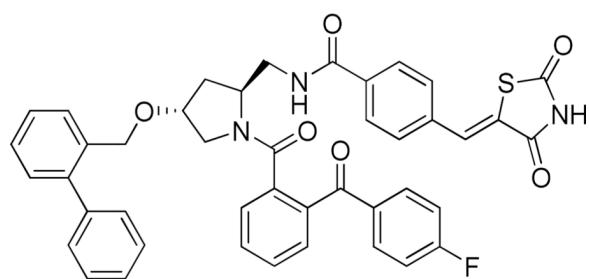


52

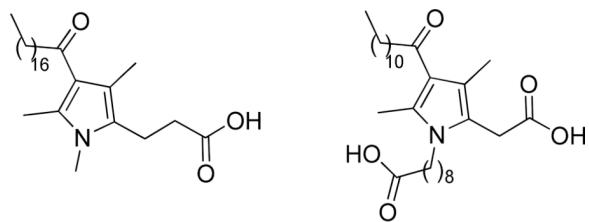
53

**54**, R¹=H, R²=F**55**, R¹=R²=F

In a continuation of the previous studies, pyrrophenone (**56**) proved to be an excellent inhibitor of human GIVA PLA₂ (IC₅₀ 4.2 nM).^{295,296} Pyrrophenone strongly inhibited arachidonic acid release, prostaglandin E₂, thromboxane B₂ and leukotriene B₄ formation in human whole blood. The magnitudes of PGE₂ and thromboxane B₂ inhibition were the same as those of indomethacine. Pyrrophenone showed reversible inhibition of GIVA PLA₂, not displaying characteristics of slow-binding inhibition. Pyrrophenone potently inhibited LT, PGE₂ and PAF biosynthesis in human neutrophils with IC₅₀s in the range of 1–20 nM.²⁹⁷ A structurally related inhibitor, pyrroxyphene (**57**), displayed anti-arthritis and anti-bone destructive action in a murine arthritis model.²⁹⁸

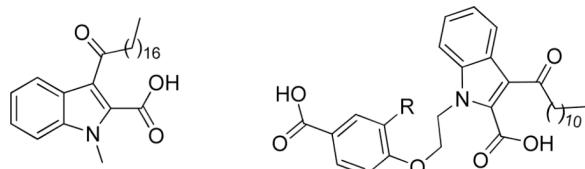
**56**, Pyrrophenone**57**, Pyrroxyphene

3.4.5 Pyrroles—A series of substituted pyrroles have been developed by Lehr.²⁹⁹ Inhibitor **58** (IC_{50} 13 μM) displayed activity similar to that of the reference inhibitor AACOCF₃ (11 μM).^{299b}

**58****59**

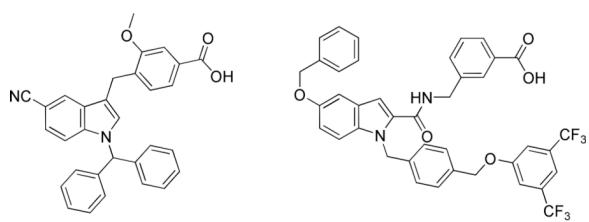
To define the structural requirements for improved cPLA₂ inhibition, Lehr varied systematically the alcanoic acid group, the acyl residue, the nature of the substituents of the pyrrole ring, as well as the pyrrole nitrogen relative to the pyrrole ring substituents. The best inhibitor of this class of compounds was compound **59** (IC_{50} 3.3 μM).^{299d} When several of these pyrrole derivatives were tested in intact bovine and human platelets using the calcium ionophore A23187 as stimulant and the results were similar for both cell types.^{299e}

3.4.6 Indoles—Lehr also developed a series of indoles. The first potent inhibitor of this series, compound **60**, presented inhibition of GIVA PLA₂ with an IC_{50} value of 8 μM .³⁰⁰

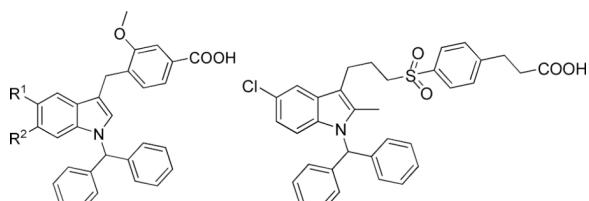
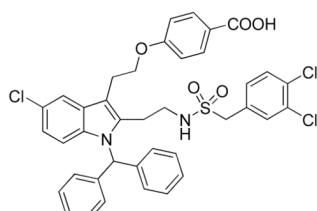
**60****61a, R=H****61b, R=F**

He later reported a series of 3-acylindole-2-carboxylic acid derivatives where the influence of the carboxylic acid group, the acyl residue, the moiety in position 1 and the substituent of the benzoic acid residue over the inhibition was investigated, along with the introduction of substituents in the phenyl ring of the indole. Compounds **61a** and **61b** showed the best inhibitory activity of the series with IC_{50} values of 0.5 μM and 0.52 μM , respectively, in a cPLA₂-mediated arachidonic acid release assay from human platelets stimulated with calcium ionophore A23187.³⁰¹ However, compounds **61a** and **b** were not found to inhibit isolated GIVA PLA₂, a fact that indicates that they do not directly interact with the enzyme.³⁰²

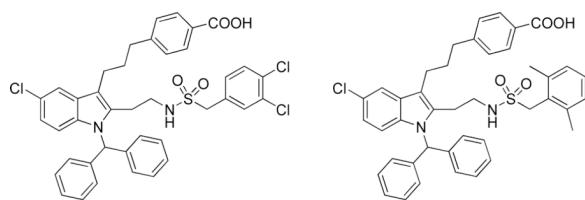
Genetic Institute developed several series of indoles as inhibitors of GIVA PLA₂.³⁰³ Compounds **62** and **63** inhibited the enzyme in both an isolated enzyme assay and in cell-based assays. In addition, they proved to be active in the rat carrageenan paw edema test.

**62****63**

Wyeth (which acquired Genetics Institute) has undertaken a tremendous effort aimed toward the discovery of novel GIVA PLA₂ inhibitors. The exploration of their application as novel pharmaceutical agents for inflammatory diseases led to their study in clinical trials. Using a substrate mimetic approach, a SAR study led to inhibitors **64a,b**.³⁰⁴ Extensive SAR data led to the conclusion that increasing the distance between the indole and the benzoic acid moieties provided more potency as illustrated with compound **65** (Table 18).³⁰⁵ A continuation of this research resulted in the discovery of ecopladib (**66**).³⁰⁶ Ecopladib displayed oral efficacy in the rat carrageenan air pouch and rat carrageenan-induced paw edema models and advanced to Phase I clinical trials.

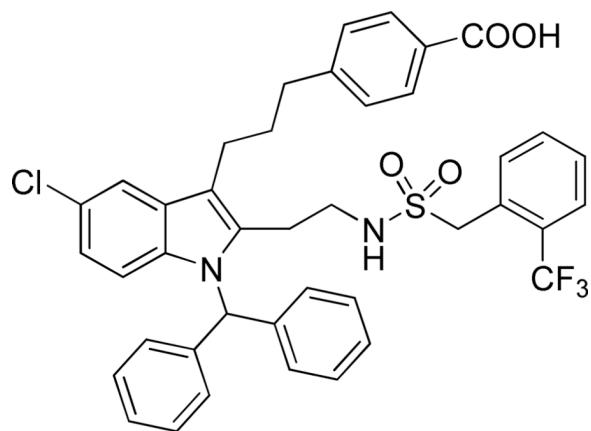
**64a, R¹=H, R²=Cl****65****64b, R¹=Cl, R²=H****66, Ecopladib**

Another study on the importance of the substituent at C3 and the substitution pattern of the phenylmethane sulfonamide region led to epipladib (**67**) and WAY-196025 (**68**).³⁰⁷ These two compounds have shown efficacy when dosed orally in multiple acute and chronic prostaglandin and leukotriene dependent *in vivo* models.

**67, Epipladib****68, WAY-196025**

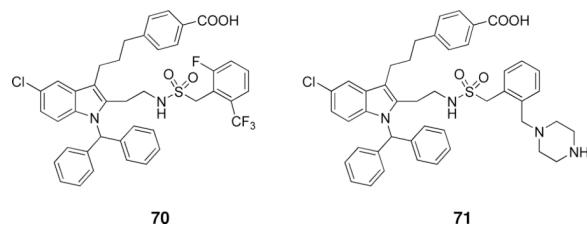
The efficacy of a similar indole inhibitor, giripladib (**69**), in two different mouse models of rheumatoid arthritis has been reported.³⁰⁸ Giripladib, also known as PLA-695, was

advanced into a phase II clinical trial for osteoarthritis, but the trial was terminated due to a failure to differentiate from the standard of care with naproxen because of gastroenterologic effects.

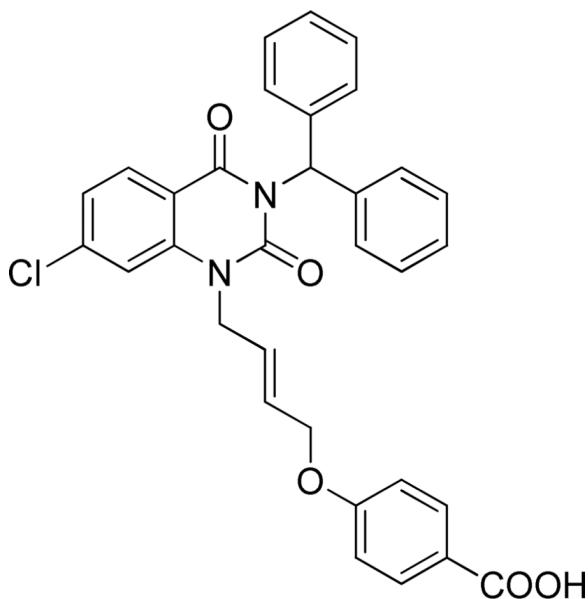


69, Giripladib

Further studies aimed at the optimization of in vitro potency and rat pharmacokinetics for oral efficacy³⁰⁹ and lowering the lipophilicity of the inhibitors.³¹⁰ Examples are structures **70** and **71**.

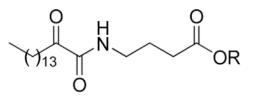


Wyeth has also reported 1,2,4-oxadiazolidin-3,5-diones and 1,3,5-triazin-2,4,6-triones³¹¹ as well as quinazoline-2,4(1H,3H)-dione GIVA PLA₂ inhibitors (for example, compound **72**) with reduced lipophilicity and improved aqueous solubility.³¹²



72

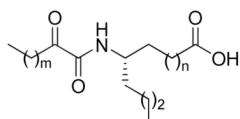
3.4.7 2-Oxoamides—In 2002, a novel class of GIVA PLA₂ inhibitors was reported, including AX006 (**73a**) and AX007 (**74a**), designed to contain the 2-oxoamide functionality and a free carboxyl group.³¹³



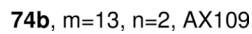
73a, R=H, AX006



73b, R=OC₂H₅, AX048



74a, m=9, n=1, AX007



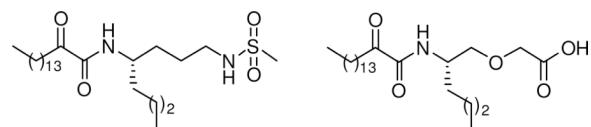
74b, m=13, n=2, AX109

Structure-activity relationship studies showed that long chain 2-oxoamides based on γ - or δ -amino acids, for example compounds **74a** and **74b**, are potent selective inhibitors of GIVA PLA₂, while the corresponding esters may inhibit both GIVA PLA₂ and GVIA PLA₂ (Table 19).³¹⁴ AX007 and AX109 presented a potent in vivo effect in the carrageenan paw edema test.^{314c} Inhibitor AX048 (**73b**) showed a potent anti-hyperalgesic effect, being able to block spinal PGE₂ release.³¹⁵

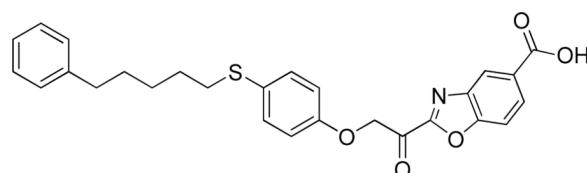
Recently, the location of the 2-oxoamide inhibitor AX007 (**74a**), as well as of pyrrophenone (**56**), in the active site of GIVA PLA₂ was determined by a combination of deuterium exchange mass spectrometry with molecular dynamics.²¹⁷ The models showed that both inhibitors interact with key residues that also exhibit changes in deuterium exchange upon inhibitor binding. Pyrrophenone was bound to the protein through numerous hydrophobic residues located distal from the active site, while the oxoamide was bound mainly through contacts near the active site as illustrated in Figure 9.

Further studies,³¹⁶ revealed that the sulfonamide group constitutes a bioisosteric group suitable to replace the carboxyl group in 2-oxoamide inhibitors (for example, compound

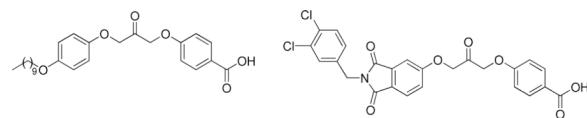
75,^{316b} while the pseudodipeptide-based inhibitor **76** inhibited the arachidonic acid release (IC_{50} 2 μ M) in RAW 264.7 macrophages.³¹⁷

**75****76**

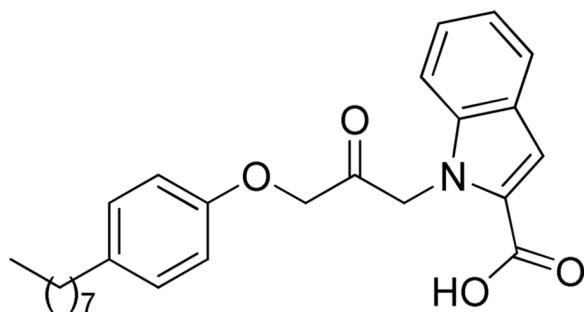
3.4.8 1,3-Disubstituted Propan-2-ones—AstraZeneca reported a series of heterocyclic compounds, such as compound **77**, able to inhibit the GIVA PLA₂-mediated arachidonic acid release from HL60 cells.³¹⁸

**77**

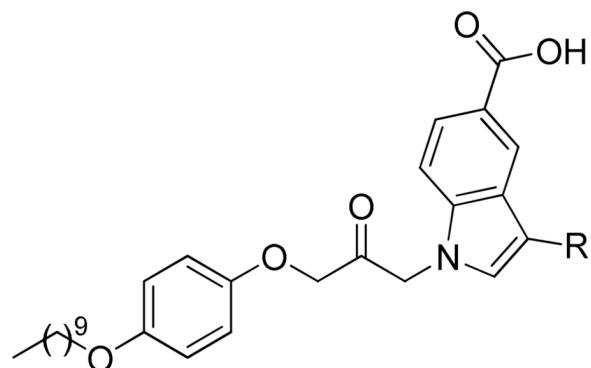
In 2002, Conolly et al. presented a series of novel potent inhibitors of GIVA PLA₂ based on a 1,3-disubstituted propan-2-one skeleton, where the electrophilicity of the carbonyl group could be altered over a wide range of structural modifications.³¹⁹ Compound **78** (AR-C70484XX), which contains a decyloxy lipophilic side chain and a benzoic acid group, inhibited the enzyme presenting an IC_{50} value of 0.008 μ M in a bilayer assay, 0.03 μ M in a soluble assay and 2.8 μ M in a whole cell assay. In an effort to reduce the lipophilicity, modifications of the decyloxyphenyl chain led to inhibitor **79** (IC_{50} 0.56 μ M in a soluble assay and 1.0 μ M in a HL60 cell assay).³²⁰

**78**, AR-C70484XX**79**

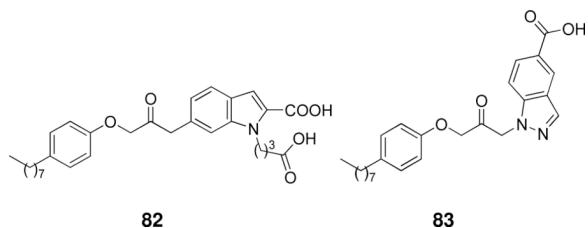
In 2006, Lehr presented several 1-indol-1-yl-3-phenoxypropan-2-ones.³²¹ Their activity was evaluated in a vesicle assay with isolated enzyme as well as in cellular assays with intact human platelets. Compound **80** presented an IC_{50} value of 8 μ M, similar to some of the indole inhibitors Lehr had presented in the past.^{300,301a}

**80**

Structure-activity relationship studies on the influence of the position of the carboxylic acid group, the nature of the substituent of the indole ring, the introduction of a second substituent in position 3 of the indole ring and the substitution of the octyl chain by a decyloxy chain led to compounds **81a** and **81b** (Table 20).³²¹

**81a, R=H****81b, R=COOCH₃**

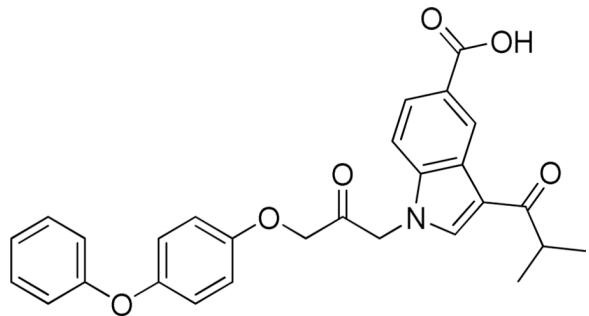
Replacement of the carboxylic acid and carboxamide moiety by other bioisosteric groups did not lead to more potent inhibitors.³²² To enhance the water solubility and metabolic stability of this class of compounds, an additional series was evaluated. Compound **82** presented interesting aqueous solubility, but also a 10-fold decrease in the inhibitory potency.³²³ The indole ring of the above inhibitors was replaced by benzimidazole, benzotriazole and indazole rings and the inhibitory activity, the metabolic stability and the water solubility were evaluated.³²⁴ Compound **83** bearing an indazole ring (IC₅₀ value of 5 nM in the vesicle assay) proved to be the most stable metabolically. Most of the above compounds were also tested for their activity against fatty acid amide hydrolase (FAAH), in order to evaluate their dual inhibitory potency.³²⁵ Compounds **83** and AR-C70484XX were found to be the best dual inhibitors for cPLA₂ and FAAH.



82

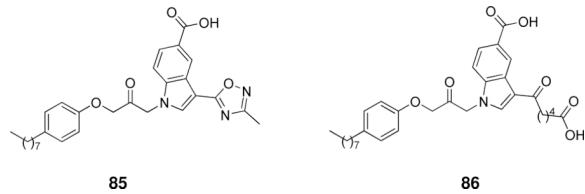
83

In an attempt to develop clinically active GIVA PLA₂ inhibitors, a series of structurally related indole-5-carboxylic acids with reduced lipophilicity was synthesized.³²⁶ The cPLA₂ inhibition, the thermodynamic solubility and the metabolic stability of the new compounds was evaluated. Compound **84** was the most potent inhibitor (IC₅₀ value of 12 nM against the isolated enzyme), and also possessed the highest water solubility (212 µg/mL at pH 7.4). Unfortunately, the po application of **84** (100 mg/kg) in mice only led to low concentrations of the substance in blood plasma and a very high plasma clearance was observed after intravenous administration (10 mg/kg). In a topical murine model of contact dermatitis, compound **84** showed a pronounced anti-inflammatory in vivo activity.³²⁶



84

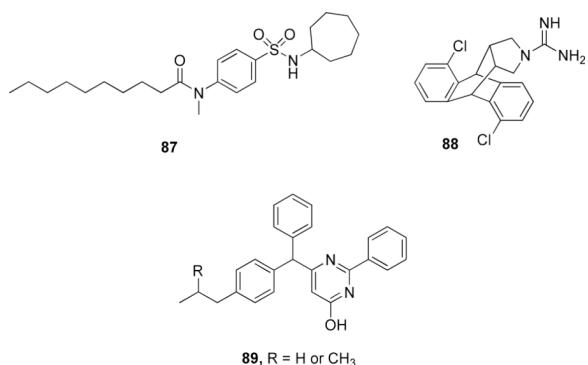
In the most recent article by Lehr *et al.*, the effect of the substituents in position 3 of the indole ring was evaluated.³²⁷ The most potent inhibitor was compound **85** bearing a 3-methyl-1,2,4-oxadiazol-5-yl-moiety with an IC₅₀ value of 2.1 nM and excellent metabolic stability (81%); however, this compound presented poor aqueous solubility. In addition, compound **86** presented inhibition of cPLA₂ with an IC₅₀ value of 22 nM, excellent metabolic stability (93%) and aqueous solubility (194 µg/mL) making this compound interesting for further investigation. Compound **86** did not inhibit at the high concentration of 10 µM calcium-independent PLA₂ (iPLA₂) from rat brain cytosol and group IB secretory PLA₂ (sPLA₂) from porcine pancreas. The bioavailability of compound **86** was disappointing, but its concentration in intestine after po administration made this compound interesting for in vivo testing in animal models of inflammatory bowel diseases.³²⁷



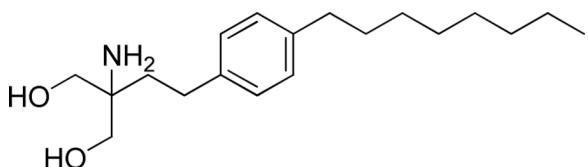
3.4.9 Other Inhibitors—Annexin V belongs to a family of proteins that interact with phospholipids in a Ca²⁺-dependent manner. Both recombinant and human placental purified

annexin V inhibited GIVA PLA₂ activity whatever the stimulus used.^{328,329} The inhibition of cPLA₂ by Annexin A6 was found to be linked to caveolin-1 export from the Golgi.³³⁰

Arylsulfonamides, such as compound **87** were reported to inhibit GIVA PLA₂ and cytokine release.³³¹ 9,10-Dihydro-9,10-ethanoanthracene derivatives were also reported to inhibit GIVA PLA₂ without affecting sPLA₂ activity.³³² Compound **88** inhibited paw swelling in the carrageenan edema model after i.p. administration with an ED₅₀ of 16 mg/kg. A series of pyrimidines, such as compounds **89**, were reported to inhibit the enzyme with IC₅₀ values <100 nM.³³³



FTY720 is a novel immunosuppressive agent that was derived from myriocin, a sphingosine-like fungal metabolite. FTY720 inhibits the egress of lymphocytes from secondary lymphoid tissues and thymus and it has recently been approved by the FDA as a first-line therapy for multiple sclerosis. It has been shown that FTY720 inhibited cPLA₂ independently of sphingosine-1-phosphate receptors.³³⁴



90, FTY720

3.4.10 Summary Status of cPLA₂ Inhibitors—A variety of different classes of GIVA cPLA₂ inhibitors have been developed in the last 20 years. The first potent inhibitor was the trifluoromethyl ketone of arachidonic acid, which has been widely used to study the role of GIVA cPLA₂ *in vitro*, in cells and *in vivo*. Even though this inhibitor does not inhibit sPLA₂s, it does inhibit GVIA iPLA₂, as well as other enzymes, so the interpretation of each studies should be taken with caution. More complex structures that also include a trifluoromethyl ketone group were presented by Bristol-Mayers Squibb as potent inhibitors of GIVA cPLA₂. Shionogi has introduced a series of pyrrolidines that are excellent inhibitors of GIVA cPLA₂, e.g., pyrrophenone, which can be used in *ex vivo* experiments. Although the above mentioned inhibitors are excellent tools for studying the role of the enzyme, none of them seems to be appropriate as a drug candidate. One of the most well studied class of inhibitors are the indoles bearing various simple or complex side chains. Indoles bearing a carboxylic acid group have been introduced as GIVA cPLA₂ inhibitors by Lehr and Genetics Institute, which was later acquired by Wyeth. Ecopladiab and later epipladiab, WAY-196025 and giripladiab are some of these indole inhibitors that have been used in *in vivo* models of inflammation, rheumatoid arthritis, etc. Another important class of

GIVA cPLA₂ inhibitors are 2-oxoamides such as AX007 and AX048 that have been shown to bind in the active site of GIVA cPLA₂ and to present potent anti-hyperalgesic activity. Finally, another significant and well studied class of GIVA cPLA₂ inhibitors are the 1,3-disubstituted propan-2-ones first introduced by AstraZeneca and later expanded upon by Lehr. A series of these inhibitors has been tested in several *in vitro* and *in vivo* experiments. To conclude, the scientific community awaits the results of the clinical trials using cPLA₂ inhibitors to determine the importance of cPLA₂ inhibition on inflammatory diseases.

