Title

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Journal

Chemical Reviews, 111(10)

ISSN

0009-2665

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Publication Date

2011-10-12

DOI

10.1021/cr200085w

Peer reviewed

Published in final edited form as:
Chem Rev. 2011 October 12; 111(10): 6130–6185. doi:10.1021/cr200085w.

Phospholipase A_2 Enzymes: Physical Structure, Biological Function, Disease Implication, Chemical Inhibition, and Therapeutic Intervention

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1. Introduction

1.1. Discovery of the Phospholipase A_2 Superfamily

Phospholipases represent one of the earliest enzyme activities to be identified and studied and the phospholipase A_2 (PLA_2) superfamily (see defining specificity[1] 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 PLA_2, 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 A_2 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 Corporation[4] independently and with much fanfare and excitement reported the purification, sequencing and cloning of the first human non-pancreatic secreted PLA_2 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 PLA_2 was designated IIA. All of these enzymes then became known as secreted or $sPLA_2$s.

It wasn't until the late 1980's that PLA_2-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 Institute[5] and Ruth M. Kramer (who had moved to Lilly Research Laboratories)[6]

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independently reported the purification, sequencing, and cloning of the first human cytosolic PLA_2 ($cPLA_2$) from the U937 macrophage cell line. The sequence was unrelated to those of the secreted enzymes. To track this new enzyme and potentially additional PLA_2s, a Group Numbering System[7] 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 PLA_2 which had been purified from bee venom; and establishing the Group IV (GIV) designation for the new cytosolic PLA_2 ($cPLA_2$).

This was fortuitous because soon thereafter a new form of secreted PLA_2 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 $sPLA_2$ 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 Diego[8] reported on another cystosolic PLA_2 purified from macrophages that differed from Group IV $cPLA_2$ in that its activity was not dependent on Ca^{2+} and Simon S. Jones from the Genetics Institute[9] reported that the cloned form from CHO cells had a very different sequence than $cPLA_2$. This new Ca^{2+}-independent PLA_2 ($iPLA_2$) was designated as Group VI PLA_2 (GVI).[10]

Earlier, investigators from the University of Utah[11] 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 ICOS[12] reported its cloning. This enzyme and other related PAF acetyl hydrolases (PAF-AH) were later recognized more broadly as PLA_2s 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 A_2 ($Lp\text{-}PLA_2$). These enzymes were designated Group VII and VIII (GVII and GVIII).[13] As additional specific PLA_2s 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 $sPla_2$s in which the sequence and/or disulfide bonding pattern varied significantly from the traditional Groups I, II, III, and V $sPla_2$s. These new forms led to the additional Groups IX, X, XI XII,XIII, and XIV $sPla_2$s 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 PLA_2 reported that did not naturally fit in the four types discussed above (secreted, cytosolic, Ca^{2+}-independent, PAF acetylhydrolases) is the lysosomal PLA_2 ($LPLA_2$) which was designated as Group XV (GXV).[14] Recently, a new PLA_2 was isolated from adipose tissue and designated as Group XVI (GXVI);[15] it appears to be a new type of PLA_2 called adipose-PLA_2 (AdPLA). The current designations are summarized in Table 1.

In this review, we will discuss in turn each of the six types of PLA_2. 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, cytosolic, Ca^{2+}-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 $cPLA_2$ are also Ca^{2+}-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 PLA_2s[16] or specific types including $sPLA_2$s,[17] $cPLA_2$s,[18] $iPLA_2$s,[19] PAF-AHs[20] and $LPLA_2$[21] have appeared as well as several review articles summarizing the classes of PLA_2 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 A_2 superfamily.

1.2 Accessing the *In Vitro* Activity of Phospholipase A_2

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 PLA_2s 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 PLA_2 activities, particularly in comparing the different enzyme forms and substrates as reviewed elsewhere[24] and illustrated in Figure 2. Thus as one compares the specific activity, specificity, and especially the inhibition[25] of each PLA_2 Group, subgroup, and species, one must note the particular assay conditions and aggregated form of substrate used including the kinetic ramifications.

2. Secreted Phospholipase A_2 (sPLA$_2$ Groups I, II, III, V, IX, X, XI, XII, XIII, XIV

2.1 Groups, Subgroups, Specificity and Mechanism

The sPLA$_2$s are small secreted proteins of 14–18 kDa (except for Group III sPLA$_2$) that usually contain 6 to 8 disulfide bonds (Table 2).[14] A schematic presentation of the sequences (Figure 3) provides an overview of this PLA_2 types. This group of enzymes uses an active site His/Asp dyad and requires mM Ca^{2+} 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 PLA_2s were sequenced and disulfide bond patterns determined, those from old world snakes (cobras and kraits) were refered to as type I and those from new world snakes (rattlesnakes) were referred to as type II. The first non-venom PLA_2, 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 sPLA$_2$, 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 sPLA$_2$ (Table 2) have been identified in mammals, insects, mollusks, reptiles, plants and bacteria. The sPLA$_2$s display a wide range of different tissue distribution patterns and distinct physiological functions.

Ten members of the sPLA$_2$ 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 sPLA$_2$s and the mouse genome contains all ten, including GIIC sPLA$_2$, which exists in the human genome only as a pseudogene.

Mammalian GIII sPLA$_2$ is a multi-domain protein with a molecular weight of 55 kDa. It contains a central domain similar to that of bee venom GIII sPLA$_2$, including a 130-amino acid N-terminal domain extension and a 219-amino acid C-terminal domain extension. GXIIB sPLA$_2$ in humans and mice (a homologue of GXIIA sPLA$_2$), has a natural mutation in the active site (H48L) and totally lacks enzymatic activity.[26] GXIIB sPLA$_2$ is highly

expressed in the liver, small intestine and kidney, both in human and mouse, and the functions of GXIIB sPLA$_2$ 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 sPLA$_2$s 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 sPLA$_2$s display a characteristic increase in activity when the substrate concentration is changed from monomers to aggregates, which is refered as “interfacial activation”.[24] The structural basis for the interfacial activation mechanism will be discussed in section 2.2. In contrast with cytosolic PLA$_2$s (cPLA$_2$s), which have a marked specificity for arachidonic acid at the *sn*-2 position of its phospholipid substrates, sPLA$_2$s 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 sPLA$_2$s show a higher activity with anionic phospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylserine (PS). GIA and GXIV sPLA$_2$s are more active against zwitterionic phosphatidylcholine (PC) vesicles. Mammalian GV and GX sPLA$_2$s 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 sPLA$_2$s contain a highly conserved Ca^{2+} binding loop (*XCGXGG*) and a catalytic site (*DXCCXXHD*). Among the sPLA$_2$s, crystal structures available are the cobra venom GIA, human, porcine and bovine pancreatic GIB, human GIIA, human GX, plant GXIB and prokaryotic GXIV sPLA$_2$s. 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 sPLA$_2$s have very similar structures, which contain three long α-helices, two-stranded β-sheets referred to as β-wings, and a conserved Ca^{2+} binding loop. The plant GXIB sPLA$_2$ has been shown to lack β-wings.[28] The prokaryotic GXIV sPLA$_2$ shows a different, all α-helical, protein folding.[29]

Although the structures of other sPLA$_2$ family members have not been reported to date, based on available structural data and sequence alignment, the GI, GII, GV and GX mammalian sPLA$_2$s are expected to share a common three-dimensional structure. The human GIII sPLA$_2$ would represent another class of fold. Its PLA$_2$ domain is expected to be similar to that of bee venom GIII sPLA$_2$, while its extra C- and N-terminal domains, as we mentioned above, are functionally unknown and structurally are not homologous to known proteins. GXIIA sPLA$_2$ comprises the third structural class, which has an unusual Ca^{2+} binding loop.