4 Calcium Independent Phospholipase A₂ [Group VI iPLA₂]

4.1 Groups, Subgroups, Specificity and Mechanism

Group VIA phospholipase A₂ is a member of the phospholipase A₂ superfamily^{13–14} and is characterized by its calcium-independent phospholipase A₂ activity. Although the activities of GIVC, GVII, GVIII and lysosomal PLA₂s are all independent of calcium, the common name of “calcium independent” PLA₂ (iPLA₂) applies only to GVI PLA₂. The first member of this family, GVIA PLA₂, was purified and characterized from macrophages in 1994.⁸ To date, the Group VI calcium independent PLA₂ (iPLA₂) includes six different members: GVIA (iPLA₂ β ; PNPLA9), GVIB (iPLA₂ γ ; PNPLA8), GVIC (iPLA₂ δ ; PNPLA6), GVID (iPLA₂ ε ; PNPLA3), GVIE (iPLA₂ ζ ; PNPLA2), and GVIF (iPLA₂ η ; PNPLA4) (Table 21).¹⁴ All of these enzymes function through a catalytic serine at the active site in a patatin-like α/β -hydrolase domain (Figure 10). Because of the homology with patatin, GVI A, B, C, D, E and F are also included in the patatin-like protein family and named PNPLA9, 8, 6, 3, 2 and 4 respectively.³³⁵

4.1.1 Group VIA PLA₂ (iPLA₂ β ; PNPLA9)—Calcium-independent PLA₂ was so named in order to differentiate it from sPLA₂ and cPLA₂, whose activities are calcium-dependent. Its gene, located at 22q13.1, expresses GVIA-1 PLA₂ as a 752 amino acid protein with a molecular mass of 85 kDa that contains eight ankyrin repeats and a catalytic domain.^{9,336} The initial reports of Ca²⁺-independent PLA₂ activity referred to a 40-kDa enzyme described as iPLA₂³³⁷. Subsequently, an 85-kDa iPLA₂ was purified and well characterized which is now known as GVIA iPLA₂ (also iPLA₂ β).⁸ The same iPLA₂ was isolated from CHO cells, sequenced, cloned and expressed^{9,336a}. The size, sequence and properties of the 85-kDa enzyme were subsequently confirmed by studies in many laboratories.³³⁸ This enzyme showed potent phospholipase, lysophospholipase and transacylase activities which could be inhibited by a variety of inhibitors including bromoenol lactone (BEL) and various fluoroketones.^{240,339} The active site contains a lipase consensus sequence (GXS⁴⁶⁵XG) located in the catalytic domain (Figure 10).⁹ GVIA-1 PLA₂ was shown to be active as an oligomer through radiation inactivation studies.^{336a} ATP binding protected GVIA-1 PLA₂ from cysteine oxidation and prevented loss of activity.^{240,336a,340}

The human Group VIA PLA₂ gene was found to express multiple splice variants, including GVIA-1, GVIA-2, GVIA-3 PLA₂, GVIA Ankyrin-1 and GVIA Ankyrin-2,^{338a,b,341} in tissue-dependent patterns.³⁴² At least two of these isoforms, GVIA-1 and GVIA-2 PLA₂ are active. GIVA-2 is the longer (88 kDa) splice variant and is composed of 7 ankyrin repeats, a linker region and a catalytic domain (Figure 10). The eighth ankyrin repeat is disrupted by a 54 amino acid insert.^{338b} GVIA-1 PLA₂ activity was reported to be unaffected by ATP, while GVIA-2 PLA₂ activity is enhanced by ATP.^{337c,341,343} GVIA-2 PLA₂ is membrane-associated when overexpressed in COS-7 cells, rat vascular smooth muscle cells,^{338a,341} and Sf-9 insect cells.³⁴⁴ The Ankyrin-1 and Ankyrin-2 splice variants are mainly the ankyrin repeats without phospholipase activity, and it has been suggested that they inhibit GVIA-2 PLA₂ by forming hetero-oligomers.^{338b}

GVI PLA₂ and GIV PLA₂ both use a serine active site to catalyze the cleavage of the *sn*-2 ester bond, but GVI PLA₂ does not show arachidonic acid specificity in the *sn*-2 position, while GIV PLA₂ does.²⁴⁰ GVIA PLA₂ also exhibits lysophospholipase activity, transacylase activity and acyl-CoA thioesterase activity.^{342,345} It has been suggested that the activity of GVIA PLA₂ is regulated through many different mechanisms, including ATP binding, caspase cleavage, calmodulin, and possible ankyrin repeat mediated protein aggregation.^{16c}

4.1.2 Group VIB PLA₂ (iPLA₂ γ ; PNPLA8)—The GVIB PLA₂ gene at 7q31 was cloned in 2000 from heart, skeletal muscle,³⁴⁶ and lymphocyte cDNA³⁴⁷ and the transcript was detected in heart, placenta, kidney, liver, brain, and skeletal muscle.^{346–347} GVIB PLA₂ is a 90 kDa protein with 782 amino acids.³⁴⁷ The region 445–640 is a patatin-like lipase domain and the GXSXG motif implies that the active site is at Ser-483 (Figure 10).³⁴⁸ The catalytic core shares approximately 25% identity with GVIA-1 PLA₂.¹³ GVIB PLA₂ protein activity has been identified in membrane fractions^{346–347} and was later found to associate with mitochondria and peroxisomes.³⁴⁹ Typical localization motifs of mitochondria and peroxisomes were identified.³⁴⁹ GVIB PLA₂ exhibits both PLA₁ and PLA₂ activity and shows low specificity to *sn*-2 fatty acids for PC hydrolysis.^{346,350} Research in GVIB PLA₂-transgenic and GVIB PLA₂-deficient mice show that GVIB PLA₂ is critical to cardiac phospholipid homeostasis and mitochondrial function.^{349,351} This enzyme may be responsible for cardiolipin remodeling in mitochondria.³⁵¹

4.1.3 Group VIC PLA₂ (iPLA₂ δ ; PNPLA6)—Group VIC PLA₂ is commonly known as a neuropathy target esterase (NTE), since its discovery in 2002.³⁵² The human Group VIC PLA₂ gene is located at chromosome 19p13.3-13.2. It is expressed in neurons and is localized to the endoplasmic reticulum (ER) and Golgi apparatus.³⁵² The expressed enzyme is a membrane protein with 1366 amino acids (146 kDa) and exhibits esterase activity in phenyl valerate substrate. The patatin-like catalytic domain is in the region 933–1100 and the active site Ser-1005 is in the GXSXG motif (Figure 10).³⁴⁸ The recombinant esterase domain (residues 727–1216) also demonstrates calcium-independent phospholipase and lysophospholipase activities.³⁵² Inhibition of NTE lysophospholipase activity may be responsible for the accumulation of its physiological substrate lysolecithin, leading to delayed toxicity.³⁵³ Inhibition of NTE by organophosphorus esters can initiate axonal degeneration syndrome. NTE knockout mice are embryonically lethal and the NTE inhibitor caused hyperactivity in NTE(+/−) mice.³⁵⁴ NTE deletion mice have exhibited neuronal pathology in the hippocampus and thalamus, cerebellum defects, and abnormal cellular features, including disruption of the ER, vacuolation of nerve cell bodies, and abnormal reticular aggregates.³⁵⁵ Human patients with NTE mutations were found to suffer from the severe NTE-related motor neuron disease.³⁵⁶

4.1.4 Group VID PLA₂ (iPLA₂ ϵ ; PNPLA3)—GVID PLA₂ was identified in adipocytes in 2001, and is also called adiponutrin because of its transcript responses to a nutritional diet.³⁵⁷ Human adiponutrin is a transmembrane protein that is expressed in adipocytes.³⁵⁷ Its gene is located at 22q13.31 and the protein has 481 amino acids/ 52 kDa. It contains a patatin-like lipase domain in residues 10–180 and Ser-47, within this domain, is predicted to be the active site (Figure 10).³³⁵ The phylogenetic tree shows the closest relation as GVIE PLA₂ among the GVI PLA₂s.³³⁵ Human GVID PLA₂ has exhibited triacylglycerol hydrolase, transacylase, and calcium-independent PLA₂ activity.³⁵⁸ The adiponutrin gene has been strongly associated with nonalcoholic fatty liver disease,³⁵⁹ liver dysfunction,³⁶⁰ insulin secretion and obesity.³⁶¹

4.1.5 Group VIE PLA₂ (iPLA₂ ζ ; PNPLA2)—PNPLA₂ was identified in 2004^{358a,362} and was named adipose triglyceride lipase (ATGL), iPLA₂ ζ , GVIE PLA₂ and PNPLA₂, due to

its functions and structural homology.^{14,358a,362} The gene is located at chromosome 11p15.5 and translates to a 55 kDa protein with 504 amino acids.^{335b,358a,362} The protein is expressed mostly in white and brown adipocytes and is localized to the membrane and lipid droplets.^{362–363} Similar to GVID PLA₂, it also contains a patatin-like lipase domain in residues 10–180 and Ser47 is expected to be an active site residue (Figure 10).³³⁵ ATGL exhibits triglyceride lipase, transacylase and phospholipase A₂ activity in vitro.^{358a,362b,363a,364} The phospholipase activity is independent of calcium and ATP.^{358a} An ATGL knockout mouse study has confirmed its importance in triglyceride hydrolysis.³⁶⁵ Human mutations in the ATGL gene cause accumulation of triacylglycerol in multiple tissues and neutral lipid storage disease in patients, along with skeletal- and cardiomyopathy.³⁶⁶ The polymorphisms in PNPLA₂ have also been associated with decreased plasma fatty acid and triacylglycerol levels and increased risk for type 2 diabetes.³⁶⁷

4.1.6 Group VIF PLA₂ (iPLA₂η; PNPLA4)—Human PNPLA4 was identified in 1994 and was named gene sequence-2 (GS2)³⁶⁸. The gene at xp22.3 expresses ubiquitously in all tissues examined.^{348,358b,368–369} GVIF PLA₂ is a 27 kDa protein comprised of 253 amino acids. The region 6–177 contains a patatin-like catalytic domain and Ser-43 is the expected active site in the GXSXG motif (Figure 10).³⁴⁸ GS2 has shown retinylester hydrolase,³⁶⁹ acylglycerol and retinol transacylase,^{358a,369} TG hydrolase,^{358b,369} and PLA₂ activity.^{358a}

4.2 Structural Characteristics and Activation Mechanisms

There are currently six forms of the GVI PLA₂. Among them, GVIA is the most well studied and recognized iPLA₂. In this section, we will use the long form splice variant GVIA-2 PLA₂ as our main example for discussing the structural characteristics of iPLA₂. As described in section 4.1.1, all GVI PLA₂ isoforms contain a patatin-like lipase domain. GVID, E and F PLA₂s are all small enzymes primarily composed of only the patatin-like domain.^{335,348} GVIB PLA₂ shows the highest homology to GVIA PLA₂, but does not contain the ankyrin repeats.^{335,348} GVIC PLA₂ is a transmembrane protein and also the largest protein in GVI.³⁵² It shares low similarity with all of the other GVI PLA₂s, except for the patatin-like lipase domain. The 85 kDa human GVIA-2 PLA₂ (806 amino acids) contains seven ankyrin repeats (residues 152–382), a linker region (residues 383–474) with the eighth repeat disrupted by a 54-amino acid insert, and a catalytic domain (residues 475–806) (Figure 11). Definition of the segments of GVIA PLA₂ is still vague. To avoid confusion, the regions that show high homology to the patatin protein were defined as the catalytic domain. And the rest of the region was defined as a regulatory domain, which contains the N-terminal region, ankyrin repeats and the linker region. A computational homology model based on homology structures was constructed.³⁴⁴ This model was validated by comparing the deuterium exchange results with the predicted structure.

The regulatory domain is specific for GVIA PLA₂ only. The region with a disrupted ankyrin repeat becomes the linker region in splice variant 2. The ankyrin repeats show a recurring 30–40 residue repeat with a helix-turn-helix-loop structure and has sequence homology with the human Ankyrin-R D34 protein.^{240,370} Although no crystal structure of the ankyrin repeats in GVIA PLA₂ had been obtained, the identified ankyrin repeats seem to all fold in a similar pattern. A homology model of the ankyrin repeats has been built based on the human Ankyrin-R D34 protein (Figure 11).^{344,370c} The ankyrin repeats are packed side by side and form a curved structure. While two additional ankyrin repeats in the n-terminal region fit in the model, they do not have a conventional ankyrin repeat sequence identity. We have shown that the linker region is a highly flexible region in our H/D exchange experiments.³⁴⁴

The catalytic domain is also called a patatin-like lipase domain. The published homology model shows 40% homology to patatin (Figure 11) and is obviously larger than the 170 residue conserved core.^{344,371} Comparison of our GVIA PLA₂ model to the GIVA PLA₂ crystal structure shows similar folding of the α/β -hydrolase domain to GIVA PLA₂ without the cap region, but the overall sequence homology is low.^{202a,344,371} The active site serine of the GVIA PLA₂ lies within a lipase consensus sequence (Gly-X-Ser519-X-Gly) on top of the catalytic domain (Figure 11). Unlike the GIVA PLA₂ active site, which is under the cap region,^{202a} the active GVIA PLA₂ site, Ser519, is quite exposed to solvent.³⁴⁴ This suggests that the cap region contributes to the GIVA PLA₂ AA specificity and that the exposed serine active site results in low substrate specificity. We further found that GVIA PLA₂ also has Asp-652 near the active site, which has been identified as the active site dyad in patatin.^{344,371} Moreover, there is no histidine near the active site Ser-519. The homology model strongly suggests GVIA PLA₂ is also a Ser519/Asp652 catalytic dyad, similar to the dyad in GIVA PLA₂ and patatin.

This enzyme is known to be regulated by ATP binding, caspase cleavage, oligomerization and calmodulin binding. They all take part in modulating GVIA PLA₂ activity and function. We will further discuss GVIA PLA₂ regulation mechanisms in the following section.

4.2.1 ATP Activation—Regulation of ATP is critical for various cellular functions, especially in mitochondria.³⁷² GVIA PLA₂ has been shown to localize in mitochondria in various cell types.³⁷³ GVIA PLA₂ is the only PLA₂ reported to be regulated by ATP and ADP binding.²⁴⁰ GVIA PLA₂ can be activated by ATP, but ATP is not a substrate or cofactor for GVIA PLA₂.²⁴⁰ The binding of ATP by the GVIA enzyme either stabilizes the enzyme's structure or activates the enzyme.^{8,240,341} Its activation likely results from a conformational change in the enzyme. ATP has also been shown to prevent loss of activity in GVIA PLA₂.²⁴⁰ Research has shown that ATP binding can prevent the dimerization and cysteine oxidation of GVIA PLA₂, enabling it to maintain its activity over time.³⁴⁰ Because of GVIA PLA₂'s homology to protein kinases, it has been suggested that the binding site is in the G485XGXXG motif,³⁷⁴ close to the active site serine within a lipase consensus sequence (Gly-X-Ser519-X-Gly) in the α/β hydrolase domain. However, this proposed ATP binding site is in a highly negatively charged environment in the homology model of the catalytic domain of GVIA PLA₂, based on the crystal structure of patatin.³⁴⁴ Although the homology model may not provide accurate electrostatic potentials, it is unlikely that ATP would bind to a region surrounded by negatively charged residues. In addition, the proposed ATP binding sequences may not be similar to the glycine rich loop of protein kinases. Recently, the crystal structure of the ankyrin repeats of the transient receptor potential cation channel TRPV1 showed that ATP can bind to ankyrin repeats and regulate the calcium channels.³⁷⁵ This process utilized a completely different ATP binding mechanism than that found in protein kinases, which does not require metal ions.

4.2.2 Caspase Cleavage—GVIA PLA₂ plays an important role in homeostasis, maintaining a constant level of lysoPC in resting P388D1 macrophages cells.^{342,376} Imbalances in the homeostasis can seriously damage a cell. Caspase proteolysis of the GVIA enzyme in apoptosis produces a truncated protein in which the first of the ankyrin repeats has been clipped, resulting in a hyperactive form of the protein.³⁷⁷ In the U937 system, both the GIVA and GVIA PLA₂s are targets of caspase-3 proteolysis, but only the GVIA enzyme remains active following cleavage.³⁷⁷ Three forms of the caspase cleaved GVIA PLA₂ have been identified, 70 kDa, 26 kDa and 32 kDa^{19a} The preferred cleavage site is DVTD¹⁸³, while DLFD⁵¹³ and MVVD⁷³³ are minor cleavage sites.^{377–378} Thapsigargin-induced apoptosis results in caspase-3 cleavage and generate an active 62-kDa protein localized to the nucleus.³⁷⁹ During apoptosis, active GVIA PLA₂ may participate in membrane damage and provide bioactive lipid metabolites, such as lysoPC, for phagocytosis.^{19a,380}

4.2.3 Calmodulin Inhibition—Calmodulin is activated by binding to four Ca²⁺ ions, which induces a conformational change.³⁸¹ Activated calmodulin can interact with calmodulin binding proteins to change their subcellular localization or enzymatic activity.³⁸¹ Calmodulin binding proteins that are not directly regulated by Ca²⁺ can respond to intracellular Ca²⁺ through calmodulin. Calmodulin associates with GVIA PLA₂ and was used to purify GVIA PLA₂.^{338c} No evidence of Ca²⁺ binding to GVIA PLA₂ has ever been reported, and we have shown membrane penetration by GVIA PLA₂ in the absence of Ca²⁺.³⁴⁴ Although GVIA PLA₂ activity is independent of Ca²⁺, Ca²⁺-activated calmodulin has been reported to bind to the IQ motif in the tryptic C-terminal 15 kDa region of GVIA PLA₂, inhibiting its activity.³⁸² The IQ motif is located in residues 694–705 of the catalytic domain of GVIA-1 PLA₂.

4.2.4 Oligomerization—Radiation inactivation studies of murine GVIA PLA₂ suggest that it is active as an oligomer of 340 kDa.⁸ Early work suggested that the 340 kDa complex was a tetramer of the 85 kDa GVIA enzyme.⁸ It was assumed that the eight ankyrin repeats near the N-terminus of the protein played a role in this aggregation. The requirement of the ankyrin repeats for GVIA PLA₂ activity is identified by the truncation mutation.^{338b} The splice variant GVI Ankyrin-1 was proposed to be a potential negative regulator of GVIA PLA₂ by blocking oligomerization.^{338b} GVIA PLA₂ may also associate with other regulatory proteins to form active complexes.³⁷⁷ GVIA PLA₂ has been shown to form a signaling complex with the calcium/calmodulin-dependent protein kinase IIβ that is expressed in pancreatic islet beta-cells.³⁸³ Additionally, the ankyrin repeats are reported to be involved in protein-protein interactions, such as 53BP2-p53, GA-binding protein α-GAbinding protein β, p16INK4a-CDK6, and IκBα-NFκB.^{336b}

4.2.5 Membrane Interaction—The activity of phospholipases depends critically on the interaction of the protein with phospholipid membranes. GVIA-2 PLA₂ is composed of seven consecutive N-terminal ankyrin repeats, a linker region, and a C-terminal phospholipase catalytic domain.^{8,240,341} No crystal structure of GVIA PLA₂ has ever been published, and no information is known about the membrane binding surface. Deuterium exchange experiments on GVIA-2 PLA₂ in the presence of phospholipid substrate (PAPC) was carried out to locate regions in the protein that change upon lipid binding (Figure 12A).³⁴⁴ The region with the greatest change was region 708–730, which showed a >70% decrease in deuteration levels at numerous time points. No decreases in exchange due to phospholipid binding were seen in the ankyrin repeat domain of the protein. The homology model, combined with the deuterium exchange results in the presence of lipid substrate, has led to the first structural model of GVIA-2 PLA₂ as well as the interfacial lipid binding region (Figure 12B).

Only one of these regions was located in the regulatory domain. Region 378–389, located in the middle of the linker region, had a 2.2 deuteron increase in exchange at 5 min of on-exchange.³⁴⁴ When deuterium exchange experiments were carried out with the isolated ankyrin repeat linker construct, the entire linker region had increased exchange compared with the intact enzyme. This implies that the linker region is in contact with the catalytic domain. The increase in exchange at 378–389 may imply that upon lipid binding there is a conformational change in the orientation of the catalytic domain relative to the ankyrin repeat linker region. GVIA-2 PLA₂ was found to be membrane-associated when overexpressed in COS-7 cells, and this was further confirmed in rat vascular smooth muscle cells.^{338a,341} The other active splice variant, GVIA-1, is cytosolic and not specific in targeting membrane surfaces,^{338a,341} indicating that these two splice variants use two different regulatory mechanisms. The 54-residue insertion in the eighth ankyrin repeat alters the property of GVIA-2 PLA₂ for membrane association.

Four of the regions that showed changes in deuterium exchange are localized to the catalytic domain, and all showed decreases in exchange (Figure 12A).³⁴⁴ The decreases in exchange on the catalytic domain occurred in areas that have numerous different hydrophobic residues. The region with the greatest decrease in exchange is 708–730. This region had a 13.2 deuteron decrease in exchange at the 10s time point but only a 3.5 deuteron decrease at 166 min. This region is the most highly solvent-exposed region in the protein in the absence of vesicles. The almost total absence of exchange at 10s of on-exchange in the presence of lipid vesicles implies that this region is penetrating into the membrane surface. This region contains hydrophobic residues Val-708, Phe-709, Trp-715, Leu-717, Val-721, Phe-722, and Leu-727 that may mediate penetration into the lipid surface.

The negatively charged region 773–778 also shows a decrease in exchange (Figure 12A).³⁴⁴ This region consists of an α -helix that is part of the catalytic core, which explains the low rates of H/D exchange. There are no hydrophobic residues in this region, and it is most likely that decreases here are due to either electrostatic interactions between the charged membrane head group and the charged residues Asp-771, Glu-772, and Asp-775 or conformational changes induced by substrate binding. Regions 631–655 and 658–664 are both spatially very close to the active site Ser-519 and are on the same face of the enzyme as the proposed membrane penetration region, 708–730. Neither of these regions has hydrophobic regions and are also most likely interacting with the charged surface of the membrane rather than mediating penetration.

The model of GIVA PLA₂ membrane binding suggests penetration of the 708–730 region into the membrane surface with regions 631–655, 658–664, and 773–778 interacting with the charged head group of the phospholipid substrate (Figure 12B).

4.3 Biological Functions and Disease Implications

4.3.1 Biological Functions—The Group VI enzymes are diverse in terms of their structure and function, even though they all have calcium-independent phospholipase A₂ activity and a patatin-like lipase domain.^{13–14,335a} Most of these enzymes have multiple catalytic activities and localize to different organelles. The GVID, GVIE and GVIF are all shorter forms of GVI PLA₂.^{14,335a,358a} All of them were found to localize to adipose cells.^{358a} It is suggested that fatty acid acylation is the main cellular function of these enzymes.^{358a} GVIA and GVIB have closer relationships than the other GVI enzymes in the phylogenetic tree and both have been shown to localize to mitochondria.^{335b,348} GVIC, neuropathy target esterase, is quite different from the rest of the GVI enzymes.³⁵² We have briefly described the general functions of all GVI enzymes in the previous sections. Here we focus on representative GVIA enzymes to discuss their biological functions and disease implications.

GVIA-1 has been shown to be important in membrane homeostasis and remodeling^{376b}, and it appears that this enzyme is the primary PLA₂ for day to day metabolic functions within the cell. GVIA PLA₂ is involved in cell proliferation,³⁸⁴ cell cycle progression,³⁸⁵ apoptosis,^{376b,386} bone formation,³⁸⁷ sperm development,³⁸⁸ glucose-induced insulin secretion,³⁸⁹ cardiolipin acetylation,³⁹⁰ and monocyte recruitment,^{384a,391} which also shows that its function may vary by cell and tissue. Interestingly, GVIA PLA₂ has been involved in both cell growth and cell death in various cell types. On one hand, GVIA PLA₂ promotes cell cycle progression and proliferation by GVIA PLA₂-generated lipid mediators, including free fatty acids, eicosanoids and LPA.^{317,384c,d,385,392} On the other hand, GVIA PLA₂ has been shown to be involved in various stimuli-induced apoptosis, including Fas-ligand, thapsigargin, H₂O₂, ROS generation, and chemotherapeutic drugs.^{19b,377,393} Several studies of insulin secretion, cell proliferation and apoptosis in beta-cells have established the involvement of GVIA PLA₂ with diabetes.³⁹⁴

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Several lines of evidence have shown that GVI PLA₂ is involved in regulating store-operated calcium channels in glial cells, astrocytes, endothelial cells, epithelial cells and smooth muscle cells, and also mediates store-operated calcium entry.³⁹⁵ GVI PLA₂ has also been shown to be activated during ischemia in rabbit myocardium and human coronary artery endothelial cells³⁹⁶. Although the GVI PLA₂ knockout mice do not exhibit the myocardial function defect, they are relatively refractory to ischemia-induced cardiac arrhythmias, which can be completely suppressed by the administration of GVI PLA₂ inhibitor BEL^{19e,388}.

Several lines of evidence also show the significant role of GVIA PLA₂ in muscle functions in muscle degeneration,^{395b} skeletal muscle contractility,³⁹⁷ cardiomyopathy,³⁹⁸ drip formation in muscle,³⁹⁹ skeletal muscle fatty acid oxidation,^{345b} vascular smooth muscle cell contraction⁴⁰⁰ and Barth syndrome with cardinal disorder characteristics. GVIA PLA₂ function is also strongly related to function and dysfunction in the nerve system, such as neurotransmission and nerve degeneration.⁴⁰¹ Researchers have shown a correlation of GVIA PLA₂ activity with several diseases including neurodegeneration with brain iron accumulation (NBIA) disorders,⁴⁰² infantile neuroaxonal dystrophy,⁴⁰³ and memory loss.⁴⁰⁴ GVIA PLA₂ also plays an important role in Wallerian degeneration and axon regeneration in nerve injury in mouse models.^{165,405}

4.3.2 Diabetes—GVIA PLA₂ has been related to diabetes due to its participation in β-cell apoptosis, superoxide production and glucose-induced RhoA/Rho kinase activation. GVIA PLA₂ genetically-modified mice and various cellular studies suggest that GVIA PLA₂ participates in β-cell apoptosis.^{379,406} β-cell apoptosis and ceramide production may be responsible for the loss of beta-cell mass that is associated with the onset and progression of type 1 and type 2 diabetes mellitus.^{406a} In diabetes mellitus patients, GVIA PLA₂ enhanced superoxide generation in neutrophils and siRNA knockdown inhibited superoxide generation.⁴⁰⁷ GVIA PLA₂-deficient cell and inhibitor studies have found that GVIA PLA₂ participates in the high glucose-induced RhoA/Rho kinase/CPI-17 activation pathway, which has been shown to contribute to diabetes-associated vascular smooth muscle hypercontractility.⁴⁰⁸

4.3.3 Barth Syndrome—Barth syndrome is associated with mutations of the X-linked tafazzin gene (TAZ).⁴⁰⁹ Cardiolipin is a distinct phospholipid component of the mitochondrial membrane.⁴¹⁰ Tafazzin is responsible for cardiolipin homeostasis in mitochondria.^{390a,411} GVIA PLA₂ localizes in mitochondria, maintains the integrity of the mitochondrial membrane and prevents the rupture of mitochondria and the release of cytochrome c under oxidative stress.³⁷³ Recent research suggests that GVIA PLA₂ participates in cardiolipin remodeling in cardiomyocytes.^{390a} The other isoform, GVIB PLA₂, shows alterations to hippocampal cardiolipin content in transgenic and deficient mice⁴¹² and a role in cardiac phospholipid homeostasis and mitochondrial function.^{349,351} This enzyme may be responsible for cardiolipin remodeling in mitochondria.³⁵¹ GVIA PLA₂ is also responsible for cardiolipin deacylation and monolyso cardiolipin accumulation in Barth syndrome.^{390b} Genetic inactivation of GVIA PLA₂ can suppress the phenotype of tafazzin knockout drosophila.^{390b} GVIA PLA₂ inhibition is a possible treatment for Barth syndrome patients.

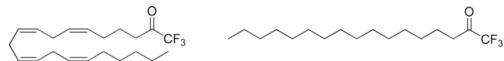
4.3.4 NBIA/Neuroaxonal Dystrophy—Recent clinical studies have shown that GVIA PLA₂ mutations are associated with “neurodegeneration with brain iron accumulation” (NBIA) disorders and infantile neuroaxonal dystrophy.^{389b,402,413} Neither the active site, Ser-519, nor the potential active site, Asp-652, was found to be mutated in the more than 40 mutation sites occurred in patients. These GVIA PLA₂ mutants may retain different level of activities, except for some truncation mutants. Double mutations of GVIA PLA₂ tend to

correlate to earlier age of disease onset, suggesting that GVIA PLA₂ activity is critical in development.^{389b,402}

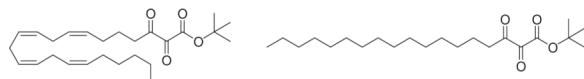
4.4 Chemical Inhibitors and Therapeutic Intervention

In comparison to the secreted and cytosolic Ca²⁺-dependent PLA₂ enzymes, the research on inhibitors of GVI PLA₂ is much more limited. However, a summary of GVIA PLA₂ inhibitors is included in two review articles.^{22c,22f}

4.4.1 Fatty Acid Trifluoromethyl Ketones and Tricarbonyls—In 1995, the inhibition of macrophage Ca²⁺-independent PLA₂ by AACOCF₃ (**43**) and palmitoyl trifluoromethyl ketone (**44**) were reported.⁴¹⁴ In contrast to the case with GIVA PLA₂, for GVIA PLA₂ the saturated derivative was found to be 4-fold more potent than AACOCF₃ (Table 22). Fatty acyl tricarbonyls **47** and **91** also inhibited GVIA PLA₂ but appeared to be much poorer inhibitors than the corresponding trifluoromethyl ketones.²⁷⁹

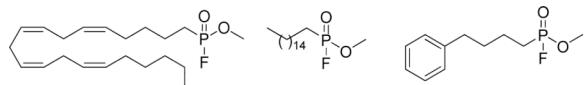


43, AACOCF₃ **44**, PACOCF₃



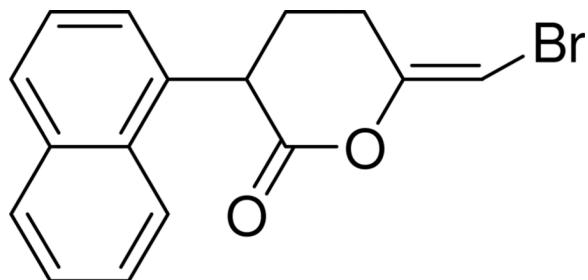
47 **91**

4.4.2. Methyl fluorophosphonates—MAFP (**48**) was found to irreversibly inhibit GVIA PLA₂.⁴¹⁵ In addition, the saturated fluorophosphonates **92** and **93** inhibited GVIA PLA₂ showing similar potencies and considerably higher potency than that of MAFP.



48 **92** **93**

4.4.3. Bromoenol lactone



94, BEL

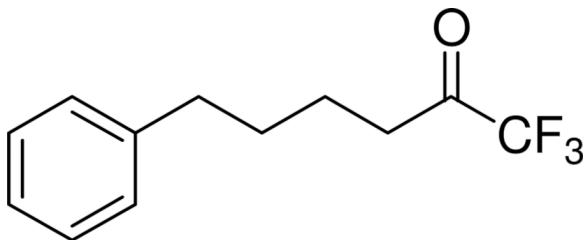
Bromoeno lactone (**94**, BEL) is an irreversible, covalent inhibitor of GVIA PLA₂,^{414,376b} inhibiting the enzyme at concentrations far lower than those required to inhibit sPLA₂ or cPLA₂ family members. As a result, BEL is commonly used to selectively inhibit GVIA PLA₂ in cellular systems. However, although BEL can distinguish GVIA PLA₂ among other PLA₂s, it may also inhibit other enzymes, for example the magnesium-dependent phosphatidate phosphohydrolase-1,⁴¹⁶ and was first identified as a serine protease inhibitor.⁴¹⁷ Thus, studies involving iPLA₂ inhibition by BEL are ambiguous and require confirmation by other experiments.

Initially, BEL had been characterized as a suicide inhibitor of canine myocardial calcium-independent PLA₂.⁴¹⁸ Using BEL it has been suggested that PGE₂ generation upon stimulation may be partially mediated by iPLA₂ in addition to sPLA₂.⁴¹⁹ In a number of studies, Turk et al. showed that GVIA PLA₂ (iPLA₂ β) played a signalling role in β -cells that differed from housekeeping functions in PC biosynthesis and degradation in P388D1 and CHO cells.³⁷⁹ BEL was found to decrease arachidonic acid release and PGE₂ production in 3T6 fibroblast cultures stimulated by fetal calf serum.⁴²⁰ Prosynaptic injection of BEL selectively increased AMPA receptor-mediated synaptic transmission.⁴²¹ Intracerebroventricular injection of BEL significantly reduced responses to von Frey hair stimulation after facial carrageenan injection in both C57BL/6J (B6) and BALB/c mice.²⁸⁶

The effect of inhibition of iPLA₂ β on chemotherapeutic-induced death and phospholipid profiles in renal cells was studied.^{393c} Inhibition of iPLA₂ by BEL decreased prostate cancer cell growth by p53-dependent and independent mechanisms.⁴²² Alterations in Mdm2 and epidermal growth factor receptor activation following BEL exposure suggested novel roles for iPLA₂ in prostate cancer cell signaling. Most recently, it was shown that iPLA₂ inhibition by BEL activated p38 MAPK signaling pathways during cytostasis in prostate cancer cells.⁴²³

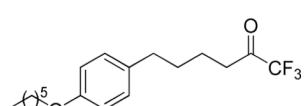
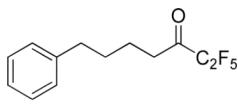
Interestingly, in a number of studies it has been demonstrated that (*R*)- and (*S*)-enantiomers of BEL present different inhibitory properties.⁴²⁴ GVIA PLA₂ (iPLA₂ β) and GVIB PLA₂ (iPLA₂ γ) have been found to be selectively inhibited by (*S*)- and (*R*)-enantiomers of BEL, respectively.^{424a,b} Turk et al. have reported that BEL inactivates GVIA PLA₂ by generating a diffusible bromomethyl keto acid that alkylates cysteine thiols, rather than creating an acyl-enzyme intermediate with the active-site serine.⁴²⁵

4.4.4. Polyfluoroketones—In 1999, a variety of trifluoromethyl ketones were studied for the inhibition of GVIA PLA₂ in a mixed-micelle assay.²⁶⁹ Trifluoromethyl ketone **95** was found to be a potent inhibitor of GVIA PLA₂ (Table 22), 10-fold more potent in comparison to GIVA PLA₂. In 2008, a series of polyfluoro ketones for the selective inhibition of GVIA PLA₂ was synthesized.^{405b} Such a task is very challenging, because both the intracellular GVIA PLA₂ and GIVA PLA₂ share the same catalytic mechanism and cross-reactivity is expected for inhibitors targeting the active site serine.



95

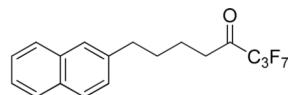
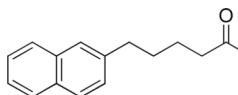
It was found that the pentafluoroethyl ketone functionality favored selective inhibition of GVIA PLA₂. FKGK11 (**96**) was found to be a selective GVIA PLA₂ inhibitor, while the trifluoromethyl ketone FKGK2 (**97**) can be considered as a pan-inhibitor inhibiting GVIA PLA₂, GIVA PLA₂, and even GV sPLA₂ (Table 23).



96

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In a continuation of SAR studies on polyfluoro ketones and using an improved assay for GVIA PLA₂, compound FKGK18 (**98**) was identified as the most potent GVIA PLA₂ inhibitor yet reported (Table 24).^{405c} Being 195 and >455 times more potent for GVIA PLA₂ than for GIVA PLA₂ and GV sPLA₂, respectively, makes it a valuable tool to explore the role of GVIA PLA₂ in cells and in vivo models. Heptafluoro derivative **99** also presented interesting inhibition of GVIA PLA₂, inhibiting GIVA PLA₂ and GV sPLA₂ at least 90 times less potently (Table 24).

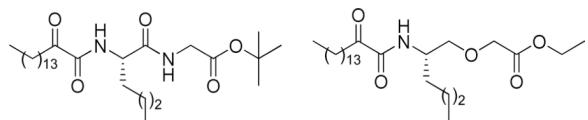


98

99

Selective PLA₂ inhibitors contributed to the clarification of the role of each PLA₂ class in neurological disorders.^{405a,426} Using the selective GVIA PLA₂ inhibitor FKGK11, the selective GIVA PLA₂ AX059 and the pan-inhibitor FKGK2, the role of the various classes of PLA₂ in EAE, the animal model of multiple sclerosis, was studied.⁴²⁶ The results suggested that GIVA PLA₂ plays a role in the onset of the disease, while GVIA PLA₂ plays a key-role both on the onset and the progression of the disease. Thus, it seems that GVIA PLA₂ is a target enzyme for the development of novel therapies for multiple sclerosis.⁴²⁶

4.4.5. 2-Oxoamides—As discussed in the section on GIVA PLA₂ inhibitors, 2-oxoamides based on esters of amino acids may inhibit both GVIA PLA₂ and GIVA PLA₂. Recently, two 2-oxoamides based on esters of dipeptides or pseudodipeptides (**100** and **101**) were reported to preferentially inhibit GVIA PLA₂ (Table 25).³¹⁷



100

101

4.4.6 Summary Status of iPLA₂ Inhibitors—Research on GVIA iPLA₂ inhibitors is relatively limited, due partially to its lack of a crystal structure, but also to the fact that only recently has it emerged that GVIA iPLA₂ plays a significant role in a number of medical conditions. As with GIVA cPLA₂, the first series of potent reversible GVIA iPLA₂ inhibitors were trifluoromethyl ketones of fatty acids. Bromoenol lactone (BEL) is the most important irreversible inhibitor of GVIA iPLA₂ and it has been used in a number of *in vitro*, *ex vivo* and *in vivo* studies to elucidate the role of GVIA iPLA₂. One should note that even though BEL is selective against GVIA iPLA₂ when compared to the other PLA₂ groups, it also inhibits other serine enzymes and therefore the data obtained from *ex vivo* and *in vivo* studies of its inhibitory activity should be carefully considered. The most potent, selective and reversible inhibitors of GVIA iPLA₂ are polyfluoroketones bearing an aromatic ring and a small aliphatic chain as a spacer between the two functional groups. The most potent and selective polyfluoroketone inhibitor, namely FKGK11, has been used to show the important role that GVIA iPLA₂ plays in EAE, the animal model of multiple sclerosis. Most recently, it has been reported that a combination therapy of GVIA iPLA₂ inhibitors (BEL as well as FKGK11) with the anticancer drug paclitaxel is highly effective at blocking development of ovarian cancer.⁴²⁷ As the significance of GVIA iPLA₂ emerges and efforts to acquire its crystal structure advance, it seems essential to persist in developing potent and selective GVIA iPLA₂ inhibitors.

5. PAF Acetylhydrolases (GVII and GVIII PAF-AH PLA₂s)

5.1 Groups, Subgroups, Specificity and Mechanism

Platelet-activating factor (PAF) is a potent phospholipid activator and mediator of many leukocyte functions, particularly as a mediator of inflammation.⁴²⁸ Two groups of PLA₂s, designated GVII and GVIII (Table 26), can catalyze the hydrolysis of the acetyl group from the *sn*-2 position of PAF to produce lyso-PAF and acetate, which is why the enzymes were originally named as PAF acetylhydrolases (PAF-AH).¹⁴ GVIIA PLA₂ is a secreted enzyme with a molecular weight of 45 kDa that associates with both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in human plasma⁴²⁹. Therefore, the enzyme is also known as plasma PAF-AH (pPAF-AH) or lipoprotein-associated PLA₂ (Lp-PLA₂). GVIIB PLA₂, also referred to as PAF-AH II, is an intracellular protein with a molecular weight of 40 kDa that has an N-terminal myristylation site and which shares 41% amino acid sequence identity with GVIIA PLA₂.⁴³⁰ GVIII PLA₂ is a brain intracellular heterotrimeric protein complex that is also referred to as PAF-AH Ib.⁴³¹ GVIII PLA₂ consists of two 26 kDa catalytic subunits (α subunits) and one 45 kDa non-catalytic regulatory subunit (β subunit or LIS1).⁴³² The catalytic subunits GVIIIA and GVIIIB PLA₂, also termed α_1 and α_2 , form catalytically active homo- and hetero-dimers. The α_1 and α_2 subunits share 69% amino acid sequence identity with each other and do not show significant amino acid sequence identity with GVII PLA₂s.

PAF acetylhydrolases are calcium independent PLA₂s. These enzymes all feature the same catalytic triad, Ser/His/Asp.^{20a} The secreted GVIIA Lp-PLA₂ shows broad substrate specificity. In addition to PAF, GVIIA Lp-PLA₂ can also hydrolyze phosphatidylcholines with short chain *sn*-2 residues.⁴³³ Lp-PLA₂ hydrolyzes PCs with decreased efficiency when the *sn*-2 residue is lengthened. The C5 homologue was 60% as efficient as PAF, the C6

homologue was 20% as efficient, and the C9 homologue was only 2% as efficient as PAF.⁴³³ However, the enzyme activity is dramatically increased when the *ω*-end contains an oxidized functional group, such as an aldehyde or a carboxyl.⁴³³ When the *sn*-2 residue terminates with an aldehydic functional group, residues up to C9 are effective substrates for Lp-PLA₂. Several studies indicate that Lp-PLA₂ can use oxidized phospholipids as substrates.^{433–434} A mass spectrometry based analysis of the effects of *in vitro* oxidation in the absence and presence of an irreversible Lp-PLA₂ inhibitor on the PC compositions of human LDL has shown that oxidized PCs are recognized as substrates by Lp-PLA₂ during LDL oxidation.^{434b} The study shows that oxidatively modified di-unsaturated and poly-unsaturated fatty acid-containing PC species are efficient substrates for Lp-PLA₂ and that truncated oxidized PCs are major substrates.^{434b} In a recent study, both Lp-PLA₂ and the intracellular GVIIB PAF-AH II have been shown to have the ability to release F₂-isoprostanes from esterified phospholipids, though at a much slower rate than they hydrolyze PAF or POVPC.⁴³⁵ Interestingly, both GVII PLA₂s show about a 200- to 1000-fold higher affinity for esterified F₂-isoprostanes than for PAF and POVPC.⁴³⁵ GVIIB PAF-AH II shows very similar substrate specificity to the plasma form of Lp-PLA₂.⁴³⁶ Both forms of GVII PLA₂ display PLA₁ activity and transacetylase activity that transfers acetic acid from PAF to lysophospholipids.^{436–437} Neither enzyme distinguishes between an ester and an ether at the *sn*-1 position of PAF or PAF analogues.^{20a}

Compared with GVII PLA₂s, GVIII PLA₂ is more restricted at the *sn*-2 position, selecting only for acetyl groups, but not selective at all for the head groups at the *sn*-3 position.⁴³⁸ GVIII catalytic homodimers and heterodimers show different activities towards PAF and PAF analogues. The α_2/α_2 homodimer hydrolyzes PAF and 1-O-alkyl-2-acetyl-*sn*-glycerol-3-phosphorylethanolamine more efficiently,⁴³⁸ while the α_1/α_1 homodimer and α_1/α_2 heterodimer exhibit higher activity towards 1-O-alkyl-2-acetyl-*sn*-glycerol-3-phosphoric acid.⁴³⁸ The β subunit possesses a regulatory role for catalytic activity in a catalytic dimer composition-dependent manner. The β subunit accelerates PAF hydrolysis of α_2/α_2 homodimers up to 4-fold, slightly suppresses the activity of α_1/α_1 homodimers, and has little effect on the activity of the α_1/α_2 heterodimers.⁴³⁸

5.2 Structural Characteristics and Interaction with Membranes

5.2.1 GVIIA PLA₂ (Lp-PLA₂)—Lp-PLA₂

Lp-PLA₂ is a 45 kDa, Ca²⁺-independent PLA₂ that contains a GSXG motif, which is a characteristic fingerprint for neutral lipases and serine esterases.⁴²⁹ The first 20 residues comprise a hydrophobic signal peptide¹². The N-terminus of this protein was found to be heterogeneous in human blood (it can be Ser-35, Ile-42 or Lys-55); the C-terminus is Asn-441.⁴²⁹ While removal of 21 amino acids from the C-terminal residues caused a slight loss of activity, a 30 residue deletion reduced catalysis to below the limit of detection.⁴²⁹ Lp-PLA₂ may contain heterogeneous asparagine-conjugated sugar chains at residues Asp-423 and/or Asp-433.⁴³⁹ Though N-linked glycosylation does not affect secretion of the enzyme or its catalytic activity, it may hinder the enzyme's ability to associate with HDL particles.^{439a}

The crystal structure shows that Lp-PLA₂ contains a classic lipase/esterase α/β hydrolase fold and features a catalytic serine/histidine/aspartic acid triad (Figure 13 A).⁴⁴⁰ A mutagenesis study has shown that residues Trp-115, Leu-116 and Tyr-205 are important for enzyme binding with LDL particles.⁴⁴¹ An Lp-PLA₂/LDL binding assay using LDL from transgenic mice expressing truncated apoB-100 lipoproteins has demonstrated that the C-terminus of apoB-100 (residues 4119–4279) could be important for LDL binding to Lp-PLA₂.⁴⁴¹ In fact, in humans who are apoB-100 deficient, Lp-PLA₂ was found to be entirely associated with HDL.⁴⁴² A recent study has identified a domain (residues 367–370) that mediates Lp-PLA₂/HDL association.⁴⁴³ The data also shows that residues Met-368 and

Leu-369 are necessary for binding to HDL and His-367 and Lys-370 may participate in the association as well.⁴⁴³ Based on the above experiments and the solved crystal structure, two surface exposed hydrophobic α helices (residues 114–126 and residues 362–369) (Figure 13A) have been suggested as important for mediating the enzyme's association with lipoproteins and/or lipid membranes.⁴⁴⁰ However it's still possible that other regions may also participate in the lipoprotein association. There is a large patch of carboxylate residues together with three basic residues on the protein surface that may play a role in LDL/HDL lipoprotein partitioning.⁴⁴⁰

Although it has been earlier suggested that Lp-PLA₂ acts on the substrate PAF by a non-interfacial mechanism,⁴⁴⁴ considering that the surfaces of both LDL and HDL are enriched with phospholipids, it has been expected that under physiological conditions Lp-PLA₂ would bind to its substrate(s) from the lipid membrane phase, as is the case for all classic membrane-associating PLA₂ enzymes. A recent study by Pande⁴⁴⁵ has shown that the lipid composition of membrane vesicles affects Lp-PLA₂ activity and that membrane binding of Lp-PLA₂ increases enzyme activity, which suggests that Lp-PLA₂ may also operate by an interfacial mechanism. Recently peptide amide hydrogen-deuterium exchange mass spectrometry (DXMS) was employed to characterize, at the molecular level, the association of Lp-PLA₂ with lipid membranes.⁴⁴⁶ It was found that specific residues 113–120 in one of the enzyme's surface-exposed hydrophobic α -helices likely mediate liposome binding (Figure 13 B).⁴⁴⁶ In the resulting model, the active site opens to the solvent, but faces the interfacial surface that accesses substrates from the lipoprotein particles. The active site would theoretically allow substrates to enter from the aqueous phase as well as substrates partitioning into the lipoproteins.

The distribution and location of Lp-PLA₂ in LDL/HDL lipoproteins have been suggested to affect Lp-PLA₂ function and/or its physiological role.⁴⁴⁷ An abnormal distribution of the enzyme may correlate with diseases.⁴⁴⁷ The enzyme localized in LDL was shown to be more active than the same enzyme localized on HDL.⁴⁴² In vitro study using assays at low PAF concentrations that mimic physiological levels showed that the enzyme associated with HDL particles is inactive.⁴⁴⁸ Thus the enzyme localized in LDL and HDL may have different functions. One suggestion is that the enzyme associated with HDL could serve as a reservoir for plasma Lp-PLA₂ under the circumstance that excess enzyme is present.⁴⁴⁹ This suggests that the Lp-PLA₂ interaction with HDL could be different for the same enzyme interacting with LDL and the enzyme associated with HDL may form a catalytically unactive conformation. In order to access the substrate from the lipid membrane, Lp-PLA₂ would have to either undergo a conformational change or dissociate from the HDL.

Additionally, Lp-PLA₂ shows preferential association with dense LDL and with the very high density lipoprotein-1 (VHDL-1) subfraction in human plasma.⁴⁵⁰ The electronegative LDL subfraction [LDL(–)] has shown 5-fold higher PAF-AH activity than the nonelectronegative LDL subfraction; Lp-PLA₂ is mainly associated with the LDL(–) subfraction.⁴⁵¹ Lipoprotein(a) [Lp(a)] also accounts for Lp-PLA₂ activity to a small extent.^{20d} In addition to lipoproteins, Lp-PLA₂ may also associate with microparticles in human plasma.⁴⁵² Furthermore, different lipoprotein carriers of Lp-PLA₂ may result in different roles of the enzyme in atherosclerosis.⁴⁴⁷

5.2.2 GVIII PLA₂ (PAF-AH Ib)—Several three-dimensional crystal structures of GVIII PAF-AH Ib were determined, including the α_1/α_1 homodimer, the α_1/α_2 heterodimer and the α_2/α_2 homodimer complex with LIS1.^{431,453} PAF-AH Ib contains a single α/β domain with a similar fold to that found in GTPases⁴³¹. The side chains of Thr-103, Leu-48 and Leu-194, which are all conserved in α_1 and α_2 isoforms, form a hydrophobic pocket which could only fit a substrate's acetyl moiety (Figure 14).⁴³¹ Mutants replacing Thr-103 or Leu-48 or

Leu-194 with an alanine residue showed higher relative activity against phospholipids with an *sn*-2 acyl chain longer than an acetyl^{453a}. This structural characteristic can explain why PAF-AH Ib shows much more strict substrate specificity than other PAF-AHs.

The crystal structure of the LIS1 complex with the α_2/α_2 homodimer shows that one LIS1 homodimer binds symmetrically to one α_2/α_2 homodimer via the highly conserved top faces of the LIS1 β propellers.^{453c} A site-directed mutagenesis study has shown that Glu-39 of the murine PAF-AH Ib α_2 subunit is crucial for LIS1 binding and the E39D mutation results in a complete loss of LIS1 binding.⁴⁵⁴ Comparing the α_2/α_2 structure complexed with LIS1 with α_1/α_1 and α_1/α_2 dimer structures, no major changes were found.^{453c} This indicates that the α_2/α_2 dimeric structure is not impacted significantly when it associates with LIS1. The mechanism of the β subunit's regulation of PAF-AH Ib activity requires further investigation.