The cobra venom GIA sPLA$_2$ is one of the most extensively studied PLA$_2$ enzymes and it has been considered an important model for phospholipase A$_2$ enzymology. Therefore, we will use GIA sPLA$_2$ as an example to show the unique structural characteristics of sPLA$_2$ enzymes. GIA sPLA$_2$ 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 sPLA$_2$s 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 H_2O, 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 $sPLA_2$ 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 $sPLA_2$s 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 A_2 family members depends critically on the interaction of the protein with large lipid aggregates. The interfacial activation mechanism of PLA_2 enzymes has long been an interesting topic in membrane protein enzymology.[24,31] GIA $sPLA_2$ 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 $sPLA_2$/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 $sPLA_2$ 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 $sPLA_2$ to lose its ability to bind to zwitterionic PC vesicles.[34] A similar result was found in GX $sPLA_2$, 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 $sPLA_2$, 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 $sPLA_2$ 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 $sPLA_2$ surface could be the reason that this enzyme has highly selective binding to anionic vesicles. The recently defined crystal structure of GIB $sPLA_2$ 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 $sPLA_2$ have shown that the interaction occurs predominantly through a nonelectrostatic mechanism.[38] When the five basic residues on the bee venom $sPLA_2$ 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 $sPLA_2$ 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 $sPLA_2$ was recently reported.[40] The trimeric form shows a much more positively charged interfacial surface. The authors suggested that human GIB $sPLA_2$ may use a different activation mechanism in which GIB $sPLA_2$ 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 venom[41] and *Naja naja* GIA $sPLA_2$s.[42] Anion-assisted dimer crystal structures were reported for other pancreatic

sPLA$_2$s.[43] While the existence of dimeric and trimeric forms may be a functionally important feature of these sPLA$_2$s, the higher oligomeric states of sPLA$_2$ structures are found under high concentration crystallization conditions, so they may not be representative of the real physiological state. Whether the trimeric or dimmeric form is important for sPLA$_2$ catalysis or not requires further investigation.

2.3 Biological Functions and Disease Implications

sPLA$_2$s 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] sPLA$_2$s also show antiviral activity.[45] sPLA$_2$s are expressed and released by human inflammatory cells including macrophages, monocytes, T cells, mast cell and neutrophils and increased concentrations of different isoforms of sPLA$_2$s have been detected in the blood of patients with inflammatory and autoimmune diseases.[46] sPLA$_2$s also play a role in the hydrolysis of oxidized lipids in low- and high-density lipoproteins ontributing 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 sPLA$_2$ family. However, due to the large number of sPLA$_2$ 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 sPLA$_2$.

2.3.1 Antibacterial and Antiviral Functions of sPLA$_2$s (sPLA$_2$ Groups I, II, III, V, X)—There is a considerable body of evidence supporting the antibacterial functionality of sPLA$_2$. GIIA sPLA$_2$ 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 sPLA$_2$ are found in tears, where the majority of bactericidal action is due to GIIA sPLA$_2$.[50] The concentration of GIIA sPLA$_2$ increases up to 500-fold in the serum samples of patients with severe acute diseases compared with healthy controls.[49b] High concentrations of GIIA sPLA$_2$ have also been found in seminal plasma, inflammatory exudates, bronchoalveolar lavage and intestinal lumen.[17e,51] Overexpression of GIIA sPLA$_2$ 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 sPLA$_2$ and mice that have had recombinant human GIIA sPLA$_2$ administered *in vivo* are resistant to *B. anthracis* infection.[53]

The antibacterial activity of GIIA sPLA$_2$ is calcium-dependent and is negated in the presence of EGTA.[44,54] The antibacterial activity also depends on PLA$_2$ activity to hydrolyze the cell membrane.[55] To kill the bacteria, GIIA sPLA$_2$ 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 sPLA$_2$ also enables it to bind with the lipoteichonic acids of Gram-positive bacteria.[57] The bactericidal effect of GIIA sPLA$_2$ 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 sPLA$_2$s also have antibacterial activity.[49a,56,59] The ranking of most to least potent sPLA$_2$s 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 sPLA$_2$ depends significantly on the

highly positively charged protein surface. For example, the GIIA $sPLA_2$ shows more antibacterial function than other $sPLA_2$s-this may be related to its highly cationic nature (pI > 10.5). Antibacterial activity was also found in snake venom GIA $sPLA_2$ 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 lipopolysacchride, which makes it difficult for the enzyme to access and hydrolyze the plasma membrane phospholipids. GIIA $sPLA_2$ 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 $sPLA_2$ can be converted into an enzyme active against *E. coli* treated with BPI.[62] GV $sPLA_2$ was also found to hydrolyze phospholipids from *E. coli* in the presence of serum, but not as efficiently as GIIA $sPLA_2$.[59] GV $sPLA_2$ 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, $sPLA_2$s also display antivirus activity. GIII, GV and GX $sPLA_2$s are capable of preventing host cells from being infected with adenovirus.[63] The antivirus activity of GV and GX $sPLA_2$s 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 $sPLA_2$ has a different antivirus mechanism, which requires the presence of both the catalytic domain and the N-terminal domain for the anti-adenovirus effect.[63b] By degrading the virus membrane and recognizing the virus envelop, human GX $sPLA_2$ is capable of neutralizing HIV-1.[64] Bee and snake venom $sPLA_2$s and a peptide derived from bee venom $sPLA_2$ 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 $sPLA_2$ and Inflammation ($sPLA_2$ Groups I, II, V, X)—The $sPLA_2$s appear to play a role in several inflammatory diseases. The first evidence was from GIIA $sPLA_2$, which is present at high concentrations in the synovial fluid of patients with rheumatoid arthritis.[3] GIIA $sPLA_2$ deficient mice have shown reduced signs of arthritis when compared with wild-type mice. Recently, GV $sPLA_2$ has also been found in rheumatoid arthritis synovial fluid, but the expression was notably lower than GIIA $sPLA_2$.[66] However, GV $sPLA_2$ may play an anti-inflammatory role rather than the normal pro-inflammatory role.[66] GV $sPLA_2$ deficient mice were protected from K/BxN arthritis when treated with exogenous recombinant GV $sPLA_2$.[66] Increased levels of $sPLA_2$s have also been detected in the plasma or serum of patients with acute pancreatitis, septic shock, Crohn's diseases and ulcerative colitis.[47] Furthermore, $sPLA_2$s seem to be involved in adult respiratory distress syndrome (ARDS) and inflammatory bowel disease.[67]