5.3 Biological Functions and Disease Implications

5.3.1 Lp-PLA₂ in Atherosclerosis—Lp-PLA₂ is secreted predominantly by macrophages.^{11,455} Its expression and secretion increase significantly as human monocytes differentiate into macrophages, and increase even more dramatically during activation of macrophages in the atherosclerotic lesion.⁴⁵⁶ Thus, Lp-PLA₂ is considered a very important enzyme for atherosclerotic progression. However, since its discovery, the role of Lp-PLA₂ in atherosclerosis has always been a controversial issue. By inactivating PAF and PAF-like lipid mediators and hydrolyzing oxPC in the oxidized LDL particles, Lp-PLA₂ could act as a potent anti-atherogenic enzyme. However, growing evidence has shown that Lp-PLA₂ may also play a pro-atherosclerotic role. This is because Lp-PLA₂ generates the pro-inflammatory and proapoptotic lipid mediators lyso-PC and oxidized nonesterified fatty acids, which play an important role in the development of atherosclerotic necrotic cores. In the current review, we summarize progress in understanding the anti-atherosclerotic and pro-atherosclerotic roles of Lp-PLA₂.

Lp-PLA₂ was initially considered to be an anti-inflammatory enzyme. Recombinant Lp-PLA₂ markedly decreases vascular leakage in pleurisy and paw edema and blocks inflammation.¹² Probably the strongest evidence is from a loss of function mutation study. A missense mutation (V279F) found in 4% of the Japanese population leads to a complete loss of enzyme activity⁴⁵⁷ and Lp-PLA₂ deficiency is shown to be an independent risk factor for cardiovascular disease and stroke⁴⁵⁸. Minimally oxidized LDL obtained from Japanese subjects with this mutation consistently induced greater monocyte adhesion.⁴⁵⁵ Several other experimental data from either *in vitro* or animal models also support the protective role of Lp-PLA₂. Lp-PLA₂-treated mildly oxidized low density lipoprotein (MM-LDL) lost the ability to induce endothelial cells to bind to monocytes.⁴⁵⁹ Lp-PLA₂ destroys the action of MM-LDL by facilitating hydrolysis of bioactive oxidized phospholipids to lysophospholipids.⁴⁵⁹ Macrophages in both human and rabbit atherosclerotic lesions express Lp-PLA₂ and modulation of Lp-PLA₂ activity could lead to anti-atherogenic effects in the vessel wall.⁴⁵⁵ Transgenic apoE deficient mice expressing human Lp-PLA₂ have shown increased Lp-PLA₂ enzyme activity, decreased oxidized lipoprotein accumulation in the injured vessels, and reduced macrophage homing.⁴⁶⁰ The same mouse model has also shown that gene transfer of Lp-PLA₂ inhibits injury-induced neointima formation and reduces spontaneous atherosclerosis in the absence of mechanical injury.⁴⁶¹ In nonhyperlipidemic rabbits the expression of Lp-PLA₂ reduces oxLDL accumulation in arteries and exerts anti-inflammatory, antithrombotic, and antiproliferative effects.⁴⁶² All together, the potential anti-atherosclerotic role of Lp-PLA₂ may be attributed to hydrolysis of the oxidized phospholipids and therefore a reduction in the accumulation of oxidized lipoproteins. However, Lp-PLA₂ associates mainly with HDL in either mice or rabbits; even in pigs,

which have a similar lipoprotein profile to that of humans, 90% of the Lp-PLA₂ is associated with HDL and only 5% with LDL.⁴⁶³ In fact, humans are the only mammals where Lp-PLA₂ predominantly associates with LDL. So the results from animal models may not be the case in humans and the human enzyme may not protect against atherosclerosis.

Increasing evidence suggests that Lp-PLA₂ plays a critical role in the development and progression of atherosclerosis. Epidemiological studies that began in 2000 with about 80,000 participants have shown that increased Lp-PLA₂ activity and mass in plasma are associated with increased risk of coronary disease, stroke, and mortality.^{20e,464} Both the lyso-PC and nonesterified fatty acids that are produced by hydrolyzing oxidized LDL are pro-inflammatory and atherogenic, and they play a critical role in atherosclerosis. Increased levels of Lp-PLA₂ and lyso-PC were found in symptomatic carotid artery plaques and the increase is correlated with markers of tissue oxidative stress, inflammation, and instability.⁴⁶⁵ A study in hypercholesterolemic pigs also shows increased Lp-PLA₂ activity is associated with increased levels of lyso-PC, oxidized LDL and inflammation.⁴⁶³ Thus increased Lp-PLA₂ levels further accelerate atherosclerosis in the hypercholesterolemic minipig model.⁴⁶³ A recent study has shown that the ratio of Lp-PLA₂ to oxLDL is higher in carotid atherosclerotic tissue and plasma than it is in normal tissue and plasma.⁴⁶⁶ The oxPC/apoB 100 ratio has been shown to be a significant risk factor for cardiovascular disease (CVD) and when associated with high levels of Lp-PLA₂ activity, the risk for CVD is increased.⁴⁵⁶ More evidence suggesting a pro-atherogenic role for Lp-PLA₂ comes from an inhibitor study. *In vitro* experiments using GlaxoSmithKline (GSK) inhibitors SB-222657 (see section 5.4) and SB-677116 demonstrate reduced generation of lyso-PC and oxidized nonesterified fatty acids.^{434a,467} Lp-PLA₂ activity inhibited by SB-222657 showed reduced atherosclerotic plaque development in a 3-month rabbit model.⁴⁶⁸ A more specific and efficient inhibitor from GSK, SB-480848 (Darapladid), can selectively inhibit Lp-PLA₂ and hence reduce the development of advance coronary atherosclerosis in diabetic and hypercholesterolemic swine.⁴⁶⁹ Thus Lp-PLA₂ is now considered a risk factor, a potential biomarker, and a target of therapy in the treatment of cardiovascular disease.

A non-functional V279F allele was first discovered in the Japanese population and was later found in other East Asian populations.⁴⁷⁰ This null allele is rarely found in Middle Eastern populations and is almost absent in Europeans.⁴⁷¹ A recent study of the V279F null mutation population in Japan has shown that there is no reduced risk of Alzheimer's disease with genetic deficiency of Lp-PLA₂.⁴⁷² However, another study in South Korean men has shown that the V279F null allele carriers are protected from coronary artery disease.⁴⁷³ In addition to V279F, another inactive mutant Q281R is also found in humans.⁴⁷⁴ Residue 281, near the active site Ser-273, may affect the active site folding and/or substrate binding and therefore causes a loss of enzymatic activity. Other polymorphisms, such as R92H, I198T and A379V, have also been identified, and their presence may correlate with CVD.⁴⁷⁵ Details regarding these mutations and polymorphisms are in a recent published review.⁴⁷⁶

5.3.2 Lp-PLA₂ and Neonatal Necrotizing Enterocolitis—Several studies have indicated that Lp-PLA₂ may play an important role in the pathogenesis of neonatal necrotizing enterocolitis (NEC), which afflicts premature newborn infants and is characterized by an acute onset of intestinal inflammatory necrosis. Human infants with NEC have systemic accumulation of PAF and decreased serum Lp-PLA₂ levels.⁴⁷⁷ A neonatal rat model treated with human recombinant Lp-PLA₂ shows a reduced incidence of NEC compared with controls.⁴⁷⁸ Enteric Lp-PLA₂ administration resulted in significant intestinal Lp-PLA₂ activity but no serum Lp-PLA₂ activity.⁴⁷⁸ Lp-PLA₂ knockout mice show lower mortality rates before 24 h of life compared with wild type controls in response to bacterial exposure, formula feeding, and asphyxia.⁴⁷⁹ However, the knockout mice have a significantly higher incidence of NEC after 24 h and showed increased expression of

intestinal pro-inflammatory mediators compared with wild type controls.⁴⁷⁹ Therefore, Lp-PLA₂ may play a protective role in the development of NEC and exogenous Lp-PLA₂ supplementation may help to reduce the incidence of NEC in premature infants.

In addition to atherosclerosis and neonatal necrotizing enterocolitis, Lp-PLA₂ may also be related to severe anaphylaxis. The PAF hydrolysis activity of Lp-PLA₂ was found to be significantly lower in patients with fatal anaphylactic reaction to peanuts than control groups and Lp-PLA₂ enzymatic activity was inversely correlated with the severity of the anaphylactic response.⁴⁸⁰

5.3.3 GVIIIB PLA₂ (PAF-AH II)—The intracellular form of PAF-AH II is predominantly expressed in epithelial cells, such as kidney proximal and distal tubules, intestinal column epithelium, and hepatocytes.⁴⁸¹ PAF-AH II is thought to have an antioxidant function. During oxidative stress, PAF-AH II translocates from cytosol to the membrane and protects the cell against oxidative stress induced cell death.⁴⁸² Overexpression of PAF-AH II suppresses the oxidative stress-induced cell death.⁴⁸² The antioxidant function may depend on the enzyme's ability to hydrolyze oxidized phospholipids. The N-myristoylated property of PAF-AH II makes it possible for it to be present both in the cytosol and membranes.

PAF-AH II knockout mice are not phenotypically distinguishable from wild-type mice, although PAF-AH activity was almost abolished in the liver and kidney of knockout mice.⁴⁸¹ However, the knockout mice showed a delay in hepatic injury recovery when injected with carbon tetrachloride.⁴⁸¹ Moreover, the levels of F₂-isoprostane esterified phospholipids in the liver are higher in knockout mice than wild type mice after the injection of carbon tetrachloride.⁴⁸¹ As discussed above, plasma containing both PAF-AH and PAF-AH II can efficiently hydrolyze F₂-isoprostane esterified phospholipids *in vitro*.⁴³⁵ Therefore, the accumulation of F₂-isoprostane esterified phospholipids in knockout mice should account for the loss of PAF-AH II expression. PAF-AH II may be involved in the metabolism of esterified 8-isoprostaglandin F_{2α} and may protect tissues from oxidative stress-induced injury.⁴⁸¹

Overexpression of PAF-AH II also shows protective effects on neurons in a transgenic mouse model of focal cerebral ischemia.⁴⁸³ Overexpression of PAF-AH II in neurons may protect the central nervous system neurons against ischemic damage by hydrolyzing PAF, PAF like lipids and oxidized phospholipids.⁴⁸³

In addition to mammals, PAF-AH II has also been found in *C. elegans* and it is important for epithelial morphogenesis.⁴⁸⁴ However, PAF is not present in *C. elegans* and therefore PAF-AH II seems not to function in a PAF metabolism pathway in *C. elegans*. There's really not much evidence to support the idea that GVII PAF-AHs are involved in the PAF metabolism pathway, although the enzyme was first thought to regulate PAF. Additional work must be done to determine the relevant substrate of GVII PLA₂s *in vivo*.

5.3.4 GVIII PLA₂ (PAF-AH Ib)—The β subunit of GVIII PAF-AH Ib is a product of the LIS1 gene for type I lissencephaly, a severe developmental brain disorder caused by abnormal neuronal migration. In addition to forming a complex with PAF-AH Ib catalytic subunits α₁ and α₂, LIS1 interacts with a number of other proteins, such as cytoplasmic dynein, tubulin and NudE. Both PAF-AH Ib catalytic subunits α₁ and α₂ and LIS1 are expressed at high levels in the brain and testis.⁴⁸⁵ Haploid insufficiency of LIS1 results in neuronal migration defects in mice and mice that are homozygous null for LIS1 experience early embryonic lethality after implantation.⁴⁸⁶ Thus, PAF-AH Ib was thought to be important for brain development. However, none of the α₁, α₂ knockout mice or the α₁/α₂ double knockout mice exhibit brain abnormalities.^{485,487} α₁ Knockout mice are

indistinguishable from wild type mice and neither double knockout mice exhibit brain α_2 nor LIS1 levels are changed.⁴⁸⁵ α_2 Knockout male mice show a significant reduction in testis size and both α_1 and LIS1 levels are significantly reduced compared with wild type mice.⁴⁸⁵ Unexpectedly, α_1/α_2 double knockout mice exhibit severe impairment in spermatogenesis with no significant reduction of LIS1 levels.^{485,487} These data shows that the PAF-AH Ib catalytic subunits may not be required for brain development and that the catalytic units may mediate the signaling pathway by interacting with LIS1.

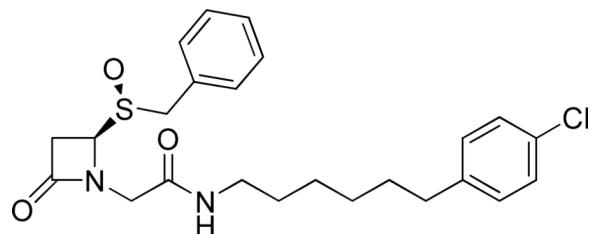
In addition to interacting with LIS1, the PAF-AH Ib catalytic α subunits are shown to interact with the Reelin very low density lipoprotein receptor (VLDLR), binding to the C terminal of VLDLR, but not with the apolipoprotein E receptor 2.⁴⁸⁸ The binding of α subunits to VLDLR is very specific and requires the NPxY domain and the presence of a leucine residue immediately following the sequence in the VLDLR.⁴⁸⁸ PAF-AH Ib may have a functional role in Reelin signaling during brain development.

PAF-AH Ib subunits expression levels are shown to be proportional to the expression levels of α -tubulin.⁴⁸⁵ Recently, Bechler and coworkers have shown that the PAF-AH Ib complex can regulate the functional organization of the Golgi complex.⁴⁸⁹ PAF-AH Ib can stimulate membrane tubules from Golgi complexes *in vitro*, and the catalytic activity is required.⁴⁸⁹ But the catalytic activity and LIS1 binding are not required for PAF-AH Ib α_1 and α_2 to associate with Golgi membranes⁴⁸⁹. Both PLA₂ enzymatic activity and LIS1 are important for maintaining the Golgi structure. Knockdown of either PAF-AH Ib α_1 and α_2 or LIS1 results in the formation of mini-stacks and inhibits tubule-mediated Golgi assembly and reduces anterograde trafficking.⁴⁸⁹

5.4 Chemical Inhibitors and Therapeutic Intervention

Although a limited number of synthetic inhibitor classes of GVIIA Lp-PLA₂ has been reported, one of them, darapladib, has reached the most advanced step of clinical trials (Phase III). The recent developments on Lp-PLA₂ inhibitors are summarized in a number of review articles.^{22e-i}

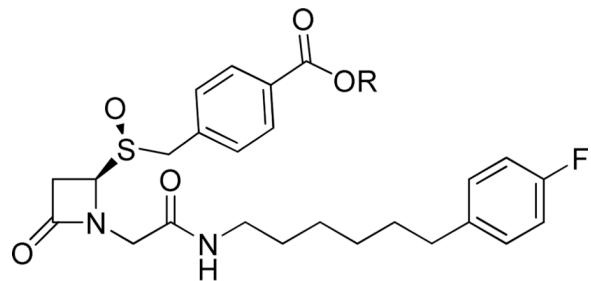
5.4.1. Azetidinones—In 1998, SmithKline Beecham presented a novel series of monocyclic β -lactams (or azetidinones) as inhibitors of Lp-PLA₂.⁴⁹⁰ Even though these compounds presented only modest inhibition, they gave way to the further investigation on Lp-PLA₂ inhibitors and to the first potent azetidinone inhibitor of Lp-PLA₂, SB-222657 (**102**).^{434a} This inhibitor was used to investigate the role of the enzyme in the oxidative modification of lipoproteins, and it was found that the inhibition was stereoselective since SB-222657 presented a K_i of 40 ± 3 nM, while its enantiomer had a K_i of 6.3 ± 0.5 μ M.⁴⁹¹



102, SB-222657

Another β -lactam, SB-245713 (**103a**), also presented inhibitory potency with an IC₅₀ of 5.2 nM, while its ethyl ester (**103b**), acting as an effective prodrug, was used in a 3 month proof of concept study in Watanabe heritable hyperlipidaemic rabbits (WHHL rabbits).^{468,492}

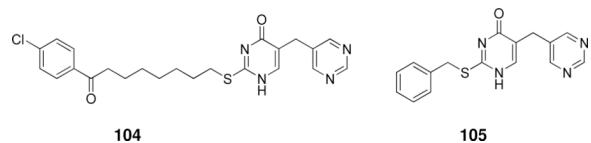
Histological analysis of aortic segments showed a decrease in both lesion cross-sectional area and thickness, particularly in segments with the most complex, raised plaque. These results supported the theory that Lp-PLA₂ plays a significant role in the development of atherosclerotic plaque and that Lp-PLA₂ inhibitors would be effective in blocking the later stages of plaque progression, including stability.^{468,492} β -Lactams acted as covalent inhibitors and presented a poor pharmacokinetic profile.



103a, R=H, SB-245713

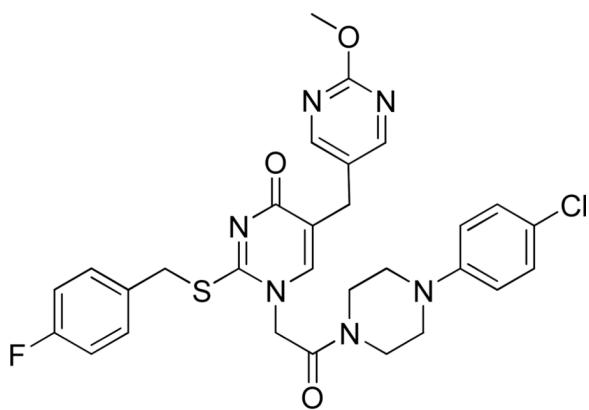
103b, R=Et, SB-244323

5.4.2. Pyrimidones—High throughput screening of a broad compound bank led to the identification of pyrimidones that were reversible inhibitors of Lp-PLA₂. For example, compounds **104** and **105** inhibited the enzyme with IC₅₀ values of 54 nM and 1.1 μM, respectively.⁴⁹³

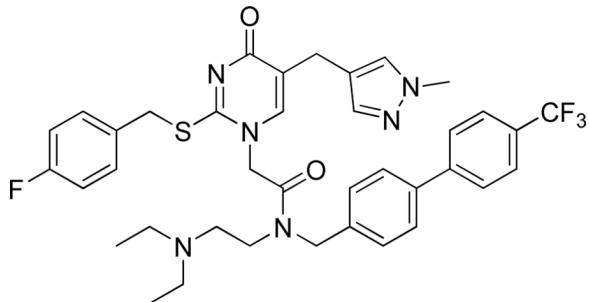


In a series of communications, various modifications were undertaken in those first pyrimidone structures in order to find the most potent inhibitor that would at the same time present excellent *in vivo* activity and oral bioavailability.⁴⁹⁴ Based on this series, novel 1-((amidolinked)-alkyl)-pyrimidones were designed as nanomolar inhibitors of human Lp-PLA₂. These compounds showed greatly improved activity in isolated plasma, while compounds **106a** and **106b** (Table 27) were orally active with a good duration of action.^{495,494a}

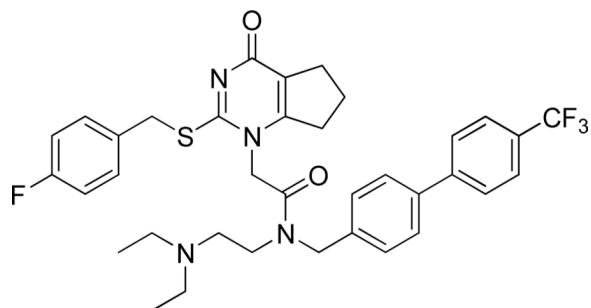
One of the most promising inhibitors was 1-(arylpiperazinylamidoalkyl)-pyrimidone **107** that presented an IC₅₀ value of 20 nM, 77% inhibition in human plasma at 100 nM and was orally active, properties that suggested that it would be an excellent lead.^{494c} In 2002, GlaxoSmithKline presented a series of 1-(biphenylmethylelamidoalkyl)-pyrimidones that were highly potent inhibitors of Lp-PLA₂ and showed excellent activity in the WHHL rabbit.^{494d}

**107**

Based on this series, they discovered a more potent, orally active inhibitor of Lp-PLA₂, SB-435495 (**108**) with an IC₅₀ of 0.06 nM and a suitable profile for evaluation in man.⁴⁹⁶

**108, SB435495**

Simplification of the pyrimidone 5-substituent of compound **108** led to the inhibitor, SB-480848 (**109**, Darapladib), that demonstrated excellent in vitro and in vivo profiles and was selected for progression to man.⁴⁹⁷ SB-480848 presented IC₅₀ 0.25 nM against rhLp-PLA₂, showed prolonged inhibition of plasma Lp-PLA₂ and a good correlation of pharmacodynamic and pharmacokinetic effects. Mechanistic studies indicated this compound to be a freely reversible, non-covalently-bound inhibitor of rhLp-PLA₂. Furthermore, the presence of SB-480848 during the copper catalyzed oxidation of human LDL prevented the production of lysoPC (IC₅₀ 4 ± 3 nM). Additional in vivo studies showed that SB-480848 had good oral bioavailability, but also presented excellent inhibition of Lp-PLA₂ within atherosclerotic plaque after oral administration of **109** to the WHHL rabbit.⁴⁹⁷



109, SB-480848, Darapladib

The results of a large case-control study provided strong evidence for an independent and clinically relevant relationship between elevated concentrations of Lp-PLA₂ and risk of stable coronary artery disease (CAD) and thus further support to the hypothesis that Lp-PLA₂ may be considered as a novel risk marker for CAD.^{464f} The effect of darapladib on plasma Lp-PLA₂ activity and cardiovascular biomarkers in patients with stable coronary heart disease or coronary heart disease risk equivalent was studied.⁴⁹⁸ Darapladib produced sustained inhibition of plasma Lp-PLA₂ activity in patients receiving intensive atorvastatin therapy and caused changes in IL-6 and hs-CRP after 12 weeks of darapladib 160 mg suggesting a possible reduction in inflammatory burden. A study compared the effects of 12-month treatment with darapladib or placebo on coronary atheroma deformability and plasma high-sensitivity C-reactive protein in 330 patients with angiographically documented coronary disease.⁴⁹⁹ The necrotic core volume continued to expand among patients receiving placebo, while Lp-PLA₂ inhibition with darapladib prevented necrotic core expansion, a key determinant of plaque vulnerability. In another study, selective inhibition of Lp-PLA₂ with darapladib inhibited progression to advanced coronary atherosclerotic lesions and confirmed a crucial role of vascular inflammation independent from hypercholesterolemia in the development of lesions implicated in the pathogenesis of myocardial infarction and stroke.⁴⁶⁹

A number of review articles published in 2010 summarize the completed preclinical and early phase clinical studies with darapladib.^{22g-i,500} Two phase III clinical studies are in progress and are expected to be completed by the end of 2012. The Stabilization of Atherosclerotic Plaque by Inhibition of Darapladib Therapy Trial (STABILITY, <http://clinicaltrials.gov/>, Identifier: NCT00799903) is a phase III randomized, double-blind, parallel-assignment, safety/efficacy study. The Stabilization of Plaques using Darapladib – Thrombolysis in Myocardial Infarction 52 (SOLID-TIMI 52, <http://clinicaltrials.gov/>, Identifier: NCT01000727) trial will test whether daily administration of darapladib (160 mg po) versus placebo when treatment is initiated within 30 days after an ACS will reduce the risk of CVD death, nonfatal MI or nonfatal stroke.

5.4.3 Summary Status of Lp-PLA₂ Inhibitors—Darapladib is a potent, selective inhibitor which is in a phase III trial and should also be useful for mechanistic studies.

6. Lysosomal Phospholipase A₂ [Group XV LPLA₂]

6.1 Groups, Subgroups, Specificity and Mechanism

GXV PLA₂ was first identified in the soluble fraction of Madin-Darby canine kidney (MDCK) cells⁵⁰¹ and was subsequently purified from bovine brain.⁵⁰² The genes encoding

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this enzyme were also identified in mouse, rat, cow and human (Table 28).⁵⁰³ GXV LPLA₂ has high protein sequence identity to a human lecithin: cholesterol acyltransferase-like lysophospholipase (LLPL).⁵⁰⁴ However GXV LPLA₂s do not show significant lecithin cholesterol acyltransferase or lysophospholipase activity under acidic or neutral conditions.⁵⁰⁴ Instead, the protein possesses Ca²⁺ independent PLA₂ and transacylase activity as well as 1-O-acylceramide synthase (ACS) activity, which esterifies an acyl group with the hydroxyl group in the C-1 position of ceramide using phospholipids as the acyl group donor.⁵⁰² Therefore, this enzyme was also named ACS. Hiraoka et. al. proposed that the hydrolyzed acyl group is transferred through an enzyme-acyl intermediate to ceramide or water, resulting either in the production of 1-O-acylceramide (ACS activity) or the release of free fatty acids (PLA₂ activity).⁵⁰⁴⁻⁵⁰⁵ Thus, ACS activity may be related to PLA₂ activity. GXV PLA₂ shows optimal enzymatic activity at pH 4.5 and the protein co-localizes with betahexosaminidase, suggesting that the enzyme localizes to lysosomes. Hence, the enzyme is known as a lysosomal PLA₂ (LPLA₂). In addition, LPLA₂ also shows PLA₁ activity.

GXV LPLA₂ is a water-soluble glycoprotein with a molecular mass of 45 kDa. GXV LPLA₂ has a signal sequence cleavage site and several N-linked glycosylation sites.⁵⁰⁴ Although the enzymatic activity of GXV LPLA₂ does not require divalent cations, such as Ca²⁺ and Mg²⁺, millimolar Ca²⁺ or Mg²⁺ does enhance the activity.⁵⁰² LPLA₂ has shown specificity toward phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and also has positional specificity. LPLA₂ is able to hydrolyze the acyl chain at both the *sn*-1 and *sn*-2 positions of PC or PE. LPLA₂ has a higher preference for the *sn*-2 position of 1-O-palmitoyl-2-unsaturated PCs and PEs.⁵⁰⁶ However this is not true if the substrate is PAPC or PAPE. LPLA₂ has demonstrated a higher specificity for the *sn*-1 position over the *sn*-2 position of PAPC or PAPE.⁵⁰⁶ The authors suggested that the polyunsaturated acyl chains affect the lipid bilayer packing structure and hence weaken the *sn*-2 preference of LPLA₂. In addition, LPLA₂ has shown preference for an unsaturated acyl group over a saturated acyl group of PC. In the cases of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) or 1-oleoyl-2-steraryl-*sn*-glycero-3-phosphocholine (OSPC) and 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC) or substrates, LPLA₂ favors the oleoyl group at the *sn*-2 position of SOPC and POPC or the *sn*-1 position of OSPC and OPPC.⁵⁰⁶

LPLA₂ contains a *GXSXG* motif, which is a characteristic fingerprint of neutral lipases and serine esterases. LPLA₂ belongs to the α/β hydrolase superfamily and contains the Ser-198, Asp-360 and His-392 catalytic triad. All three residues are required for enzymatic activity and replacement of the catalytic triad with alanine residues totally eliminated the transacylase activity.⁵⁰⁷ Four cysteine residues (Cys-65, Cys-89, Cys-330, and Cys-371) are conserved between LPLA₂ and lecithin cholesterol acyltransferase (LCAT)⁵⁰⁷. A site-directed mutagenesis study has shown that there is one disulfide bond between Cys-65 and Cys-89 and that there are free cysteine residues at Cys-330 and Cys-371, which are required for full expression of LPLA₂ activity. Quadruple mutations at all four cysteine residues and double mutations at Cys-65 and Cys-89 and a single mutation at Cys-65 or Cys-89 have been shown to cause total loss of LPLA₂ activity.⁵⁰⁷ However, the double mutations at Cys-330 and Cys-371 and the signal mutations at Cys-330 or Cys-371 only show partially reduced activity.⁵⁰⁷

LPLA₂ shows increased activity towards zwitterionic phospholipids that contain negatively charged lipids, e.g., PA, PE and PS, under acidic conditions.⁵⁰⁵ This may be due to the electrostatic interaction between LPLA₂ and the negatively charged lipid, and the interaction could promote the association of LPLA₂ with lipid vesicles. LPLA₂ operates using the same interfacial mechanism as the classic membrane-associating PLA₂s where substrate exclusively originates from the lipid vesicle. Adding NaCl or increasing the pH can

markedly reduce the increase in LPLA₂ activity due to negatively charged lipids.⁵⁰⁵ The increased Na⁺ concentration could destroy the electrostatic interaction between LPLA₂ and the lipid-water interfacial surface.

6.2 Biological Functions and Disease Implications

LPLA₂ plays an important role in lysosomal phospholipid degradation. LPLA₂ knockout mice (LPLA₂^{-/-}) showed an accumulation of PC and PE in alveolar macrophages as well as peritoneal macrophages and spleen, compared with wild type mice.⁵⁰⁸ This is consistent with the preference of the enzyme for hydrolysis of PC and PE.⁵⁰⁸ Recombinant LPLA₂ protein from HEK293 cells was added to LPLA₂^{-/-} mice alveolar macrophages. The uptake of exogenous LPLA₂ significantly decreased phospholipid accumulation.⁵⁰⁹ In contrast, the catalytically inactive LPLA₂-protein treated LPLA₂^{-/-} mice alveolar macrophages do not show a decrease in phospholipid accumulation, which suggests that LPLA₂ enzymatic activity is responsible for the reduction in phospholipid.⁵⁰⁹

LPLA₂ may be involved in surfactant phospholipid catabolism in alveolar macrophages. LPLA₂ is highly expressed in alveolar macrophages.⁵⁰³ Granulocyte macrophage colony stimulating factor (GM-CSF) knockout mice, a model of impaired surfactant catabolism, were found to have six times lower LPLA₂ activity than wild type mice.⁵⁰³ One year old LPLA₂^{-/-} mice showed marked splenomegaly, foam cell formation, and increased lung surfactant phospholipid levels.⁵⁰⁸ Thus, LPLA₂ may be a major enzyme that is responsible for pulmonary surfactant phospholipid degradation.

Several lines of evidence suggest that LPLA₂ is involved in phospholipidosis. In addition to the accumulation of phospholipid, LPLA₂ knockout mice show the formation of foam cells with lamellar inclusion bodies, which is a hallmark of cellular phospholipidosis.⁵⁰⁸ LPLA₂ has also been shown to be involved in the phospholipidosis induced by cationic amphiphilic drugs (CAD).⁵¹⁰ Treatment of MDCK cells with two CADs, 2-[4-(2-butyl-1-benzofuran-3-yl) carbonyl]-2,6-diiodophenoxy ethyl diethylamine (AMIOD) and D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), shows the formation of numerous multi-lamellar inclusion bodies.⁵¹⁰ AMIOD and PDMP both inhibited the transacylase activity of the soluble fraction from LPLA₂-overexpressed COS-7 cells in a concentration dependent manner.⁵¹⁰ CAD induced phospholipidosis may be due to the decreased phospholipid catabolism caused by inhibition of LPLA₂ activity.

In apolipoprotein E (apoE) knockout mice, LPLA₂ was found in foam cells in severe atherosclerotic lesions.⁵¹¹ LPLA₂ and apoE double knockout mice showed increased lesion formation but little effect on the plasma-lipid profile when fed on a normal diet. However the double knockout mice did not show a significant difference in the extent of atherogenesis when fed an atherogenic, Western-style diet.⁵¹¹ Thioglycolate-elicited peritoneal macrophages from the double knockout mice were more sensitive to apoptosis induced by oxLDL.⁵¹¹ All together, this suggests that LPLA₂ may play an important role in atherogenesis.

7. Adipose-Specific Phospholipase A₂ [GXVI AdPLA]

7.1 Groups, Subgroups, Specificity and Mechanism

Adipose-specific phospholipase A₂ (AdPLA) was initially cloned and characterized as a tumor suppressor.⁵¹² The enzyme has been considered to be a member of the lecithin retinol acyltransferase (LRAT) family.⁵¹³ Recently, it was found to exhibit phospholipase A₂ activity^{15a,513-514} and was designated as GXVI PLA₂.^{15a} Human AdPLA is expressed ubiquitously in various tissues and is highly expressed in adipose tissue.¹⁵ The enzyme contains 162 amino acids and is detected as an 18 kDa protein in an immunoblot.^{15b,514} In

3T3-L1 adipocytes, AdPLA showed perinuclear localization and was partially co-localized with the endoplasmic reticulum.^{15a}

AdPLA exhibits calcium-independent phospholipase A₁ and A₂ activities toward PC and PE, but lacks acyltransferase activity.^{15a,514} Its PLA₁ activity is higher than its PLA₂ activity.⁵¹⁴ The 18kDa AdPLA is distinct from iPLA₂ and cPLA₂ in size. The GXSXG or GXSGS motif in the active sites of iPLA₂ and cPLA₂ is not found in any of the LRAT family members. Instead, the LRAT members adopted a conserved His23/Cys113 dyad as the catalytic site.^{15a,514} Although it uses His in the active site, as does sPLA₂, AdPLA does not contain the highly disulfide bonded features or calcium dependency of sPLA₂s. Note that other members of the LRAT family, such as the calcium-independent N-acyltransferase (iNAT), also exhibit phospholipase A₁/A₂ activity.⁵¹⁴

7.2 Biological Functions and Disease Implications

AdPLA has been categorized as a Class II tumor suppressor.⁵¹² Researchers have found down-regulation of H-Rev107 in human ovarian carcinomas and involvement of H-Rev107 in interferon-dependent cell death.⁵¹⁵ AdPLA is also involved in eicosanoid production in adipose tissue. AdPLA-null mice showed reduced adipose tissue mass, triglyceride content and adipose PGE2 levels.^{15b} These knock-out mice exhibit a high rate of lipolysis and increased fatty acid oxidation in adipocytes.^{15b} Studies of AdPLA ablation in mice have indicated the importance of AdPLA in the development of obesity.^{15b}

8. Other Lipases Expressing Phospholipase A₂ Activities

The phospholipase A₂ superfamily had earlier been categorized into three major types, sPLA₂, cPLA₂ and iPLA₂.^{13–14} The diversity and growing number of enzymes, whose activity is independent of Ca²⁺ in addition to GVI iPLA₂, including the GVII and GVIII PAF-AHs and GXV lysosomal PLA₂, have complicated the classification and there are still some enzymes that display PLA₂ functionality or demonstrate homologous sequences to current PLA₂ enzymes that are not included in the naming system. We will discuss particularly the Otoconin-90-containing homology domain to sPLA₂, the phospholipase B1 containing homology domain to cPLA₂, the patatin domain containing proteins containing a homology domain to iPLA₂ and peridoxin-6, which is also an acidic lysosomal phospholipase (Table 29).

8.1 Otoconin-90 (sPLA₂ homology)

sPLA₂ has been characterized by the properties of its extracellular secretion, small size, highly disulfide bonded and His/Asp dyad active site. One major characteristic that stands out is the highly disulfide bonded PLA₂ domain. Otoconin-90/95 (OC90), also named as PLA₂-like (PLA₂L), contains a similar domain to Groups I, II, V, and X sPLA₂s. The human otoconin-90 was identified with 493 amino acids.⁵¹⁶ The partial murine otoconin-90 containing 453 residues and two sPLA₂ homologous domains were cloned in 1998.^{516a}

Otoconin is the major component of otoconia, which are protein-calcium carbonate crystals of the vestibular system that are indispensable for the perception of gravity in mammals.⁵¹⁶ The predominant mammalian otoconin, otoconin-90, is essential for formation of the organic matrix of otoconia by recruiting and associating with other matrix components, which includes otolin and cerebellin-1.⁵¹⁷ The arrest of otoconia genesis by NADPH oxidase organizer 1 (NoxO1) inactivation can result in an accumulation of otoconial protein, otoconin-90.⁵¹⁸ Otoconin-90 deletion leads to abnormal otoconia formation and physical imbalance but normal hearing in mouse models.⁵¹⁹ In vitro, the recombinant Otoconin 90 can facilitate nucleation and inhibit calcite crystal growth in a concentration-dependent

manner and induce morphologic changes in native otoconia.⁵²⁰ However, PLA₂ activity has not yet been demonstrated in this protein and it has not been categorized as a PLA₂.

8.2 Phospholipase B (cPLA₂ homolog)

cPLA₂ was initially characterized by the cytosolic property and calcium dependent activity. The current members of the cPLA₂ type contain a C2 domain, except for the GIVC PLA₂, which has high homology in the catalytic domain.¹⁴ Phospholipase B (PLB) was mentioned in association with the PLA₂ family in our previous review.¹³ Because its PLA₂ activity in a mammalian form has still not been clearly studied to date, and these enzymes are well known in the literature as PLB, we have still not included them as part of the GVI PLA₂ type. The PLB family contains PLB1, PLB2 and PLB3. By definition, these enzymes can hydrolyze both *sn*-1 and *sn*-2 acyl chains in phospholipid substrates. All PLBs contains the GXSXG serine lipase consensus sequence similar to the cPLA₂ group, as well as the other critical Asp active site dyad. Mutations of the Ser146 or Asp392 of the dyad abolish catalytic activity of PLB1 in cryptococcus neoformans.⁵²¹

PLB's studied from yeast, PLB1, PLB2 and PLB3 show significant PLB/lysoPL activity and PLB1 was shown to be responsible for much of the PLB and lysoPL activity.⁵²² The P. notatum PLB was shown to be highly glycosylated.⁵²³ Although PLB1, PLB2 and PLB3 have been identified in various species, PLB1 is the only human phospholipase B cloned and identified.⁵²⁴ The gene is encoded in chromosome 2p23.2 and found to be expressed in epidermis.⁵²⁴

8.3 PNPLA (iPLA₂ homolog)

iPLA₂ is currently defined as a calcium independent PLA₂. However, there are more and more groups of PLA₂ having calcium independent activity. Now, iPLA₂ is referred to as the GVI PLA₂. In GVI PLA₂, the key element of these enzymes is the patatin-like lipase domain and they are also included in the PNPLA family.^{335,348} Currently, there are 9 members PNPLA1-9 in this family and GVI PLA₂s are included in 6 of them.^{14,335a} The PLA₂ activities and functions of the other three enzymes, PNPLA1, 5 and 7 are not yet clearly determined.

Patatin is a protein from potatoes and other plants with confirmed PLA₁ and PLA₂ activity.⁵²⁵ Patatins have the lipase consensus sequence (Gly-Thr-Ser-Thr-Gly) as does the GVIA PLA₂. Its crystal structure was determined and showed the same catalytic dyad as cPLA₂.³⁷¹ The sequence alignment and structure modeling showed that patatin has high homology to iPLA₂.³⁴⁴ Current studies on PNPLA1 are very limited and are only at the transcript level.^{335a} The mRNA of PNPLA5 has been detected in both mouse and human tissues.^{335a,348,358b} PNPLA5, also named as GS2-like, shows TG hydrolase activity,^{358b} but the results are not consistent and need to be further confirmed.^{335a} The phylogenetic tree shows the PNPLA6 (NTE) and PNPLA7, NTE-related esterase (NRE), are closely related. Based on the sequence homology to NTE, NRE contains three nucleotide binding domains, a patatin-like lipase domain and a GXSXG motif. NRE exhibits lysophospholipase activity, but no phospholipase activity was detected.⁵²⁶ NRE transcript levels are strongly regulated by the nutritional diet and downregulated by indulin.⁵²⁶

8.4 aiPLA₂ (Peroxiredoxin-6)

Peroxiredoxin 6 in mammals (Prx6) contains a conserved cysteine at the active site to catalyze the reduction of hydrogen peroxide and alkyl hydroperoxides.⁵²⁷ This 25 kDa enzyme has been suggested to be a bifunctional enzyme that shows both peroxidase activity⁵²⁸ and phospholipase A₂ activity.⁵²⁹ It was shown to be a lysosomal protein and has an optimal Ca²⁺-independent PLA₂ activity at pH 4.0,⁵²⁹⁻⁵³⁰ and also named as acidic

calcium-independent PLA₂ (aiPLA₂) in 1997. It is structurally and functionally different from the GXV LPLA₂. Its function of hydrogen peroxide peroxidase activity was identified and named as 1-cysteine peroxiredoxin.⁵²⁸

Because this enzyme is a non-selenium glutathione peroxidase that can reduce oxidized phospholipid hydroperoxides with glutathione as an electron donor,⁵³¹ the role of the active site serine in a phospholipase catalytic process is questionable.¹³ However, other researches support the idea that the active site serine provides a phospholipase activity.⁵³²

9. Concluding Remarks

In summary, phospholipase A₂ has been studied for over a century, first from the venoms of a variety of snakes and later from mammalian pancreatic extracts. However, the emphasis was quite academic in understanding the biological function of PLA₂s, mainly as digestive enzymes, and then eventually as the enzymes were purified, the emphasis turned to their structure and function as proteins and their interaction with membranes and micelles. It wasn't until the mid-1980s that scientists began to appreciate the broader role of PLA₂ in inflammatory and other diseases and that they were not just digestive enzymes. By the late 1980's, there began an explosion of interest in PLA₂s due to the isolation, characterization, cloning, and general availability of the pure human recombinant enzymes. With time the specialized role of each type of PLA₂ in metabolism has become more and more appreciated. Although prior to the 1990's, many laboratories worked on developing inhibitors of the pancreatic and venom enzymes, once the pure cloned human non-pancreatic PLA₂s were available, numerous academic and industrial laboratories focused on the development of potent and selective inhibitors of these enzymes for specific disease applications.

The first comprehensively focused papers on inhibitors of sPLA₂, the oldest type of PLA₂ enzyme, appeared in 1985 and at that time the attempts were focused on synthetic phospholipid analogues and on marine natural products. Ten years later, Lilly Research Laboratories developed a class of indole inhibitors and one of them, Varespladib Methyl, was entered into clinical trials for the treatment of severe sepsis. However, the trials terminated at Phase II because the results were not robust. Years later, in 2008, Anthera Pharmaceuticals pursued the same inhibitor, now named A-002 for the treatment of cardiovascular diseases and this inhibitor is currently in Phase III trials. Apart from sPLA₂ inhibitors, much effort has been devoted to the discovery of inhibitors for cPLA₂. It has to be noted that for both sPLA₂ and cPLA₂ many structurally different classes of synthetic inhibitors have been reported. However, although cPLA₂ is considered to play the major role in inflammatory diseases, only in the mid 2000's did an inhibitor reach Phase II trials, an indole inhibitor developed by Wyeth (now part of Pfizer) for rheumatoid arthritis. Unfortunately, this trial (<http://clinicaltrials.gov/>, Identifier: NCT00396955) was terminated because of an "imbalance of gastrointestinal and lipase effects".

Although Lp-PLA₂ is the most recently recognized enzyme among the major PLA₂ types and although only two chemical classes of inhibitors have been reported for this enzyme, the pyrimidinone derivative Darapladib is at the most advanced clinical trials stage (Phase III). iPLA₂ has received little attention up to now as a therapeutic target. However, recent studies on animal models have demonstrated the importance of this PLA₂ type in a large variety of pathological conditions, for example, in neurological disorders. In conclusion, the results of the Phase III clinical trials on Darapladib and Varespladib Methyl within the next year should demonstrate whether or not PLA₂ inhibitors will become useful in clinical practice for cardiovascular diseases. In addition, we anticipate that the continuing research efforts on

cPLA₂ and iPLA₂ inhibitors may provide new chemical entities as potential novel investigational drugs that may eventually reach evaluation in clinical trials.

Identifying selective inhibitors for the various human PLA₂ groups is of paramount importance in the effort to develop PLA₂ inhibitors as pharmaceutical agents. Selective inhibitors for the major groups that have been especially useful for *in vitro* mechanistic studies have been reported, for example pyrrophenone for cPLA₂, varespladib for sPLA₂, pentafluoroketone FKGK11 for iPLA₂. However, inhibitors able to selectively inhibit the various sPLA₂ groups and subgroups (IIA, V, X) and the various cPLA₂ and iPLA₂ subgroups are still needed for use in animal and human studies.

Of course, the availability of the pure, cloned human PLA₂s covering a variety of types has opened up a large yield of basic research as to their structure and function and how they interact with substrate in the lipid-water interface presented to them as micelles or bilayer membranes. Much is still to be learned in the coming decades about the phospholipase A₂ superfamily!

Acknowledgments

We would like to thank the National Institutes of Health RO1-GM20501(EAD) for support of our work on phospholipase A₂ enzymes.

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Biographies



Edward A. Dennis is Distinguished Professor in the Department of Chemistry & Biochemistry and Department of Pharmacology at the School of Medicine of the University of California, San Diego. He received his BA from Yale University in 1963 and his PhD from Harvard University in 1968. Since completing his postdoctoral fellowship at Harvard Medical School in 1969, Dr. Dennis has been a professor at UCSD, also serving as Chair of the Department of Chemistry and Biochemistry, Chair of the Faculty Academic Senate, and on the Board of Overseers. He has authored over 330 original research publications and edited 13 books. He is currently Editor-in-Chief of the *J. Lipid Res.* and Director of the LIPID MAPS Consortium (www.LIPIDMAPS.org). He was named an inaugural Fellow of the AAAS in 1984 and was the recipient of the American Society of Biochemistry and Molecular Biology's Avanti Award in Lipid Enzymology in 2000, the European Federation for Lipid Science and Technology's European Lipid Science Award in 2008, and the Yale Medal from Yale University in 2008. Dr. Dennis' career research focus has been on the structure, function, mechanism, and inhibition of the enzyme phospholipase A₂ as well as on signal transduction, inflammation, lipid metabolism, eicosanoid action and lipidomics.



Jian Cao received his BS in applied chemistry (2000) and his MS in polymer chemistry (2003) from Jilin University, China and his PhD in biological chemistry (2008) under the supervision of Prof. Debra Dunaway-Mariano from the University of New Mexico. In 2009 he joined the Edward A. Dennis group at the University of California, San Diego as a postdoctoral fellow to study the interaction mechanisms of Group VII phospholipase A₂ (PAF-AH) with lipid membranes as well as high density and low density lipoproteins. His research interests include protein structure and function analysis, phospholipase A₂ enzymology, and hydrogen/deuterium exchange mass spectrometry.



Yuan-Hao Hsu received his BS from National Chung-Shin University in 1994 and his MS from National Taiwan University in 1996. He moved across the Pacific Ocean to the University of California, Riverside and received an MS in 2001 and a PhD in 2006, both in Biochemistry, with Dr. Jolinda A. Traugh. He joined the Edward A. Dennis group at the University of California, San Diego in 2006 to study the function, activation mechanisms

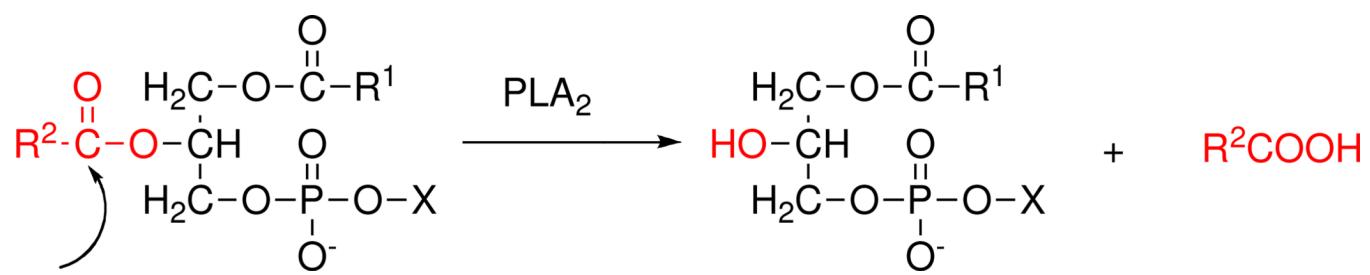
and membrane interactions of cytosolic phospholipase A₂ (cPLA₂) and calcium independent phospholipase A₂ (iPLA₂).



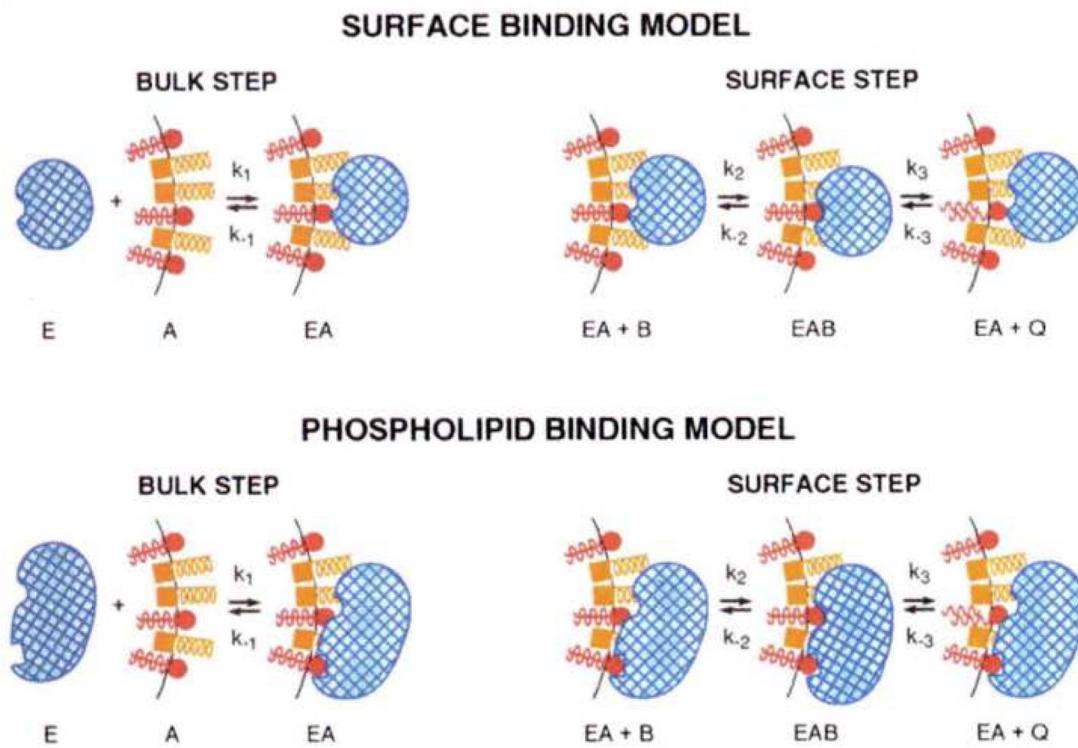
Victoria Magrioti studied Chemistry at the University of Athens. In 2003, she obtained her Ph.D. degree in Organic Chemistry under the supervision of Prof. Violetta Constantinou at the Agricultural University of Athens. During her Ph.D. studies she joined for a few months the group of Dr. Robert Verger at the CNRS, Marseille where she studied assays for lipase inhibitors. She continued her postdoctoral studies in the group of Prof. George Kokotos at the University of Athens and in the group of Prof. Alexandros Makriyannis at the Center for Drug Discovery of Northeastern University in Boston. She is currently a Lecturer in the Department of Chemistry, University of Athens. Her research is mainly focused on the design and development of novel inhibitors of lipolytic enzymes, such as human digestive lipases and phospholipase A₂s. She is also working on new synthetic methodologies for medicinally interesting enzyme inhibitors.