$sPLA_2$s 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 PLA_2 catalytic reaction is the initial and rate limiting step for the biosynthesis of eicosanoids.[68] GIVA PLA_2 has for a long time been considered the major PLA_2 enzyme to release AA for eicosanoid production.[68] However, more recent results have shown that $sPLA_2$s may also be involved in AA release and eicosanoid biosynthesis. GIIA $sPLA_2$s are

capable of mediating cytokine-induced delayed AA release and ionophoreinduced immediate AA release.[69] Exogenously added human GV $sPLA_2$ could induce AA and leukotriene C4 (LTC4) release from unprimed human neutrophils.[34] Peritoneal macrophages from GV $sPLA_2$ knockout mice show reduced production of LTC4 upon stimulation with the yeast cell wall particle zymosan which amplifies the action of GIV $cPLA_2$ in regulating eicosanoid biosynthesis.[70] In mouse peritoneal macrophages stimulated with zymosan, GV $sPLA_2$ can translocate to the phagosome and regulate phagocytosis through regulation of eicosanoid generation.[71] The exogenously added human GV $sPLA_2$ could also induce leukotriene B_4 (LTB4) synthesis in human neutrophils by the activation of GIVA PLA_2.[72] A putative mechanism has been proposed whereby human GV $sPLA_2$ 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 $cPLA_2$.[72] In addition, exogenous GV $sPLA_2$ could induce arachidonic acid release and LTC4 synthesis in isolated human peripheral blood eosinophils in a manner independent of activating $cPLA_2$.[73] Recombinant GX $sPLA_2$ has been shown to induce a substantial release of AA independent of the action of GIVA PLA_2, when added to human myeloid leukemia cells or adherent mammalian cells.[74] A GIVA PLA_2 knockout mice study further supports the results of the *in vitro* study which showed that the amount of AA released by GX $sPLA_2$ from spleen cells was not significantly altered by GIVA PLA_2 deficiency.[75] GX $sPLA_2$ knockout mice have shown a significant reduction in eicosanoid generation and this provides additional strong evidence for GX $sPLA_2$ involvement in eicosanoid-mediated inflammation.[76]

$sPLA_2$s can release AA by two mechanisms: an external plasma membrane pathway and a heparan sulfate proteoglycan (HSPG)-shuttling pathway.[77] Among $sPLA_2$s, GV and GX show high binding affinity and catalytic activity towards PCs. This would allow GV and GX $sPLA_2$s to directly act on the outer leaflet of plasma membranes, to hydrolyze PCs to release fatty acids and lysophospholipids.[77] Heparin-binding $sPLA_2$s GIIA, GIID and GV may bind to the heparan sulfate chains of glypican, 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, $sPLA_2$s also are able to activate inflammatory cells to induce the production of other proinflammatory mediators from macrophages, neutrophils, eocsinophils, monocytes and endothelia cells[78] and the function may not require enzymatic activity. GIB, GIIA, GV and GX $sPLA_2$s induce the production of proinflammatory cytokines and chemokines independent of their hydrolytic activity.[79] GIIA and GIII $sPLA_2$s 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 $sPLA_2$s have been detected in bronchoalveolar lavage fluids of ARDS patients at levels that correlate positively with the severity of ARDS.[81] The involvement of $sPLA_2$s 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 $sPLA_2$ activity ameliorated lung dysfunction by protecting against surfactant degradation.[83] Transgenic mice expressing human GV $sPLA_2$, but not GIIA or GX $sPLA_2$, show a significant reduction in the lung surfactant phospholipids phosphatidycholine and phosphatidylglycerol, a condition that leads to neonatal lethality due to lung dysfunction.[84] The overexpression of human GX $sPLA_2$ enzyme in transgenic mouse macrophages leads to massive degradation of surfactant phospholipids causing lethal lung inflammation.[85]

sPLA$_2$ may be involved in the pathogensis of inflammatory bowel disease including Crohn's disease and ulcerative colitis.[86] GIIA sPLA$_2$ protein and mRNA were detected in Paneth cells of the small intestinal mucosa in the intestine in Crohn's disease patients.[86b] GIIA sPLA$_2$ 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 sPLA$_2$ 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 sPLA$_2$s in Atherosclerosis (sPLA$_2$ Groups II, III, V, X)—There is considerable evidence to show that sPLA$_2$s play an important role in atherosclerosis. GIIA, GV and GX sPLA$_2$s 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 sPLA$_2$ 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 sPLA$_2$.[88]

Transgenic mice expressing human GIIA sPLA$_2$ 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 sPLA$_2$ 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 sPLA$_2$ is a proatherogenic factor and it may regulate the collagen production in the plaque. The macrophage-specific expression of GIIA sPLA$_2$ 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 sPLA$_2$ induces the formation of slightly smaller LDL particles, enriched with lysoPC.[93] This is because GIIA sPLA$_2$ 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 sPLA$_2$s in atherogenesis is the ability of sPLA$_2$ to hydrolyze the phospholipids on LDL particles. The modified LDL could then promote lipid accumulation and lead to enhanced macrophage uptake. Among mammalian sPLA$_2$s, GV and GX sPLA$_2$ have shown higher activity in hydrolyzing LDL and HDL than other sPLA$_2$s due to their high affinity binding to PCs.[94] Thus, they may contribute more to atherosclerosis. GV sPLA$_2$ was detected in both human and mouse atherosclerosis lesions.[95] LDL particles modified by GV sPLA$_2$ 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 sPLA$_2$ in mouse lesions and that overexpression of GV sPLA$_2$ in bone marrow cells significantly increases collagen deposition in atherosclerotic lesions, providing the first *in vivo* data showing that GV sPLA$_2$ promotes atherosclerosis.[97]

GX sPLA$_2$ shows the highest activity towards PC, which is the major lipid component of both LDL and HDL. Elevated expression of GX sPLA$_2$ 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 $sPLA_2$ enhances accumulation of cholesterol ester in macrophages,[98] where it may promote the atherosclerotic process by hydrolyzing lipoproteins. Unlike GIIA and GV $sPLA_2$, GX $sPLA_2$ does not promote LDL particle aggregation and does not bind to proteoglycans.[99] Recent research has shown GX $sPLA_2$ may suppress macrophage expression of two transport proteins, ABCA1 and ABCG1, which efflux excess cholesterols to extracellular acceptors.[100] This indicates that GX $sPLA_2$ 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 $sPLA_2$s 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 $sPLA_2$, which is distinctive among mammalian $sPLA_2$s due to its additional N- and C-terminal domains, may also play a role in atherosclerosis. Transgenic mice overexpressing human GIII $sPLA_2$ have marked alterations in the levels of plasma lipoproteins and GIII $sPLA_2$ modified LDL could promote the formation of foam cells.[103]

$sPLA_2$ 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 $sPLA_2$ 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. Lysophosphatidylchonline, a highly atherogenic lipid, can induce multiple deleterious processes in the atherosclerotic plaque.[104] In addition, GX $sPLA_2$ 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 $sPLA_2$ may function like GVIIA lipoprotein-associated PLA_2 (Lp-PLA_2), which regulates PAF in the inflammatory process.