George Kokotos is Professor of Organic Chemistry and Director of the Organic Chemistry Laboratory at the University of Athens, Greece. He studied chemistry at the University of Athens where he also obtained his Ph.D. (1984). He then conducted postdoctoral work in the Department of Pharmaceutical and Biological Chemistry at the University of London. He has spent a sabbatical leave as a visiting Professor in the Department of Chemistry and Biochemistry at the University of California, San Diego. He has authored over 110 publications in peer-reviewed journals and edited two books on Bioactive Lipids and Lipases. He is also co-inventor of more than 10 international patents. He is currently the Chairman of the Division of Organic and Medicinal Chemistry (The Association of Greek Chemists) and a member of the European Committee of the Division of Organic Chemistry (European Association of Chemical and Molecular Sciences). His research interests include the design and synthesis of bioactive compounds, in particular enzyme inhibitors, amino acid and peptide chemistry, development of new organocatalysts, and applications of enzymes in Organic Chemistry.

**Figure 1.**

The specific reaction catalyzed by phospholipase A2 at the sn-2 position of the glycerol backbone is shown. X, any of a number of polar headgroups; R1, fatty acids, or alkyl, or alkenyl groups and R2, fatty acids or acyl moieties.



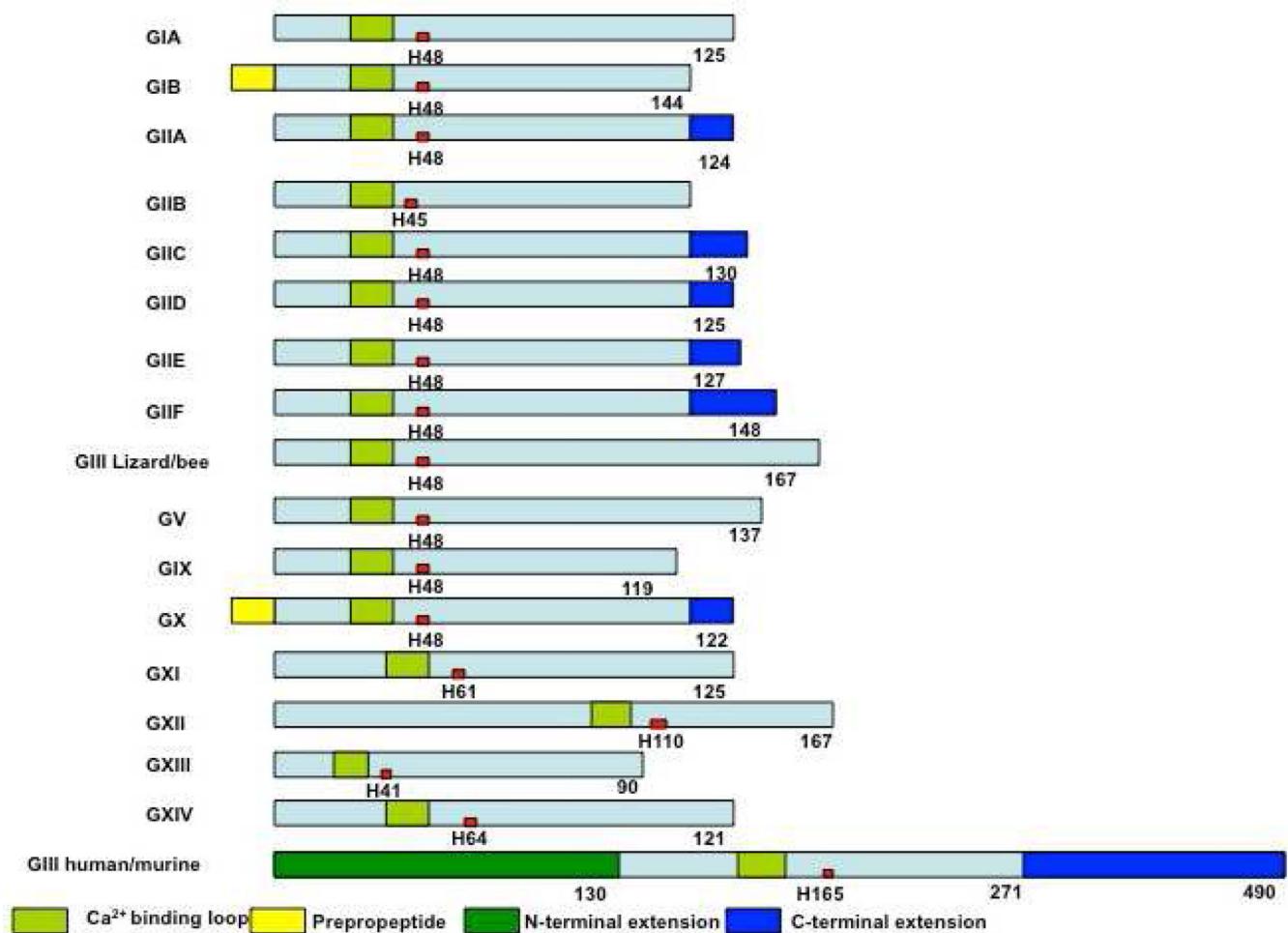
A: Triton X-100 phospholipid mixed micelle surface

B: Individual phospholipid substrate molecule

Q: Product molecules

Figure 2.

Illustration of the application of “surface dilution kinetics” to the phospholipase A₂-catalyzed hydrolysis of phospholipids contained in mixed micelles with nonionic surfactants such as Triton X-100. Two possibilities are shown: top, the “surface binding model” whereby the enzyme first associates nonspecifically with the micelle surface; and bottom, the “phospholipid binding model” whereby the enzyme first associates specifically with phospholipid in the micelle surface. In both cases, in a subsequent step, the enzyme associated with the micelle binds a phospholipid substrate molecule in the micelle in its catalytic site and carries out hydrolysis producing as products a lysophospholipid and a fatty acid, which may be released to solution or be retained in the micelle surface. Phospholipid molecules are depicted in red, detergent molecules in gold, and enzyme in blue. Adapted from Ref.²⁴

**Figure 3.**

Schematic presentation of secreted PLA₂s. Calcium binding loops, active sites (red squares), N and C-terminal extension residues are shown.

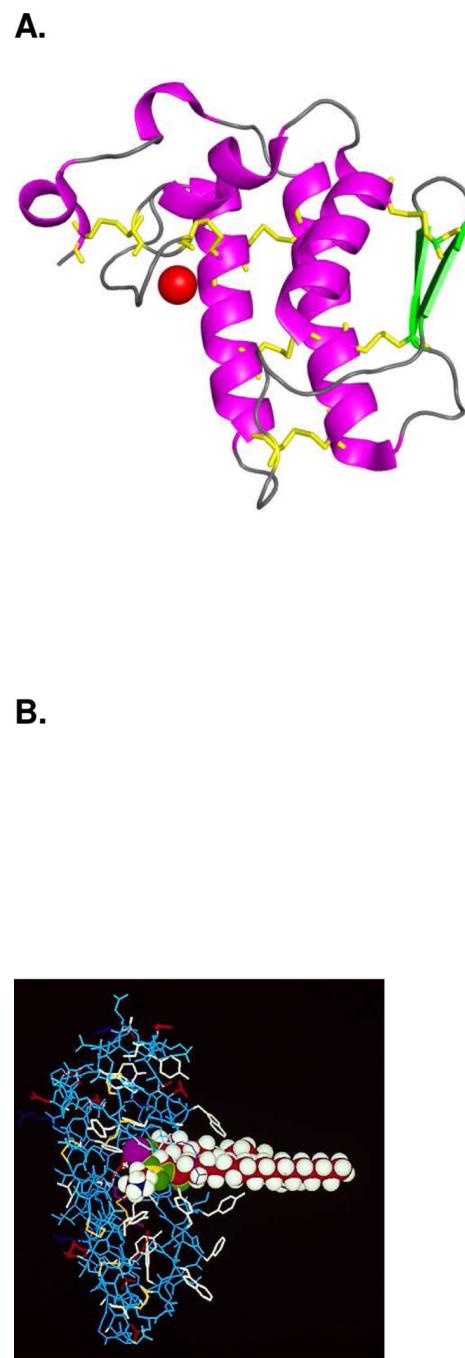
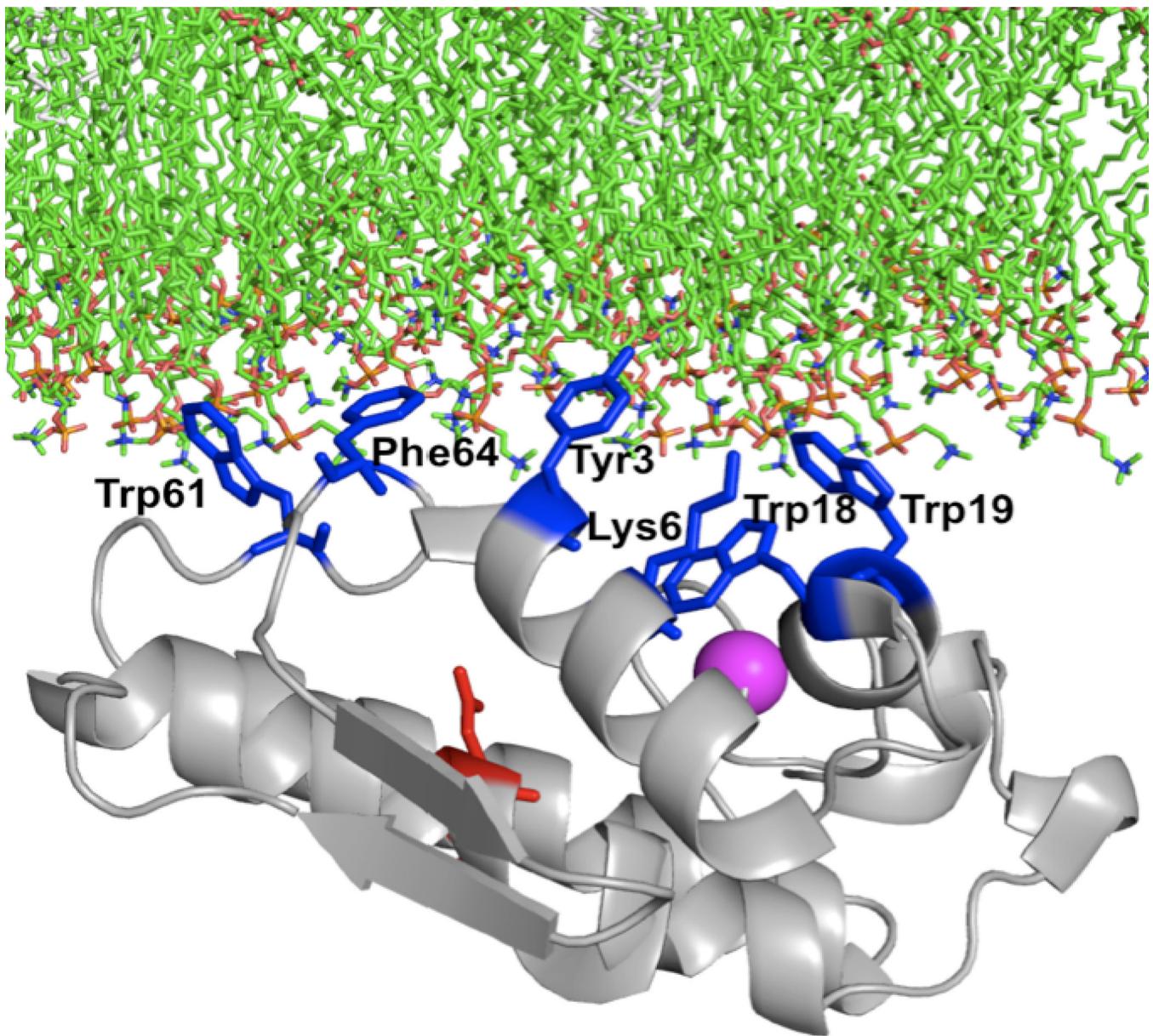
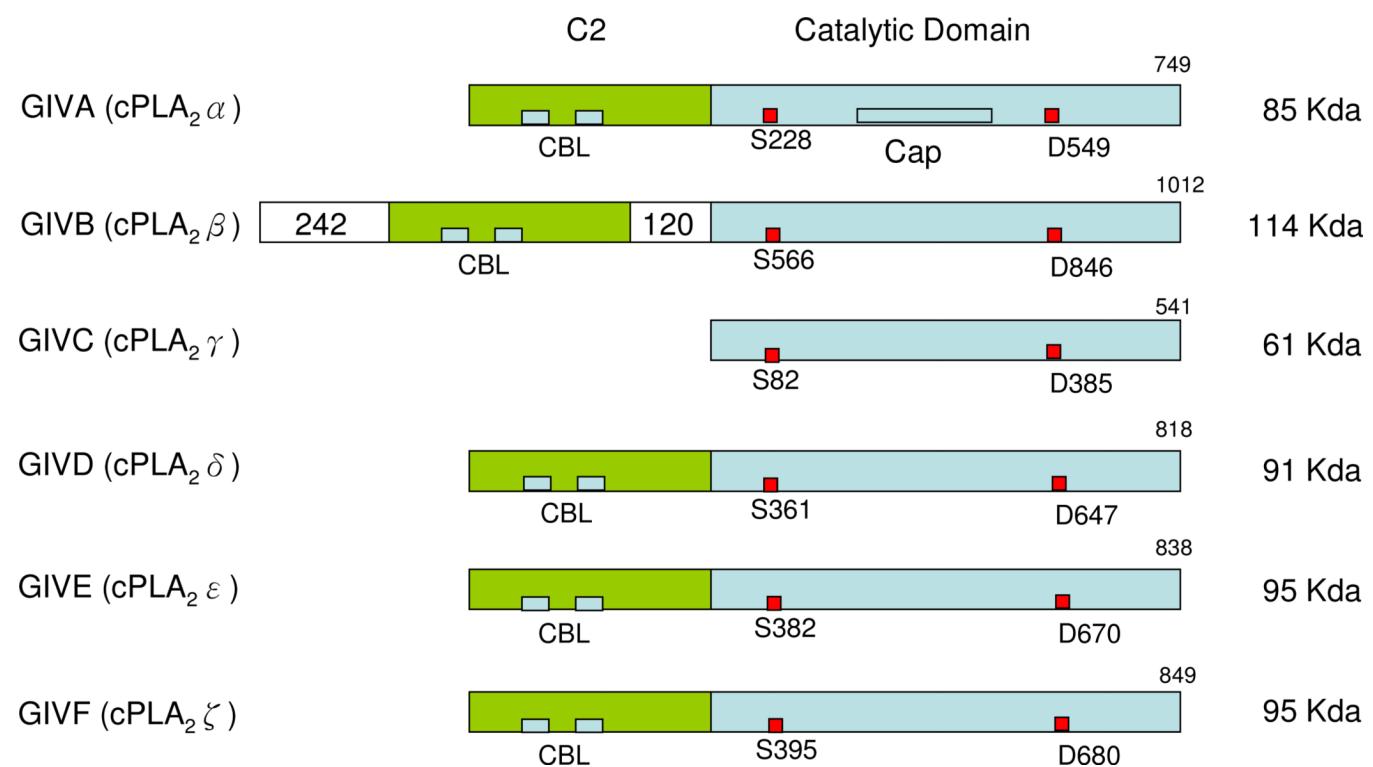


Figure 4.

A. Overall structure of Group IA sPLA₂. Helices are in magenta, β -strands are in green, calcium is shown as a red sphere and disulfide bonds are shown as yellow sticks. (PDB entry: 1PSH) **B.** The Group IA sPLA₂ with phospholipid substrate modeled in the active site as a space filling model. The active site residues His-48 and Asp-93 and the bound Ca²⁺ are shown in purple. Ca²⁺ is bound by Asp-49 as well as the carbonyl oxygens of Tyr-28, Gly-30, and Gly-32. Aromatic residues are shown in white. Adapted from Dennis.⁷

**Figure 5.**

Model of the lipid surface binding of the Group IA sPLA₂ is shown with residues on the interfacial binding surface Tyr-3, Trp-19, Trp-61, and Phe-64 shown in blue stick form. Adapted from Burke et al.³²

**Figure 6.**

Schematic presentation of Group IV cPLA₂s. Calcium binding loops (CBL), active sites (red squares), and the 242 residue- and 120 residue-inserts of GIVB PLA₂ are shown.

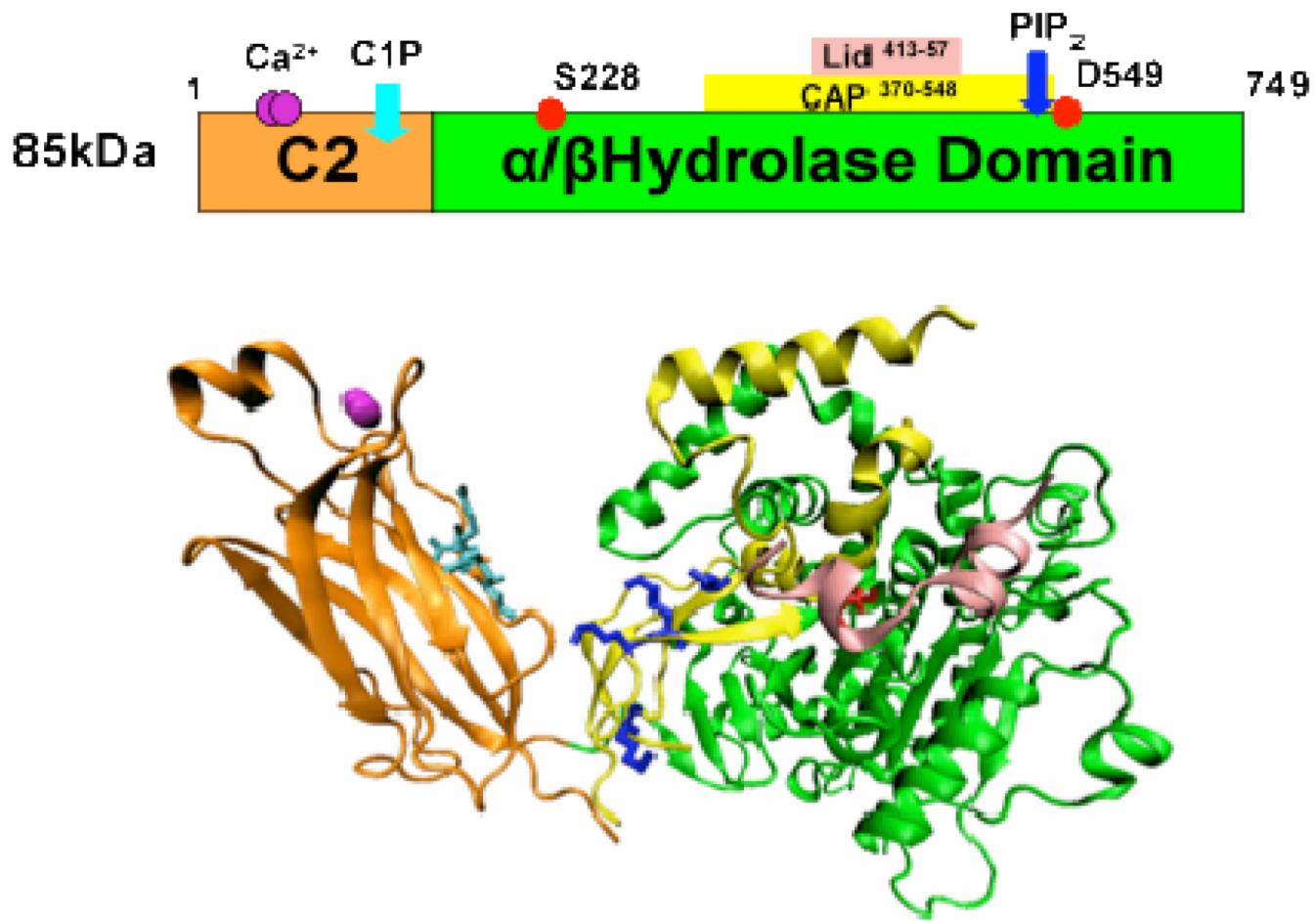
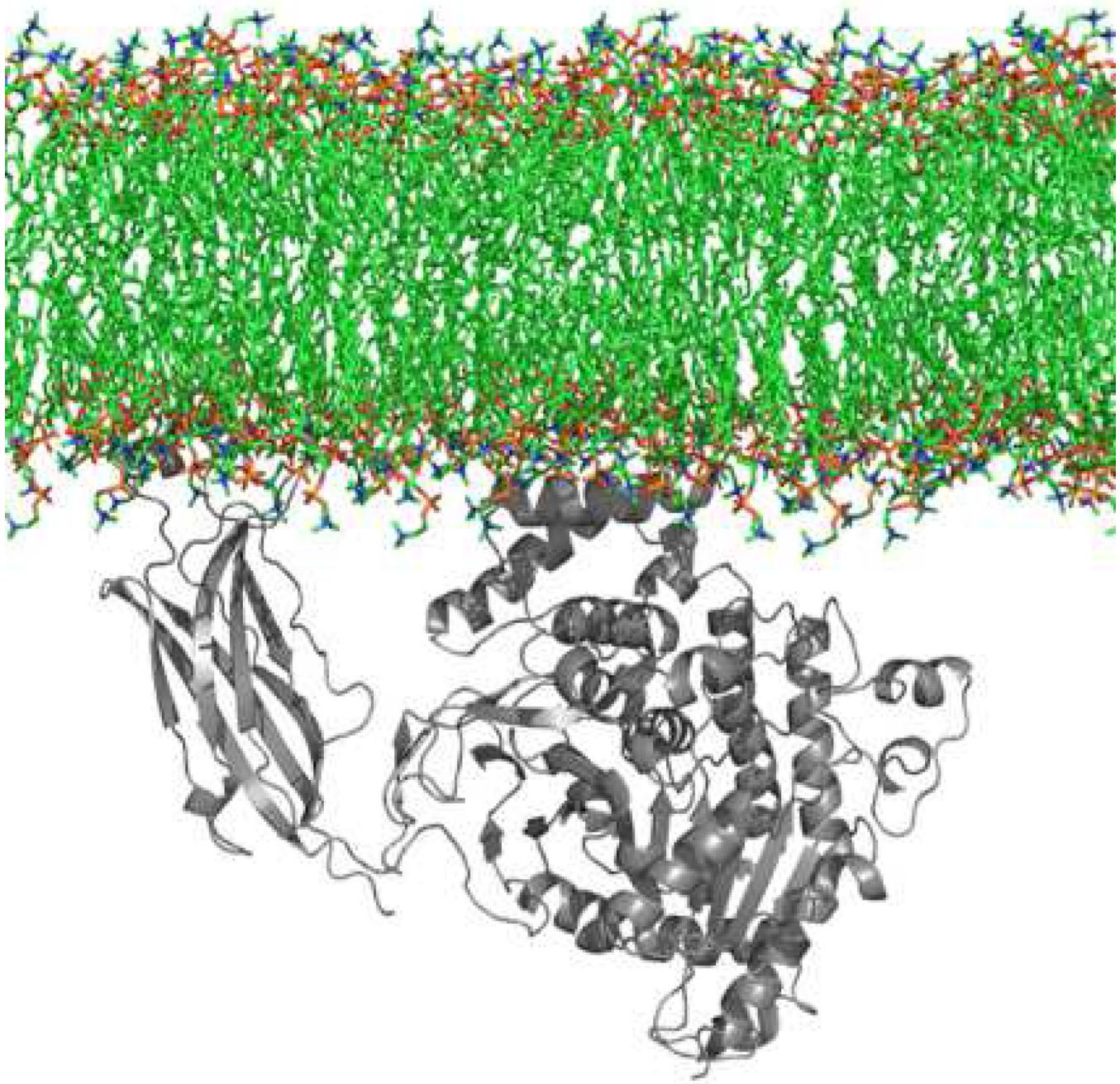


Figure 7.

Features of the Group IVA cPLA₂ crystal structure. The C2 domain is in orange, the α/β hydrolase domain is in green, the cap region is yellow, and the lid is in pink. The C1P binding site is in magenta, the PIP₂ binding site is in blue and the active site residues are in red. Adapted from Ref.²¹⁸

**Figure 8.**

Model of the lipid-binding surface of Group IVA cPLA₂. Residues interacting with the lipid membrane are based on the experiments of hydrogen/deuterium exchange in the presence of phospholipid vesicles. Adapted from Burke et al.²²²

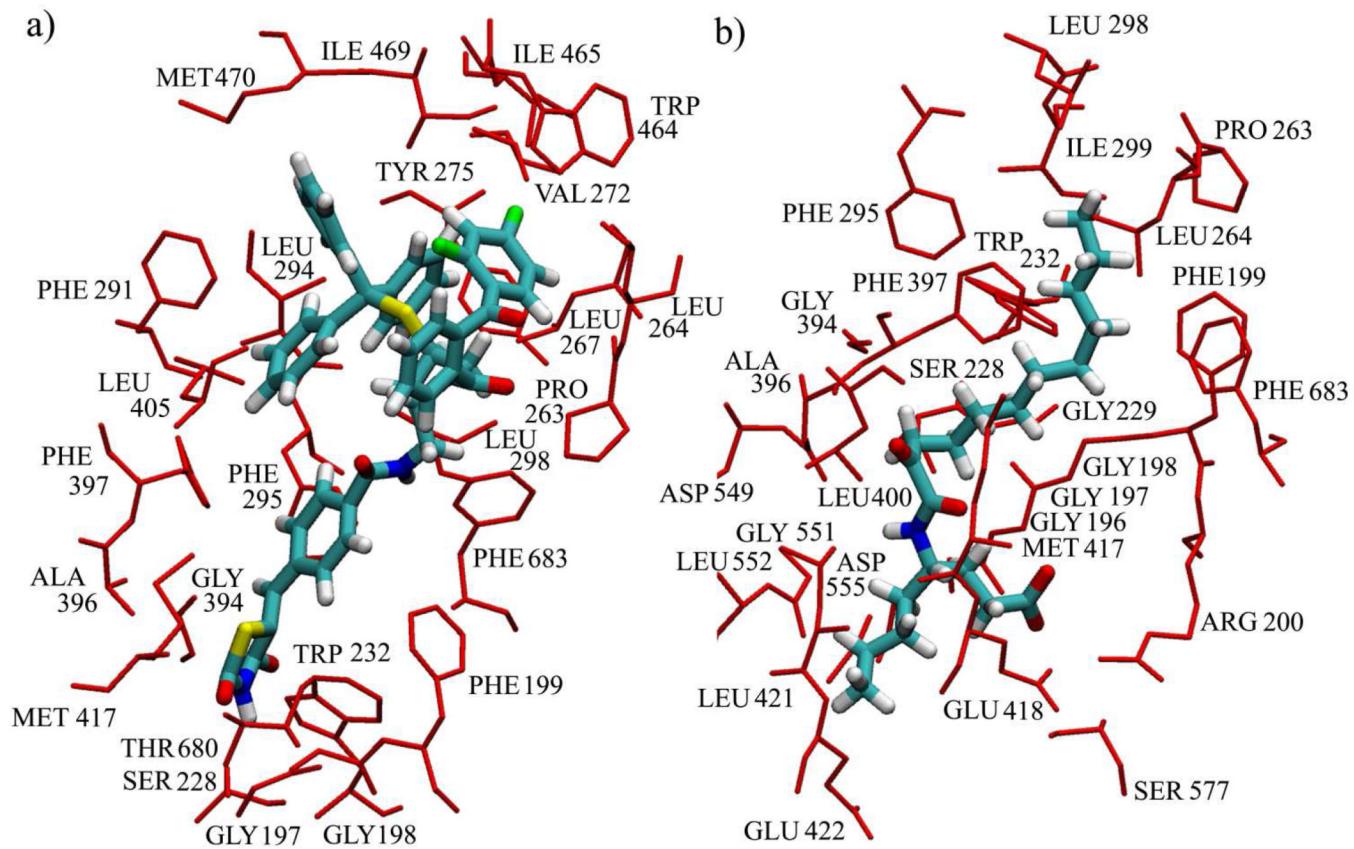
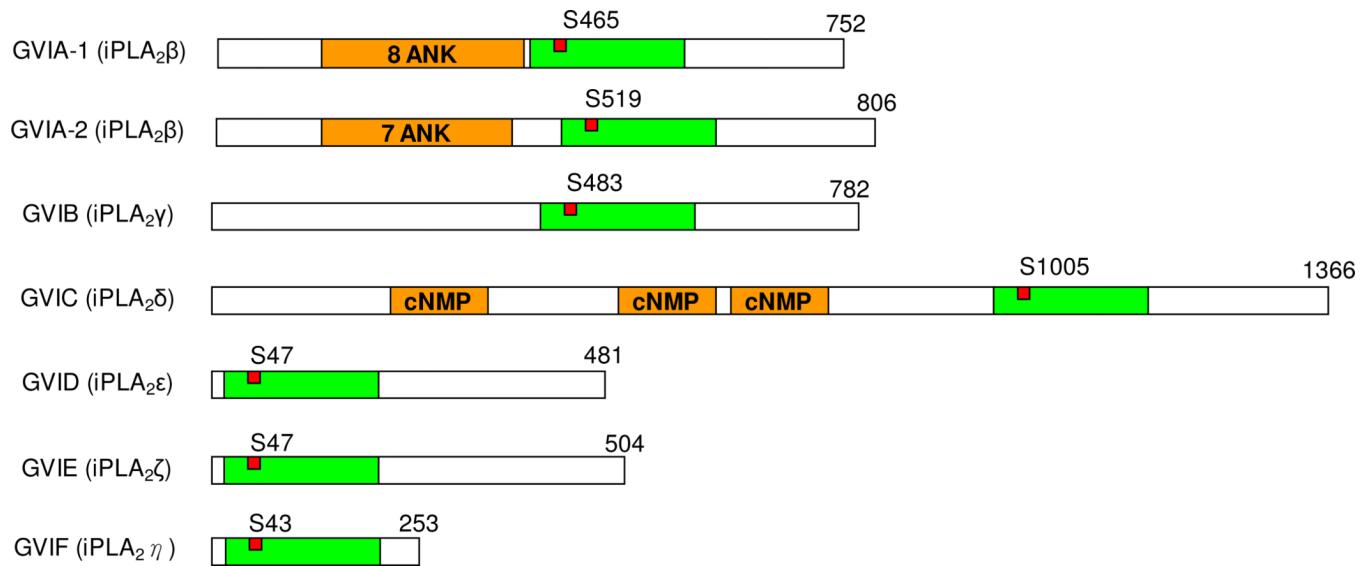


Figure 9.

A. Group IVA cPLA₂ residues involved in binding pyrrophenone. **B.** Group IVA cPLA₂ residues involved in binding oxoamide AX007. The residues that have contact with pyrrophenone or AX007 greater than 90% of the time in the molecular dynamics simulation are represented as red sticks and labeled in the figure. The inhibitor is shown in the licorice representation, with carbon, hydrogen, oxygen, nitrogen, and phosphorus atoms colored cyan, white, red, blue, and yellow, respectively. Adapted from Ref.²¹⁷

**Figure 10.**

Schematic presentation of Group VI iPLA₂. Ankyrin repeats (ANK), nucleotide phosphate binding domain (cNMP), active site residues in red squares, and patatin-like lipase domains are indicated in green.

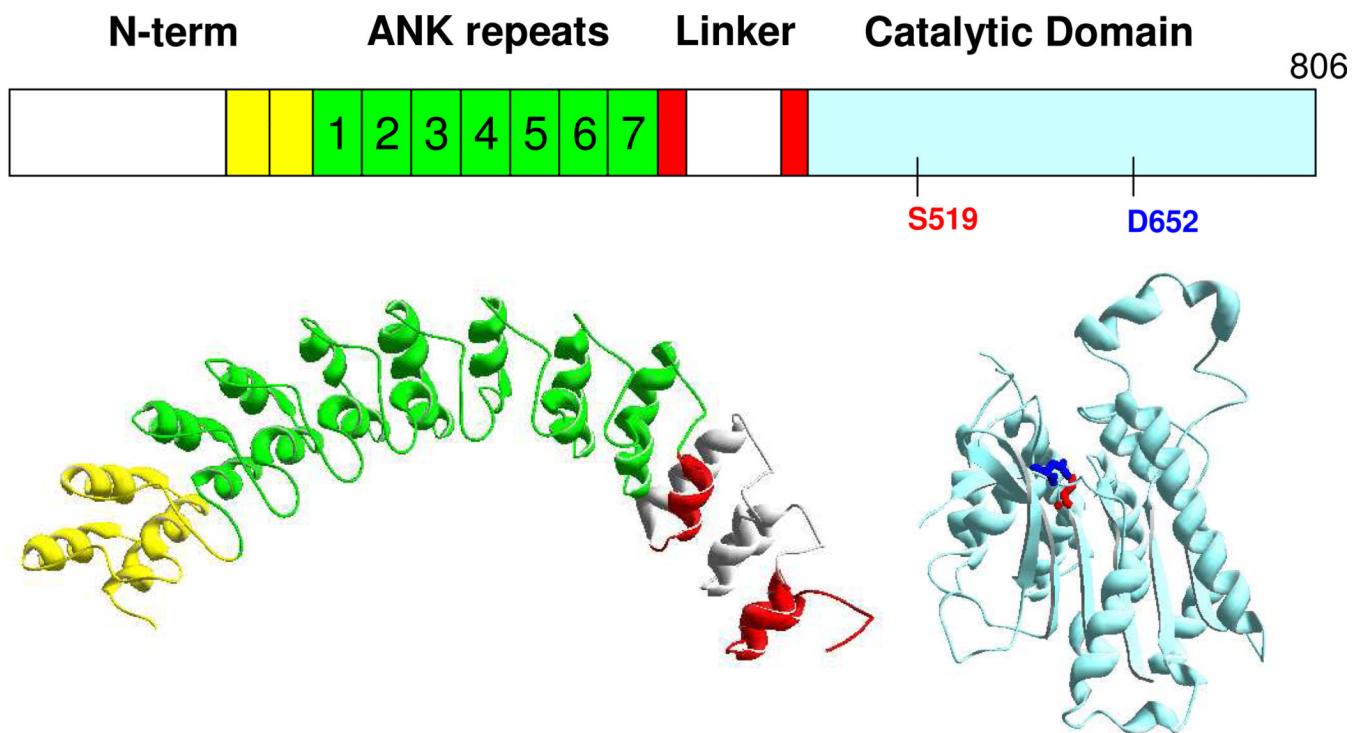


Figure 11.

Features of Group VIA PLA₂ homology models. The domains and binding sites are differentiated by colors. Adapted from Ref.³⁴⁴

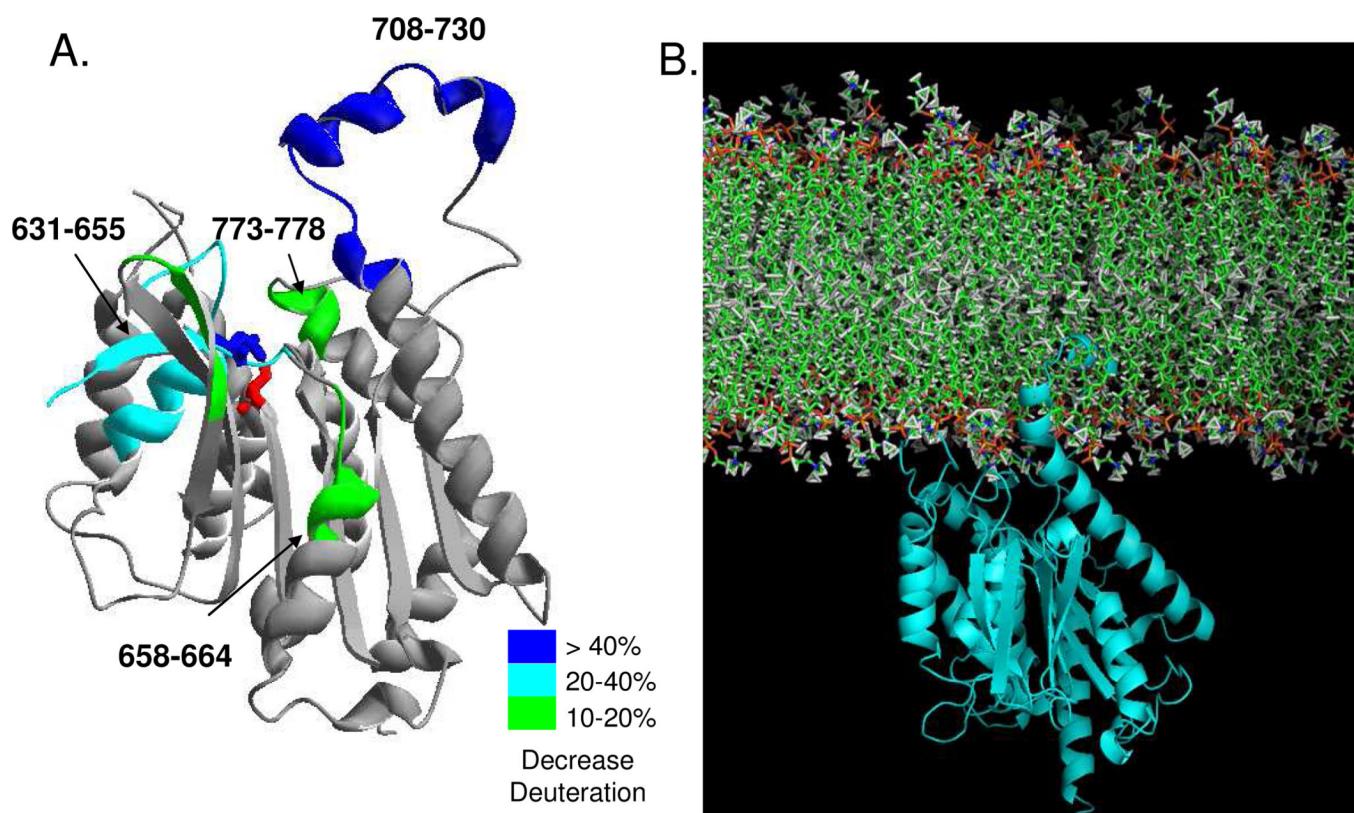
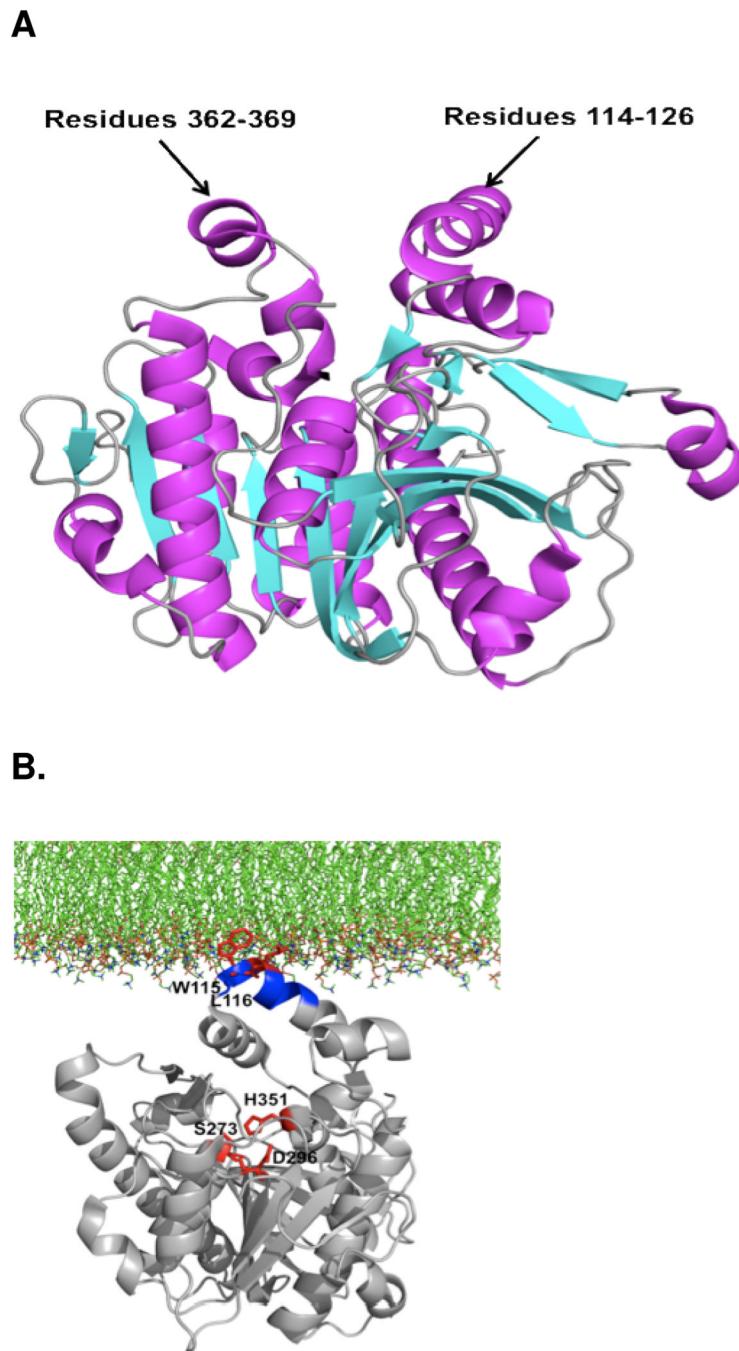


Figure 12.

Phospholipid binding of the Group VIA iPLA₂. A. Phospholipid membrane binding effects on H/D exchange of the Group VIA iPLA₂ mapped onto the catalytic domain model. B. Model of the lipid-binding surface of the Group VIA iPLA₂ based on interactions with lipid membrane. Adapted from Ref.³⁴⁴

**Figure 13.**

A. The α/β hydrolase fold of the Group VIIA PLA₂ (PAF-AH/Lp-PLA₂) crystal structure (PDB entry:3D59). Helices are shown in purple, β strands in green and loops in light gray. The predicted LDL (residues 114–126) and HDL(residues 362–369) binding surface are shown as indicated. **B.** Hypothetical model of Lp-PLA₂ association with the DMPC lipid membrane surface. The Lp-PLA₂ region implicated for Lp-PLA₂/liposome association (residues 113–120), is shown in blue and the proposed key residues for Lp-PLA₂/liposome association, Trp-115 and Leu-116 are shown in red, as are the catalytic triad residues, Ser-273, Asp-296 and His-351.⁴⁴⁶

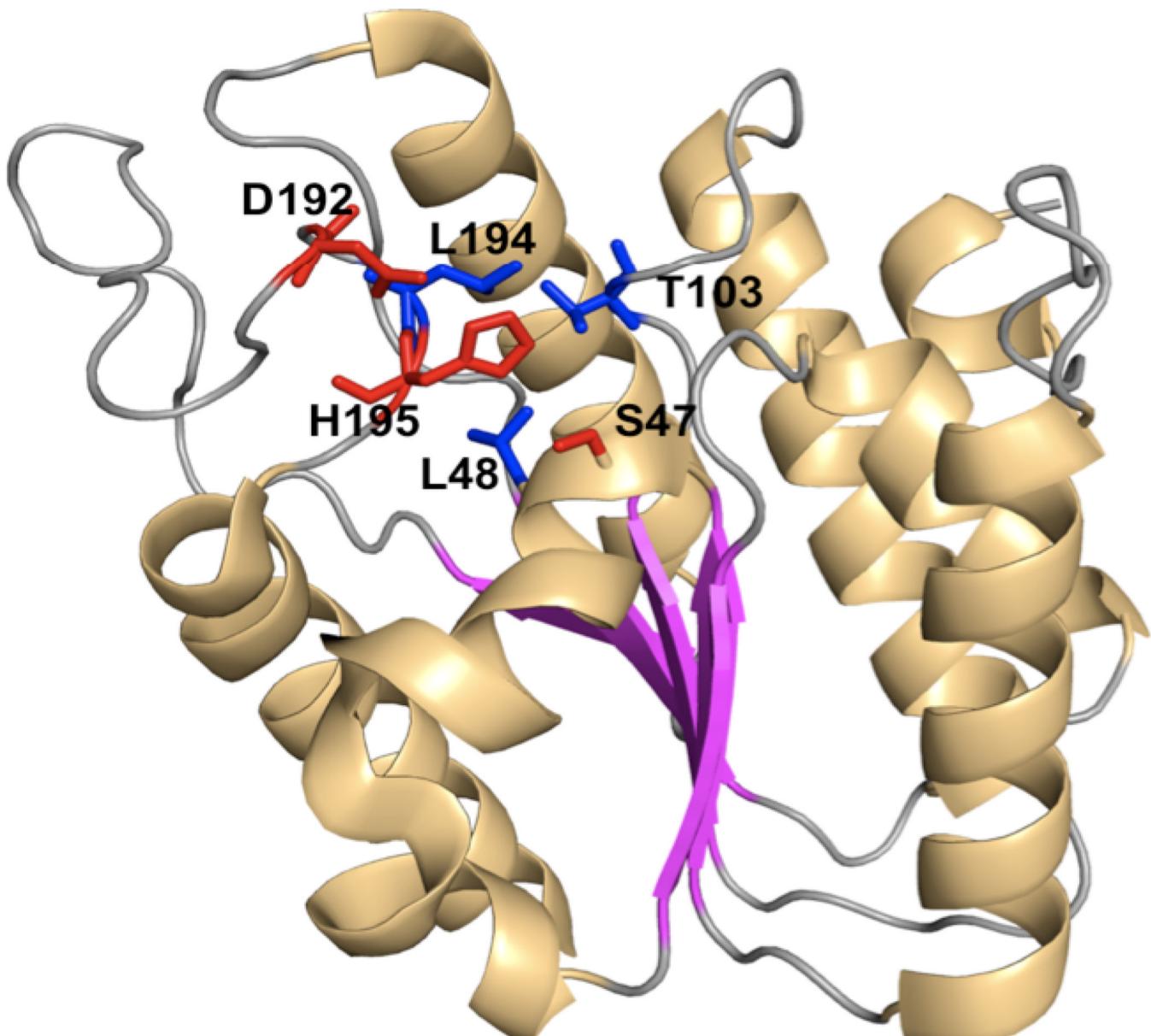


Figure 14.

The crystal structure of Group VIII PLA₂ (PAF-AH IB) α₁ subunit (PDB entry 1WAB). Helices are shown in light orange, β strands in purple and loops in light gray. The catalytic triad residues Ser-47, Asp-192 and His-195 are shown in red sticks. The specific pocket is comprised of residues Leu-48, Thr-103 and Leu-194, which defines the enzyme substrate specificity, are shown in blue sticks.

Table 1The Phospholipase A₂ Superfamily

Type	Group	Subgroup	Molecular Mass (kDa)	Catalytic Residues
	GI	A, B	13–15	
	GII	A, B, C, D, E, F	13–17	
	GIII		15–18	
	GV		14	
sPLA ₂	GIX		14	His/Asp
	GX		14	
	GXI	A, B	12–13	
	GXII	A, B	19	
	GXIII		<10	
	GXIV		13–19	
cPLA ₂	GIV	A(α), B(β), C(γ), D(δ), E(ε), F(ζ)	60–114	Ser/Asp
iPLA ₂	GVI	A(β), B(γ), C(δ), D(ε), E(ζ), F(η)	84–90	Ser/Asp
PAF-AH	GVII	A(Lp-PLA ₂), B(PAF-AH II)	40–45	Ser/His/Asp
	GVIII	A (α ₁), B(α ₂), β	26–40	
LPLA ₂	GXV		45	Ser/His/Asp
AdPLA	GXVI		18	His/Cys

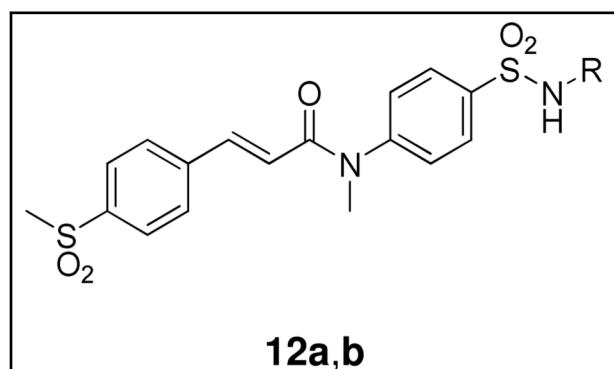
Table 2Secreted Phospholipase A₂s (sPLA₂)

Group	Source	Molecular Mass (kDa)	Disulfide Bonds
IA	Cobras and Kraits	13–15	7
IB	Human/porcine pancreas	13–15	7
IIA	Rattlesnakes; human synovial	13–15	7
IIB	Gaboon viper	13–15	6
IIC	Rat/murine testis	15	8
IID	Human/murine pancreas/spleen	14–15	7
IIE	Human/murine brain/heart/uterus	14–15	7
IIF	Human/murine testis/embryo	16–17	6
III	Lizard/bee	15–18	8
	Human/murine	55	8
V	Human/murine heart/lung/macrophage	14	6
IX	Snail venom (conodipine-M)	14	6
X	Human spleen/thymus/leukocyte	14	8
XIA	Green rice shoots (PLA ₂ -I)	12.4	6
XIB	Green rice shoots (PLA ₂ -II)	12.9	6
XIIA	Human/murine	19	7
XIIB	Human/murine	19	7
XIII	Parvovirus	< 10	0
XIV	Symbiotic fungus/bacteria	13–19	2

This table has been adapted from.¹⁴

Table 3Group IIA PLA₂ Inhibition by Diacid Inhibitors - Studies In Vitro and of Mouse Ear Edema

	IC ₅₀ (μM)	ED ₅₀ (μg/ear)
10a	8	32
10b	4	73

Table 4Intracellular Membrane Bound PLA₂ Inhibition by Benzenesulfonamides

The chemical structure of compounds 12a and 12b is shown. It features a 4-methoxybenzyl group attached to a central carbon atom. This central carbon is also bonded to a vinyl group (-CH=CH₂), a carbonyl group (-C(=O)NMe₂), and a 4-(benzenesulfonamido)phenyl group. The sulfonamide group is -NH-SO₂-Ar, where Ar is a phenyl ring. The entire structure is labeled **12a,b**.