$sPLA_2$ 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 $sPLA_2$ 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 $sPLA_2$ hydrolysis of LDL.[107]

2.3.4 Other Functions—Many of the biological functions of $sPLA_2$s appear to be independent of their catalytic activity. A variety of $sPLA_2$s exhibit potent anticoagulant activity.[67b] The mammalian GIIA, GIID and GV $sPLA_2$s, in addition to several venomous $sPLA_2$s, 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, $sPLA_2$ 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 $sPLA_2$ receptors, N-type and M-type receptors, have been identified and found to bind to both $sPLA_2$s 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 $sPLA_2$s may exert physiological roles by acting in a cytokine-like fashion and independent of their catalytic function.[79a,79c,110]

GIB $sPLA_2$, which is present at high levels in pancreatic juice, is the major enzyme responsible for the dietary phospholipid phosphatidycholine.[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 $sPLA_2$ activity were found in the bronchoalveolar lavage fluid of asthmatic patients than that of control groups.[113] GIIA and GX $sPLA_2$s were found in the airways of both asthmatic patients and controls. These two $sPLA_2$s should be responsible for the majority of PLA_2 enzymatic activity detected in the bronchoalveolar lavage fluid.[114] GX $sPLA_2$ is more highly expressed than GIIA $sPLA_2$ in the airway epithelium of asthmatic patients.[114] In a mouse asthma model, GX $sPLA_2$ deficiency was shown to potentially reduce allergen-induced airway inflammation and remodeling.[76] When transgenics expressing human GX $sPLA_2$ in GX $sPLA_2$ 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 $sPLA_2$ knockout mouse asthma model.[115] Altogether these studies suggest that GV $sPLA_2$ may play an important role in asthma.

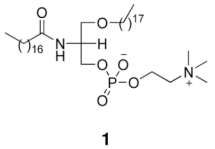
$sPLA_2$s may also play a role in tumorigenesis.[16e] GIIA $sPLA_2$ is overexpressed in almost all human prostate cancer specimens and increased levels correlate with advanced tumor grades.[116] GIIA $sPLA_2$ may stimulate tumor cell growth[117] and be involved with the progression of prostate cancer, and could therefore potentially serve as a biomarker for prostate cancer.[118] Increased expression of GIIA $sPLA_2$ was also detected in colorectal adenomas of familial adenomatous polyposis patients, and the enzyme may also be involved in human colorectal tumor development and progression[119]. However the protective role of GIIA $sPLA_2$ 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 $sPLA_2$s are expressed in various type of cancers and may also play a role in tumorigenesis.[16e] However the function of $sPLA_2$s 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

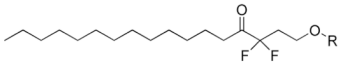
Numerous assays to evaluate the activity of inhibitors toward each PLA_2 type exist,[122] ranging from simple procedures to methods involving expensive instrumentation and from low sensitivity to highly sensitive procedures. As mentioned in section 1.2, it has to be emphasized that it makes no sense to compare the activity of various inhibitors (for example, the IC_{50} values) if they are measured in different assay systems, especially if assayed at different substrate concentrations. The IC_{50} 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 $sPLA_2$s.[22b] The most recent review article on PLA_2 inhibitors highlights the recent patent literature.[22f]

2.4.1 Early Attempts with Phospholipid Analogues—The early attempts to develop PLA_2 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 PLA_2 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

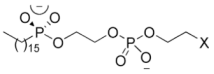
phosphatidylethanolamine was the most active in this series against cobra venom PLA_2. Phospholipid analogues (**3**) containing a phosphonate group in place of the ester at the *sn*-2 position of the glycerol backbone were found to be tight-binding inhibitors of the same enzyme.[125]



1

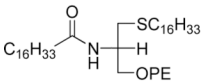


2a, R: PC
2b, R: PE



3a, X: NH_3^+
3b, X: NMe_3^+

A series of structurally modified phospholipids were used to delineate the structural features involved in the interaction between cobra venom PLA_2 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 PLA_2.[127]



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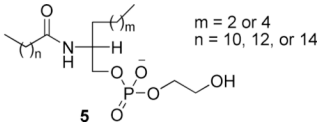
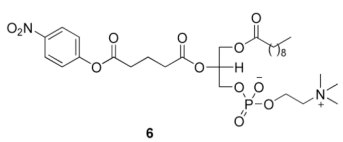


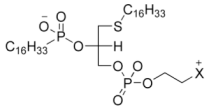
5

Some of the above mentioned inhibitors, an acylamino and a phosphonate phospholipid analogue, were useful tools to resolve the structure of various $sPLA_2$s by X-ray crystallography.[30a,33,33,128] Thus, the interfacial catalytic mechanism of $sPLA_2$ was proposed.[33] The first structure of recombinant human synovial fluid PLA_2 was reported in 1991[128] 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 invastigators alsot studied the effect of polar head groups on the interaction of cobra venom PLA_2 with phosphonate transition-state analogues **7**.[130]

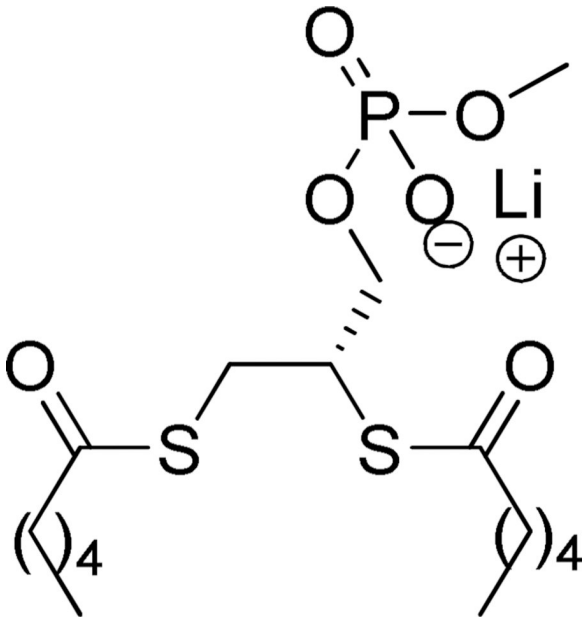


6

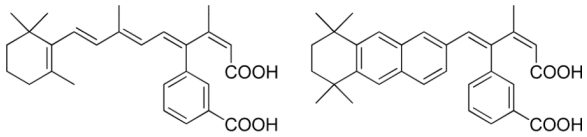


7a, X: NMe_3
7b, X: $HNMe_2$
7c, X: H_2NMe

The substrate specificity at the active site of recombinant human synovial fluid PLA_2 was investigated by using a series of short-chain phospholipid analogues such as **8**.[131]



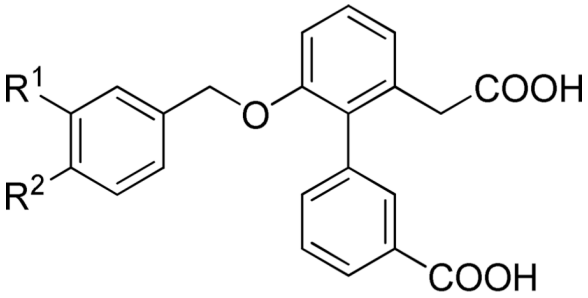
2.4.2 Dicarboxylic Acids—In 1992, Bristol-Myers Squibb presented the dicarboxylic acid **9a** (BMS-181162) as the first specific inhibitor of a 14 kDa PLA_2 (specificly compared to other types of phospholipases, PLC, PLD and PLA_1).[132] BMS-181162 blocked the arachidonic acid release (IC_{50} 10 μM) 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 disapointing 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**