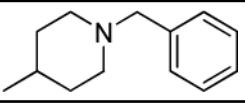
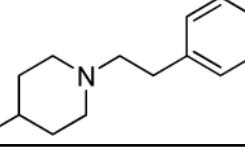
Compound	R	IC ₅₀ (μM)
12a		0.028 ± 0.012
12b		0.009 ± 0.004

Table 5Group IB and Group IIA PLA₂ Inhibition by Primary Amides of Long Chain Unsaturated Fatty Acids.^a

Compound	X _I (50)	
	pGIB PLA ₂	hGIIA PLA ₂
15a	0.0008	0.002
15b	0.0003	0.004

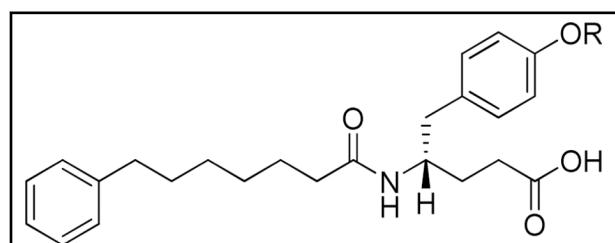
^aX_I(50) is the molar fraction of an inhibitor in the lipid layer that is required to reduce the activity of the enzyme in half.

Table 6Group IB PLA₂ Inhibition by Acylamino Phospholipid Analogues

Compound	IC ₅₀ (μM)
16a	1.4
16b	0.23
16c	4.5

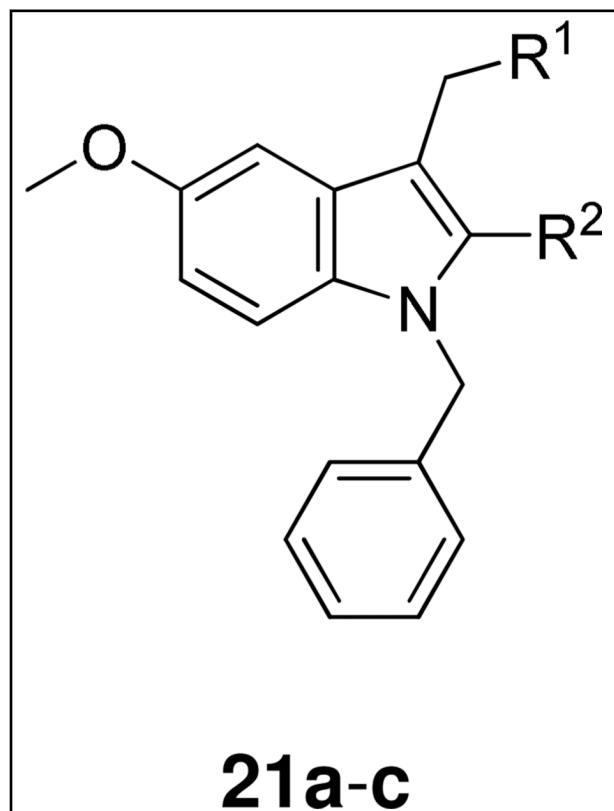
Table 7Group IB and Group IIA PLA₂ Inhibition by Non-phospholipid Amides

Compound	IC ₅₀ (μM)	
	pGIB PLA ₂	hGIIA PLA ₂
17a	0.19	NT
17b	0.023	NT
17c	0.016	1.85
18	0.015	0.021

Table 8Group IIA PLA₂ Inhibition by Amides Derived from D-tyrosine

19b

R	IC ₅₀ (μM)
benzyl-	0.029
2-picoly-	0.214
cyclopentylmethyl-	0.057
1-naphthylmethyl-	0.019
2-naphthylmethyl-	0.039
cinnamyl-	0.116
iso-butyl	0.170
n-heptyl	0.086
H	2.57

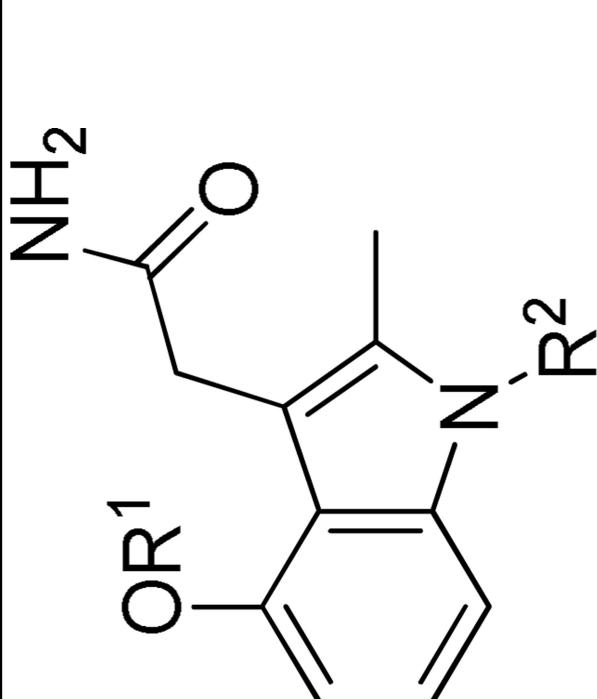
Table 9Group IIA PLA₂ Inhibition by Indoles Using the Chromogenic Assay

The chemical structure of compound 21a-c is shown. It features a central indole ring system. At position 3, there is a methoxy group (-OCH₃). At position 7, there is a phenyl group attached to a nitrogen atom. At position 2, there is a substituent R². At position 5, there is a substituent R¹.

Compound	R ¹	R ²	IC ₅₀ (μM)
21a	COOH	CH ₃	13.6 ± 4.2
21b	CONH ₂	CH ₃	0.84 ± 0.17
21c	CONH ₂	CH ₂ CH ₃	0.26 ± 0.11

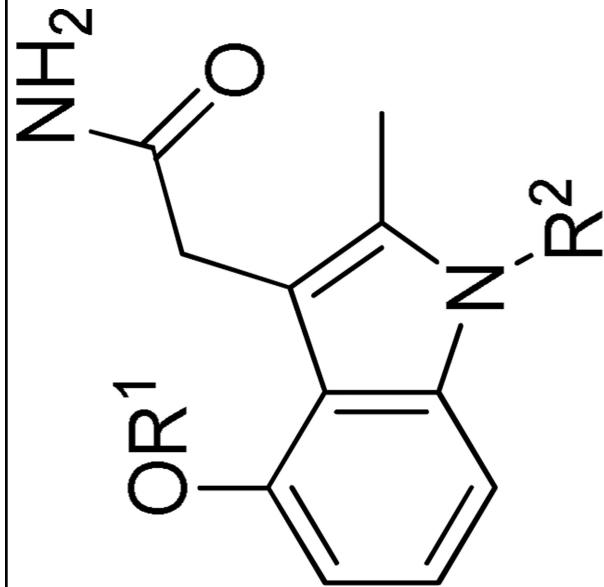
Table 10

Group IIA and Group IB PLA_2 Inhibition by Indoles Developed by SAR Study Using the Chromogenic Assay.



22a-b

Compound	R ¹	R ²	IC ₅₀ (μM)	PLA ₂ pgIB PLA ₂		
				hGIIA PLA ₂	hGIB PLA ₂	PLA ₂ pgIB PLA ₂
22a	CH ₂ COONa		0.010 ± 0.001	4.09		0.014



22a-b

Compound	R ¹	R ²	IC ₅₀ (μM)		
			hGIP PLA ₂	hGIPB PLA ₂	PLA ₂ pGIPB PLA ₂
22b	CH ₃ COOH		0.052 ± 0.010	1.4	0.15

Table 11

Comparison of GIIA and Group IB PLA₂ Inhibition by Varespladib and Analogues Using the Chromogenic Assay

Compound	R ¹	R ²	IC ₅₀ (μM)	hGIIA PLA ₂	hGIBB PLA ₂	pGIBB PLA ₂
23a	C ₆ H ₅ CH ₂	CH ₃	0.011 ± 0.004	0.761	0.015	
23b	2-(C ₆ H ₅)C ₆ H ₄ CH ₂	CH ₃	0.006 ± 0.001	0.364	0.097	
23c	3-(C ₆ H ₅)C ₆ H ₄ CH ₂	CH ₃	0.009 ± 0.001	0.57	0.007	
23d	1-naphthylCH ₂	CH ₃	0.009 ± 0.004	1.2		
23e	n-C ₈ H ₁₇	CH ₃	0.008 ± 0.003	0.78		
23f	2-(C ₆ H ₅ CH ₂)C ₆ H ₄ CH ₂	CH ₂ CH ₃	0.004 ± 0.001	0.062		
23g	3-(Cl)C ₆ H ₄ CH ₂	CH ₂ CH ₃	0.007 ± 0.002	0.360	0.003	
24	C ₆ H ₅ CH ₂	CH ₂ CH ₃	0.009 ± 0.001	0.228	0.048	

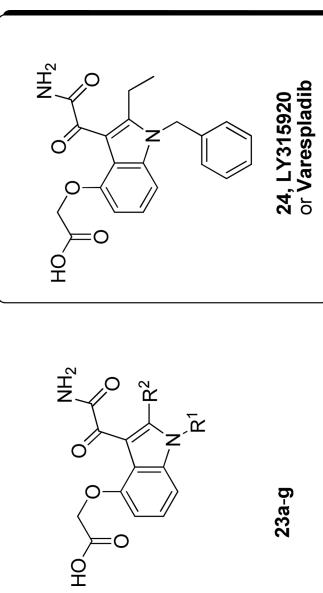


Table 12GIIA PLA₂ Inhibition by Indoxam and Analogues Using the Chromogenic Assay and a Deoxycholate Assay

The figure shows the chemical structures of compounds 26a-f and 27, Indoxam. Compound 26a-f is a substituted indole derivative where the 2-position of the indole ring is substituted with a 3-hydroxy-2-methylpropyl group and the 7-position is substituted with R¹ and R². Compound 27, Indoxam, is a more complex indole derivative with a 3-hydroxy-2-methylpropyl group, a 7-amino-2-methylpropyl group, and a 2-(4-phenylbutyl) group at the 2-position.

Compound	R ¹	R ²	IC ₅₀ (μM)	
			chromogenic assay	PC/DOC assay
26a	CH ₂ CONH ₂		0.014	0.014
26b	CH ₂ CONH ₂		0.013	0.028
26c	COCONH ₂		0.008	0.005
26d	COCONH ₂		0.005	0.024
26e	COCONH ₂		0.006	0.0013
26f	COCONH ₂		0.009	0.0042

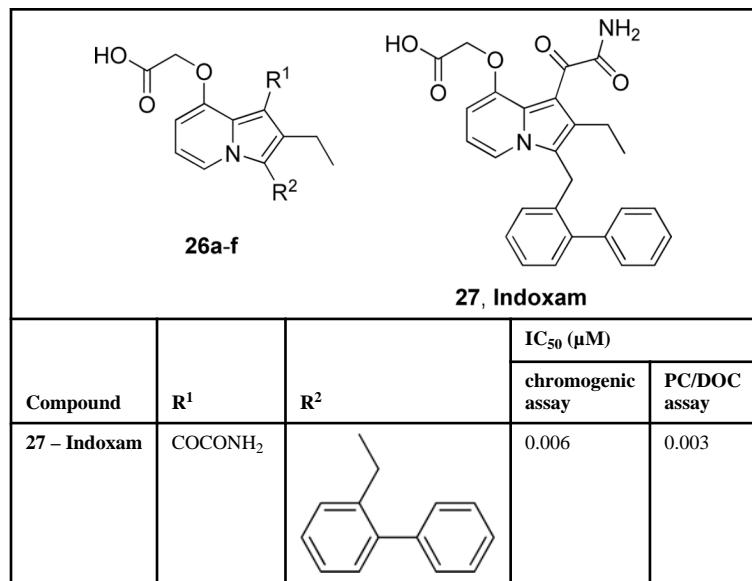


Table 13Group IB and Group IIA PLA₂ Inhibition by Oxadiazolones In Vitro and of Mouse Ear Edema

Compound	IC ₅₀ (μM)		Ear Edema
	pGIB PLA ₂	hGIIA PLA ₂	
31b	>100	4.0 ± 0.9	
32	>100	0.28 ± 0.02	
33a		9	47.83 ± 12.34 (1.0 mg/ear)
33b		0.1	
34a	7.41	0.03	
34b	>50	0.05	
Indomethacin			43.11 ± 8.79 (0.5 mg/ear)

Table 14

Group V and Group X PLA₂ Inhibition by 1,3,5-Triazepan-2,6-diones

35a-d

Compound	R ¹	R ²	R ³	R ⁴	IC ₅₀ (μM)	
					hGV PLA ₂	hGX PLA ₂
35a	CH ₂ CH=CHCOOH	H	H	CH ₂ C ₆ H ₅	12	10
35b	CH ₂ COOC(CH ₃) ₃	H	H	CH(CH ₃) ₂	12	11
35c	H	CH ₂ C ₆ H ₅	H	CH ₂ C ₆ H ₅	11	13
35d	CH ₂ COOC(CH ₃) ₃	H	CH ₂ COOC(CH ₃) ₃	CH ₂ C ₆ H ₅	11	11

35a-d

Table 15Group GIV Cytosolic Phospholipase A_{2s} (cPLA₂)

Subgroup	Initial/common sources	Residues /Molecular Mass	Domain	Activation Factor	Substrate	Activity	Post Translational Modification	Human chromosome	Swiss-Prot
GIVA (cPLA ₂ <i>a</i>)	Human macrophage-like U937 cells/ platelets/RAW 264.7/rat kidney, Ubiquitous	749/ 85 Kda	C2 ⁺ α/β hydrolase C _{ap}	Ca ²⁺ PIP ₂ CIP Phosphor ylation	PC, PE, PI High sn- 2AA specificity	PLA ₂ , PLA ₁ , Lyso-PLA transacylase	Phosphorylation	1q25	P47712
GIVB (cPLA ₂ <i>β</i>)	Human pancreas/ liver/heart/brain, Ubiquitous	1012/ 100– 114 Kda	InjC insert C2 ⁺ α/β hydrolase	Ca ²⁺ No sn-2 specificity	PC, PE Low sn-2 AA specificity	PLA ₁ , PLA ₂ , Lyso-PLA transacylase	15q11.2 q21.3	AAD321 35	
GIVC (cPLA ₂ <i>γ</i>)	Human heart/ skeletal muscle	541/ 61 Kda	α/β hydrolase	PC Low sn-2 AA specificity	PLA ₁ , PLA ₂ , Lyso-PLA	Farnesylation	19q13.3	AAC328 23	
GIVD (cPLA ₂ <i>δ</i>)	Murine placenta	818/ 91 Kda	C2 ⁺ α/β hydrolase	Ca ²⁺	PC, PE	PLA ₁ , PLA ₂ , Lyso-PLA	15q15.1	Q86XP0 .2	
GIVE (cPLA ₂ <i>ε</i>)	Murine heart/ muscle/testis/thyroid	838/ 95 Kda	C2 ⁺ α/β hydrolase	Ca ²⁺	PC, PE	PLA ₁ , PLA ₂ , Lyso-PLA	15q15.1	Q3MJ16 .2	
GIVF (cPLA ₂ <i>ζ</i>)	Murine thyroid/ stomach	849/ 95 Kda	C2 ⁺ α/β hydrolase	Ca ²⁺	PC, PE	PLA ₁ , PLA ₂ , Lyso-PLA	15q15.1	Q68DD2 .2	

Table 16Group IVA PLA₂ Inhibition by Fatty Acid Trifluoromethyl Ketones

R-COCF ₃	X _{I(50)}		
	DOPM/GLU	mixed-micelles	natural membrane
20:4	0.0050±0.001	0.036±0.006	0.05±0.02
11, 14, 17–20:3	0.0085±0.002	0.020±0.002	0.10±0.03
16:1	0.0083±0.001	0.010±0.003	0.07±0.02
16:0	0.025±0.002	0.022±0.006	0.20±0.03

Table 17Group IVA PLA₂ Inhibition by Pyrrolidines In Vitro and in THP-1 Cells

Compound	IC ₅₀ (μM)	
	PC/DOG	THP-1
54	0.031 ± 0.0006	0.052 ± 0.009
55	0.0018 ± 0.0005	0.022 ± 0.001

Table 18Group IVA PLA₂ Inhibition by Indoles

Compound	IC ₅₀ (μM)	
	GLU assay	RWB assay
65	0.5	0.8
66	0.15	0.11
67	0.04	0.07
68	0.01	0.03
70	0.065	0.10
71	0.013	0.17

Table 19
Group IVA and GVIA PLA₂ Inhibition by 2-Oxoamides

Compound	X _I (50)	
	GIVA PLA ₂	GVIA PLA ₂
AX006	0.024 ± 0.015	N.D.
AX007	0.009 ± 0.004	N.D.
AX048	0.022 ± 0.009	0.027 ± 0.009
AX109	0.005 ± 0.002	N.D.

Table 20Group IVA PLA₂ Inhibition by 1,3-Disubstituted Propan-2-ones In Vitro and in Cellular Assay

Compound	IC ₅₀ (μM)		
	vesicle assay with the isolated enzyme	cellular assay with human platelets stimulant A23187	cellular assay with human platelets stimulant TPA
81a	0.020	0.21	0.0078
81b	0.0043	0.57	0.0009

Group VI Calcium Independent Phospholipase A₂s (iPLA₂)

Table 21

Subgroup	Alternative name	Sources	Residues /Molecular Mass	Domain	Alt Splice	Activity	Post Translational Modification	Human chromosome	Swiss-Prot
GVIA	iPLA ₂ ^β PNPLA9	Human/ Murine	750/85 kDa 806/87 kDa	7-8 Ankyrin Repeats α/β hydrolase (Pataatin like Lipase)	5	PLA ₂ , Lyso-PLA transacylase acyl-CoA thioesterase	22q13. 1	O60733	
GVIB	iPLA ₂ ^γ PNPLA8	Human/ Murine	782/90 kDa	α/β hydrolase (Pataatin like Lipase)	2	PLA1, PLA ₂ , Lyso-PLA transacylase	7q31	Q9NP80	
GVIC	iPLA ₂ ^δ PNPLA6 NTE	Human/ Murine	1366/146 kDa	3 cNMP α/β hydrolase (Pataatin like Lipase)	3	PLA ₂ Lyso-PLA	Phosphorylation n(5)	19p13. 3-13.2	Q8IY17
GVID	iPLA ₂ ^ε PNPLA3 ADPN	Human	481/52 kDa	α/β hydrolase (Pataatin like Lipase)	2	PLA ₂ , TG hydrolase, transacylase	22q13. 31	Q9NST1	
GVIE	iPLA ₂ ^ζ PNPLA2 ATGL	Human	504/55 kDa	α/β hydrolase (Pataatin like Lipase)	2	PLA ₂ , TG hydrolase, transacylase	Phosphorylation n (1)	11p15. 5	Q96AD5
GVIF	iPLA ₂ ^η PNPLA4 GS2	Human	253/27 kDa	α/β hydrolase (Pataatin like Lipase)		PLA ₂ , TG hydrolase, retinylester hydrolyase acylglycerol and retinol transacylase	Phosphorylation n (1)	xp22.3	P41247

Table 22Group VIA PLA₂ Inhibition by Fatty Acyl Trifluoromethyl Ketones

Compound	IC ₅₀ (μM)	X _{f(50)}
43	15	0.028
44	3.8	0.0075
95		0.0043

Table 23Comparison of Group VIA, Group IVA and Group V PLA₂ Inhibition by Polyfluoroalkyl Ketones^a

Compound	GVIA PLA ₂	GIVA PLA ₂	GV sPLA ₂
96	>90% 0.0073 ± 0.0007	N.D.	28 ± 1%
97	>90% 0.0169 ± 0.021	>90% 0.0098 ± 0.0006	86 ± 2 %

^a Average percent inhibition at 0.091 mol fraction and $X_{I}(50)$ and standard error ($n = 3$) are reported for each compound. N.D. signifies compounds with less than 25% inhibition (or no detectable inhibition).

Table 24Comparison of Group VIA, Group IVA and Group V PLA₂ Inhibition by Polyfluoroalkyl Ketones^a

Compound	GVIA PLA ₂ <i>X_I(50)</i>	GIVA PLA ₂ % Inhibition	GV sPLA ₂ % Inhibition
98	0.0002 ± 0.0000	80.8 ± 1.5 %	36.8 ± 7.9 %
99	0.0010 ± 0.0001	55.8 ± 2.1 %	46.3 ± 10.0 %

^a Average percent inhibition at 0.091 mol fraction and standard error (*n* = 3) are reported for each compound or *X_I(50)* and standard error (*n* = 3) for those with >90% inhibition.

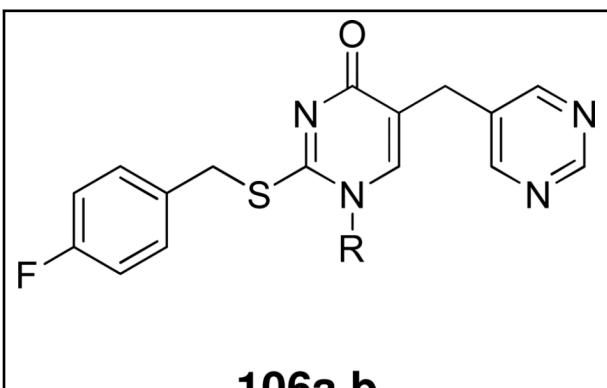
Table 25Comparison of Group VIA, Group IVA and Group V PLA₂ Inhibition by 2-Oxoamides^a

Compound	GVIA PLA ₂ $X_{I(50)}$	GIVA PLA ₂ % Inhibition	GV PLA ₂ % Inhibition
100	0.011 ± 0.001	72	59
101	0.017 ± 0.002	52	81

^a Average percent inhibition at 0.091 mol fraction are reported for each compound or $X_{I(50)}$ and standard error ($n = 3$) for those with >90% inhibition.

Table 26
Group VII and Group VIII Phospholipase A₂s (PAF-AH)

Group	Source	Molecular mass (kDa)	Features	Alternate names
VIIA	Human, murine, porcine, bovine	45	Secreted α/β hydrolase N-linked glycosylation	Lipoprotein-associated PLA ₂ (Lp-PLA ₂) Plasma PAF-AH (pPAF-AH)
VIIB	Human bovine	40	Intracellular, myristoylated, α/β hydrolase	PAF-AH II
VIIIA	Human	26	Intracellular, Homodimer or heterodimer with GVIIIB associates with regulatory β subunit	PAF-AH Ib (α_1 subunit)
VIIIB	Human	26	Intracellular, Homodimer or heterodimer with GVIIIA associates with regulatory β subunit	PAF-AH Ib (α_2 subunit)

Table 27Group VIIA PLA₂ Inhibition by Pyrimidones

The chemical structure of compounds 106a and 106b is shown. It features a pyrimidine ring substituted at the 2-position with a carbonyl group (C=O) and at the 4-position with a 2-(4-fluorophenyl)ethyl thioether group (-S-CH₂-CH₂-C₆H₄-F). The 5-position of the pyrimidine ring is substituted with an R group, which is either (E)-(CH₂)₃CONH(CH₂)₈CH=CHC₈H₁₇ or (Z)-(CH₂)₃CONH(CH₂)₈CH=CHC₈H₁₇.

Compound	R	IC ₅₀ (nM)
106a	(E)-(CH ₂) ₃ CONH(CH ₂) ₈ CH=CHC ₈ H ₁₇	0.7
106b	(Z)-(CH ₂) ₃ CONH(CH ₂) ₈ CH=CHC ₈ H ₁₇	0.4

Table 28Group XV Lysosomal Phospholipase A₂ (LPLA₂)

Group	Source	Molecular mass (kDa)	Features	Alternate names
XV	Human murine bovine	45 (deglycosylated)	Ser/His/Asp triad, glycosylated, N-terminal signal sequence	ACS, LLPL

Table 29

Summary of Human Proteins Not Traditionally Categorized as Phospholipase A₂s, but which Express Phospholipase A₂ Activity

Type	Name	Alternative name	Homology	Identification	Residues /Molecular Mass(Kd a)	PLA ₂ Activity	Other activity	Domain	Swiss-Prot
sPLA ₂	Otoconin-90	PLA ₂ L	sPLA ₂	Protein (mouse)	493/53	Predicted		sPLA ₂ -like (2)	Q02509
cPLA ₂	PLB1	Phospholipase B1		Protein	1458/163	Verified	Lyso-leithin acylhydrolase	Glycosylation	Q6P1J6
iPLA ₂	PNPLA7	NTE-related esterase (NRE)	PNPLA6	Protein	1317/145	Lyso-		Patatin-Like	Q6ZV29
PnPLA5		GS2-like (GS2L)		Protein	429/47	TG-hydrolase		Patatin-Like	Q7ZGZ6
PNPLA1			Transcript		53/257			Patatin-Like	Q8N8W4
aiPLA ₂	Peroxiredoxin-6	1-Cys peroxiredoxin aiPLA ₂		Protein	224/25	Verified	peroxiredoxin activity	Thioredoxin domain	P30041