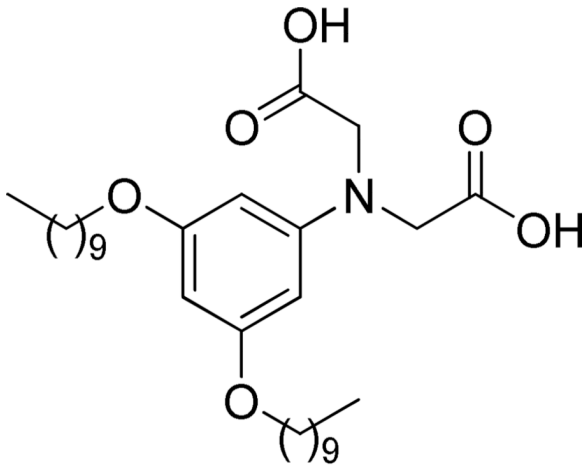
The mechanism of inhibition of GIIA $sPLA_2$ (refered to by the authors as human nonpancreatic $sPLA_2$) by the anti-inflammatory agent BMS-181162 was studied.[132c] BMS-181162 inhibited human platelet PLA_2 with an IC_{50} 40 μM and it was able to reduce mouse ear edema with an ED_{50} 160 μg/ear in a phorbol-ester induced acute inflammation assay, while BMS-188184 inhibited human platelet PLA_2 with an IC_{50} 17 μM and reduced mouse ear edema with an ED_{50} 9.37 μg/ear.[132d] It is ubclear whether the inhibtion observed in the mouse ear edema model reflected inhibtion of just GIIA $sPLA_2$ or other PLA_2s as well.

Among a series of biaryl diacid inhibitors of human $sPLA_2$, 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, $R^1=H$, $R^2=OC_{10}H_{21}$

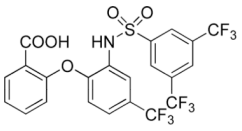
Through computer-assisted methods, Roche developed the very potent inhibitor **11** containing the iminodiacetic acid group (IC_{50} 0.23 μM for human synovial fluid PLA_2).[134] Inhibitor **11** exhibited anti-inflammatory activity in two separate animal models of inflammation.



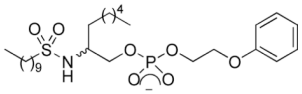
11

2.4.3 Sulfonamides—A novel series of benzenesulfonamides were prepared and evaluated as membrane-bound PLA_2 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 $sPLA_2$ to prostaglandin formation.[136] Computer-assisted methods contributed to the rational design of the sulfonamide inhibitor **14**.[137]

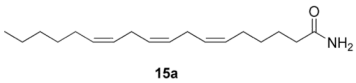


13, SB-203347

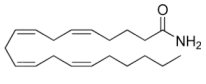


14

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 PLA_2s 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 PLA_2 (Table 5).[138]

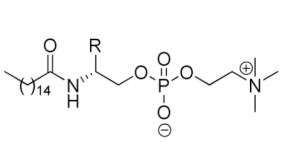


15a

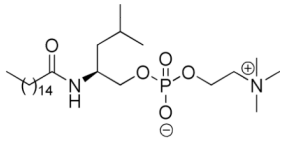


15b

Acylamino phospholipid analogues were synthesized and evaluated as pancreatic PLA_2 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.

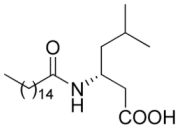


16a, R=CH_3
16b, R=$CH_2CH(CH_3)_2$

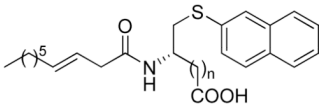


16c

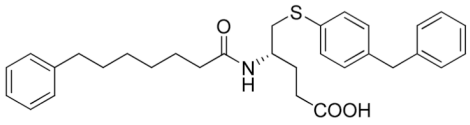
A continuation of this work reported non-phospholipid inhibitors of $sPLA_2$, where a simple carboxylic group replaced the phosphocholine moiety.[140] Structure-activity relationship studies led to interesting results (Table 7). The most potent inhibitor in this series (FPL67047XX) presented an IC_{50} value against the human platelet $sPLA_2$ of 21 ± 4 nM. The precise binding interactions of this inhibitor with the human nonpancreatic $sPLA_2$ were determined by high-resolution X-ray crystallography.[141]



17a



17b, n=1
17c, n=2

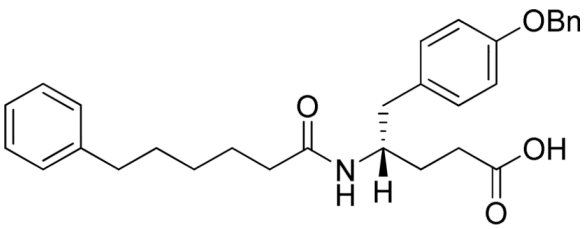


18, FPL67047XX

Most recently, new GIIA $sPLA_2$ 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 PLA_2 and the crystal structure revealed a chelation to a Ca^{2+}

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]

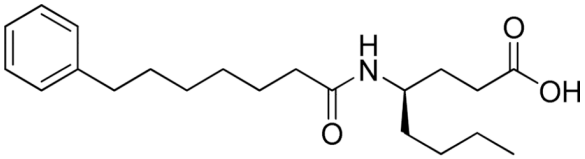


19a

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 $sPLA_2$ 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 PLA_2 enzymes showed that amide **20**, based on (*R*)-γ-norleucine, is a selective inhibitor of GV $sPLA_2$ ($X_I(50)$ 0.003 ± 0.0004) not affecting the activities of intracellular GIVA PLA_2 and GVIA PLA_2.[148]



20

2.4.5 Indoles—In 1995, researchers at Lilly reported a highly potent $sPLA_2$ 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 PLA_2 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]

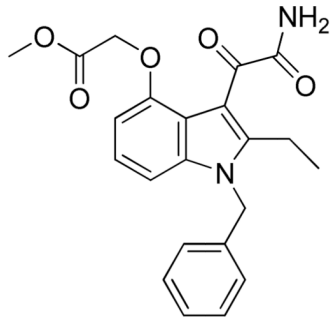
Further implementation of this structure-based design strategy and continued SAR development led to indole-3-acetamides with additional functionalities which provide increased interaction with important residues within the enzyme active site. These inhibitors **22** presented substantially enhanced potency 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 PLA_2 inhibitor.[152]

LY315920 was 40-fold less active against human GIB pancreatic PLA_2 and was inactive against $cPLA_2$ and the constitutive and inducible forms of cycloxygenase.[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 PLA_2 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 $sPLA_2$ 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]



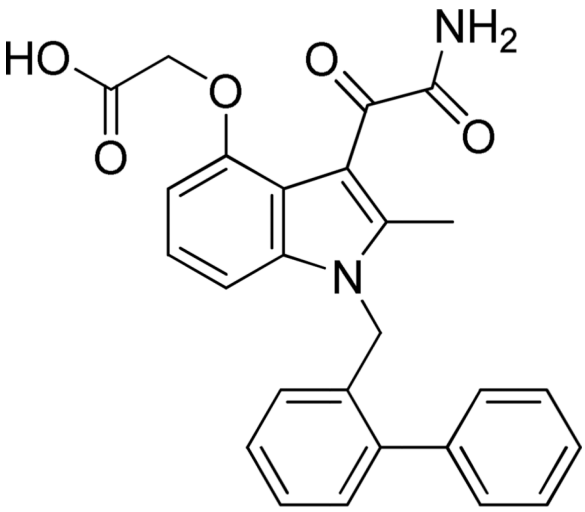
25, LY333013 or Methyl Varespladib

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 PLA_2-IIA independent mechanisms, possibly via blockade of the PLA_2R function.[161]

In 2002, the expression of the full set of human and mouse groups I, II, V, X, and XII $sPLA_2$s 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

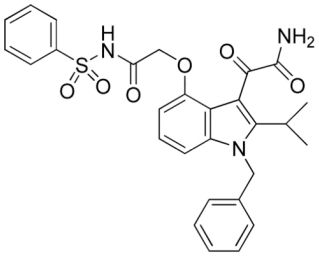
the mammalian $sPLA_2$s, with Me-Indoxam being the most generally potent $sPLA_2$ inhibitor.[27a]



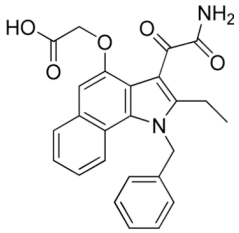
28, Me-Indoxam

A structure-guided design was employed in a search for potent and selective inhibitors of mammalian $sPLA_2$s. Although no compounds were found to be highly specific for a single human or mouse $sPLA_2$, combinations of Me-Indoxam analogues were discovered that could be used to distinguish the action of various $sPLA_2$s in cellular events.[162]

Using the X-ray structure of human GX $sPLA_2$, the first potent inhibitor of this enzyme was developed.[163] In addition, a series of inhibitors of $sPLA_2$s 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 $sPLA_2$ enzymes, while the substituted 6,7-benzoindole **29b** inhibited nearly all human and mouse $sPLA_2$s in the low nanomolar range.



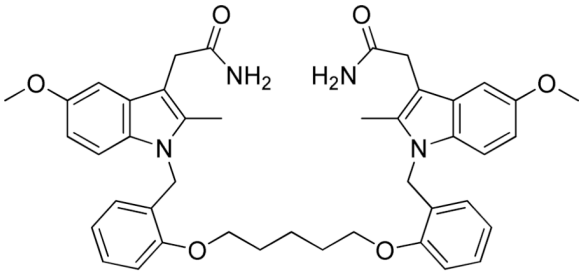
29a



29b

Most recently, molecular docking and 3D Quantitative Structure-Activity Relationship Comparative Molecular Field Analysis (3D-QSAR CoMFA) studies on indole inhibitors of GIIA $sPLA_2$ led to a model which was used for the design and evaluation of new compounds.[165]

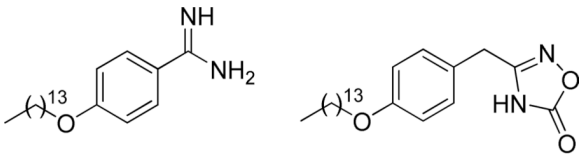
A series of bis-indole compounds were designed and synthesized on the basis of the enzyme structure of human nonpancreatic $sPLA_2$. Their inhibition activities were improved compared to that of the monofunctional protocompound. The potent compound **30** (IC_{50} 24 nM) revealed that cooperative binding interactions between the two enzyme molecules also contributed to the stability of the ternary complex.[166]



30

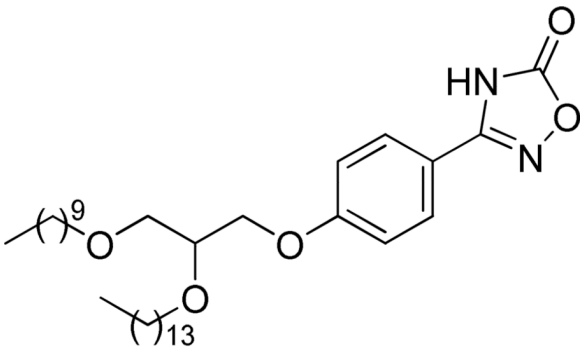
Recently, Anthera Pharmaceuticals disclosed the $sPLA_2$ inhibitors Varespladib (A-001, previously known as LY315920) and Varespladib methyl (A-002, previously known as LY333013) for the treatment of cardiovascular diseases.[167] A-002 was shown to lower levels of GIIAs PLA_2 by >90%, LDL-C by 12% to 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 $sPLA_2$ 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 $sPLA_2$ 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 IC_{50} towards three GII PLA_2s, while inactive towards four GI and one GIII enzymes in two in vitro enzymatic assay conditions.



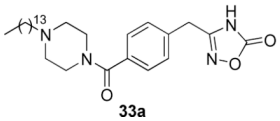
31a, PMS815 **31b, PMS1062**

In a continuation, glycerol-containing derivatives of PMS1062 were synthesized.[173] Among them, compound **32** was as potent as Me-Indoxam for hGIIA PLA_2.

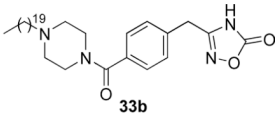


32

Replacement of the long chains by substituted piperazine derivatives led to inhibitors **33**.[174] Compound **33a** inhibited hGIIA $sPLA_2$ and in a carrageenan-induced edema test in rats showed to be as potent as indomethacin (Table 13).

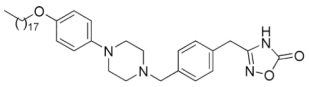


33a

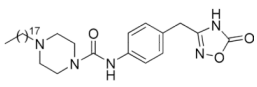


33b

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



34a



34b

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, $sPLA_2$ 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 $sPLA_2$ members (Table 14).

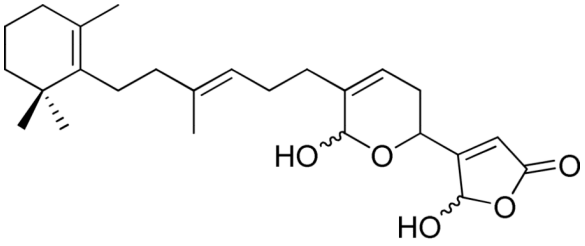
2.4.8 Aptamers and Peptides—A family of sequence-related 2'-aminopyrimidine, 2'-hydroxylpurine aptamers, developed by oligonucleotide-based combinatorial chemistry, SELEX (systematic *e*volution of ligand by *ex*ponential enrichment) technology, was found to bind human nonpancreatic $sPLA_2$ with nanomolar affinities and inhibit enzymatic activity.[177]

A number of synthetic peptides were designed and screened for $sPLA_2$ inhibition.[178] The linear peptide **36** (PIP-18) inhibited the recombinant human synovial $sPLA_2$ activity with an IC_{50} of 1.19 μM. The peptide interfered with the function of $sPLA_2$, but it also appeared to inhibit mRNA expression of $sPLA_2$ 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

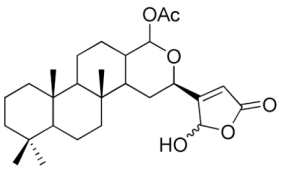
2.4.9 Natural Products—



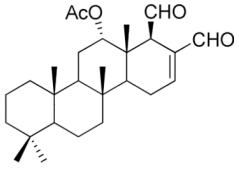
37, Manoalide

In the mid 1980s, the natural product manoalide **37** was reported to be the first inhibitor of cobra venom PLA_2.[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 PLA_2[180] and human synovial fluid PLA_2.[181] Mechanistic studies on manoalide and analogues[182] 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 sesterpenes of marine origin that contain the γ-hydroxybutenolide moiety have been studied for their anti-inflammatory activity and inhibition of PLA_2. Petrosaspongiolide M (**38**) displayed a potent inhibitory activity toward GII and GIII $sPLA_2$ 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 PLA_2 was analyzed in detail.[187] Scalaradial (**39**) is another marine metabolite, which inhibits GII and GIII PLA_2 and presents in vivo anti-inflammatory activity.[188]

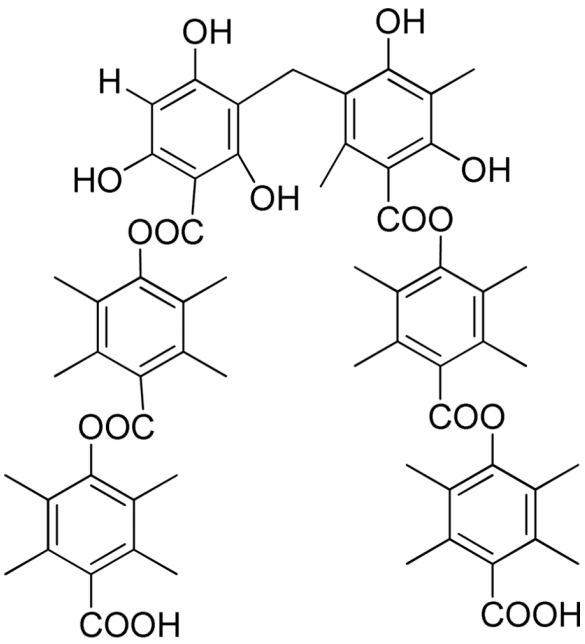


38, Petrosaspongiolide M



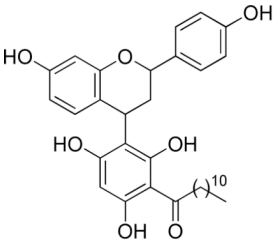
39, Scalaradial

Thielocin B3 is a very potent naturally occurring inhibitor of human nonpancreatic $sPLA_2$ (GII). Structure-activity relationship studies led to a number of analogues with potency comparable to the parent natural product.[189,190]

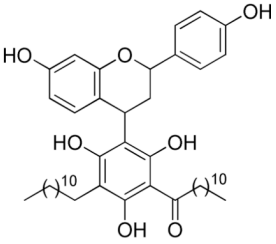


40, Thielocin B3

YM-26567 (**41**), a natural product isolated from the fruit of Horsfieldia amygdaline, is a micromolar inhibitor of rabbit platelet $sPLA_2$.[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 PLA_2[194] and the mechanism of inhibition of human $sPLA_2$ has been investigated.[195]

2.4.10 Summary Status of $sPLA_2$ Inhibitors—As presented above, a wide variety of structurally different inhibitors has been studied for their inhibition of several $sPLA_2$s. Starting in the mid 1980s, before crystal structures were widely available, the first studied inhibitors of $sPLA_2$ were substrate analogues and marine natural products. The first potent and specific (when compared to PLC, PLD and PLA_1) dicarboxylic acid inhibitor of $sPLA_2$ 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 $sPLA_2$ inhibitors. Lilly Research Laboratory presented a highly potent $sPLA_2$ inhibitor (LY315920) which was developed via molecular modeling techniques combined with chemical modification of a lead compound. This inhibitor, which is selective for GIIA $sPLA_2$, 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 $sPLA_2$ 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 $sPLA_2$ 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 $sPLA_2$s was expressed, indole-type inhibitors were developed that could somewhat distinguish the action of various $sPLA_2$s in cellular events, even though no compound was highly specific for a single $sPLA_2$. 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 $sPLA_2$ inhibitors, one should keep in mind that while selective $sPLA_2$ inhibitors can help us study the biological activity of specific $sPLA_2$s *in vitro* and *in vivo*, they are also essential for helping us to distinguish the activities of the different groups of $sPLA_2$.

3. Cytosolic Phospholipase A_2 [Group IV $cPLA_2$]

3.1 Groups, Subgroups, Specificity and Mechanism

The first cytosolic PLA_2, now attributed to GIVA PLA_2 (or $cPLA_2\alpha$), was reported as an activity by Christina Leslie and Ruth Kramer in human neutrophils[196] and platelets[197] 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 PLA_2 ($cPLA_2\beta$)[198] and GIVC PLA_2 ($cPLA_2\gamma$)[198a,199] were subsequently reported in 1998-99 by Lilly Research Laboratories and Genetics Institute investigators. GIVD ($cPLA_2\delta$), GIVE ($cPLA_2\varepsilon$) and GIVF ($cPLA_2\zeta$) PLA_2 were identified in mice by the Shimizu's group in Japan in 2004–2005.[200] Currently, the group IV PLA_2 is comprised of these six known phospholipases.[14] A summary of the characteristics of each member of the GIV PLA_2 family (Table 15) and a schematic presentation of the sequences (Figure 6) provide an overview of this PLA_2 group.

3.1.1 Group IVA Phospholipase A_2 ($cPLA_2\alpha$)—After the identification of cytosolic PLA_2 in neutrophils and platelets by cell-free assay,[196–197] the human GIVA PLA_2 was cloned and sequenced in 1991 from U937 cells.[5–6] The GIVA PLA_2 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 PLA_2 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 PLA_2 is regulated by intracellular calcium, and calcium binding to the C2 domain of GIVA PLA_2 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 PLA_2 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 phosphorylation[205] and the lipid mediators

ceramide-1-phosphate (C1P)[206] and phosphatidylinositol 4,5-bis phosphate (PIP_2)[207] were shown to further increase GIVA PLA_2 activity.

Most phospholipids, such as phosphatidylcholine (PC), phosphatidylethanoamine (PE) and phosphatidylinositol (PI), are substrates of GIVA PLA_2.[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 PLA_2 since its discovery.[5–6] GIVA PLA_2 hydrolysis of phospholipid substrates has high substrate specificity for arachidonic acid at the *sn*-2 position.[5,18a] In addition to phospholipase activity, GIVA PLA_2 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 PLA_2 isoforms and showed that they all exhibit phospholipase A_1/A_2 and lysophospholipase activities.[209]

3.1.2 Group IVB Phospholipase A_2 (cPLA$_2$β)—The full length human GIVB PLA_2 was initially cloned in 1999 and the recombinant protein was then expressed in Sf-9 insect cells.[198] The GIVB PLA_2 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 PLA_2 contains a C2 domain and a catalytic domain that are similar to those in GIVA PLA_2, with the primary sequences sharing 30% identical residues.[18a] The major difference is that the GIVB PLA_2 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 PLA_2. The α/β hydrolase domain is highly homologous to GIVA PLA_2 and the active site dyad Ser-538 and Asp-566 is conserved. When we ran a multiple sequence alignment of all six GIV PLA_2s, the alignment shows that GIVB PLA_2 does not have the phosphorylation sites, C1P binding sites, or PIP_2 binding sites, and has a significantly different cap and lid region (Figure 6). Three GIVB PLA_2 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 PLA_2 preserves the conserved calcium binding sites on the C2 domain in the sequence alignment and thus demonstrates calcium-dependent PLA_2 activity. Compared to GIVA PLA_2, its phospholipase activity is low and it has low substrate specificity for *sn*-2 fatty acids.[14,212] It also has low lysophospholipase A_1/A_2 and transacylase activities.[198b,204b,213]

3.1.3 GIVC PLA_2 (cPLA$_2$γ)—The human GIVC PLA_2 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 PLA_2 is a 61 kDa protein and has 541 amino acid residues, showing 30% homology to the catalytic domain of GIVA PLA_2 (Figure 6). The active site dyad in the catalytic domain is conserved. Because this enzyme does not contain a C2 domain, GIVC PLA_2 has no calcium-dependent phospholipase activities.[198a,199] Neither the C1P nor the PIP_2 binding sites nor the phosphorylation sites are observed. Cys-538 has shown to be farnesylated in insect cells.[214] GIVC PLA_2 is also able to hydrolyze both *sn*-1 and *sn*-2 acyl chains from a phosphatidylcholine substrate with PLA_2 and PLA_1 activity.[198b,199] It has low *sn*-2 specificity for arachidonic acid, less than the GIVA PLA_2.[199]

3.1.4 GIVD (cPLA$_2$δ), GIVE (cPLA$_2$ε) and GIVF (cPLA$_2$ζ)—The human GIVD PLA$_2$ 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 PLA$_2$ at human chromosome 15q15.1 (Table 15). The murine transcripts of all three genes are tissue specific. GIVD PLA$_2$ was detected in the placenta, GIVE PLA$_2$ was detected in the thyroid, heart and skeletal muscles, and GIVF PLA$_2$ was detected in the thyroid.[200a] The sequence lengths of GIVD, E and F PLA$_2$ 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 PLA$_2$s exhibit calcium-dependent PLA$_2$ activities.[200a] GIVF PLA$_2$ has higher activity toward phosphatidylethanolamine (PE) than phosphatidylcholine (PC), which is the opposite of GIVA PLA$_2$, suggesting preference for charged substrates. A comparison of the three enzymes shows GIVF PLA$_2$ has higher PLA$_2$ activity than GIVD PLA$_2$ and GIVE PLA$_2$. GIVF PLA$_2$ shows high phospholipase A$_2$ and lysophospholipase activities.[215] In response to ionomycin, GIVF PLA$_2$ translocates to ruffles and dynamic vesicular structures, which is different from how GIVA PLA$_2$ 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 PLA$_2$ 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 PLA$_2$ are still limited, we utilize the well-studied GIVA PLA$_2$ enzyme to represent the structural characteristics and activation mechanisms of the entire GIV PLA$_2$ type in this section. The early work showed that GIVA PLA$_2$ is activated by calcium, and determined that the C2 domain is responsible for the calcium binding and GIVA PLA$_2$ 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 PLA$_2$[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 PLA$_2$, and is not conserved in other members of the GIV PLA$_2$ family. The homologies among GIVA, B and C PLA$_2$ in the cap region are very low. The homologies in the cap region among GIVD, E and F PLA$_2$ are higher than the rest of GIV PLA$_2$s. The cap region of GIVA PLA$_2$ 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 PLA_2 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 lysophsopholipid.[202a,217] Although the determination of the crystal structure has led to an understanding of the GIVA PLA_2 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 exhchange 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 PLA_2 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-phosphate[219] and phosphatidylinositol (4, 5) bisphosphate[220] 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 Ca^{2+} concentration increases, and the GIVA PLA_2 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 PLA_2.[202a] The GIVA PLA_2 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 Ca^{2+} 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 PLA_2. The catalytic domain of GIVA PLA_2 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 domains[218]. 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

PLA_2 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 PIP_2/C1P Activation—GIVA PLA_2 is also activated by binding to the lipid second messenger phosphatidylinositol-4,5-bis phosphate (PIP_2) in a Ca^{2+} independent manner.[207b,220] An increase of intracellular PIP_2 levels caused the increase of arachidonic acid release by the calcium-independent activation of GIVA PLA_2.[223] GIVA PLA_2 has a high binding affinity and specificity toward PIP_2.[207b] The lysine cluster, Lys-488, Lys-541, Lys-543, and Lys-544, have been identified as PIP_2 binding sites and critical for PIP_2-mediated GIVA PLA_2 activation[220,224] and translocation of the enzyme to phagosomes.[225] The lysine cluster is located in the intradomain contact region (Figure 7).[218] PIP_2 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 PLA_2 and increase cell toxicity.[228] Ceramide kinase, which phosphorylates cermide to synthesize C1P, was also found to be an activator of GIVA PLA_2.[226,229] Ceramide kinase is also involved in the biogenesis of lipid droplets through GIVA PLA_2 activation.[230] C1P binds directly to the C2 domain of GIVA PLA_2 at Arg-57, Lys-58, and Arg-59 (Figure 7).[219] It is also a required bioactive lipid for GIVA PLA_2 to translocate to intracellular membranes in response to inflammatory agonists.[231] Unlike PIP_2 activation, the C1P activation mechanism is Ca^{2+} dependent. Interestingly, recent research showed that C1P activates GIVA PLA_2 by decreasing the dissociation constant to increase the residence time on membranes, while PIP_2 activates GIVA PLA_2 by increasing membrane penetration for higher catalytic efficiency.[232] More recently, GIVA PLA_2 was also shown to be inhibited by sphingomyelin at the biomembrane interface.[233]

3.2.3 Phosphorylation—GIVA PLA_2 phosphorylation regulates the enzymatic activity at the phospholipid membrane and its cellular functions. GIVA PLA_2 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 PLA_2 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 PLA_2 is already activated by calcium, Ser-505 phosphorylation increases activity by 30–60%.[237] In low (2.5 μM) Ca^{2+} concentration, membrane binding showed a 60-fold increase in membrane affinity.[238] Interestingly, this effect was also observed in the PIP_2 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 PLA_2 and inhibit the membrane binding. Phosphorylation of Ser-727 has been

shown to disrupt the binding of GIVA PLA_2 to heterotetramer (A2t) to activate GIVA PLA_2.[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 PLA_2 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 PLA_2 to the membrane is mediated through three mechanisms: Ca^{2+}-mediated translocation, binding of secondary lipid messengers, and phosphorylation. Each of these mechanisms increases GIVA PLA_2 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 PLA_2 to the membrane and causes the catalytic domain to approach the membrane.[202b,203] The catalytic sites in GIVA PLA_2 must maintain the correct orientation toward the membrane, although calcium activated, C1P/PIP_2 bound, or phosphorylated GIVA PLA_2 may have slightly different phospholipid interactions. The intradomain interaction may also be affected by the conformational change in the C2 domain, phosphorylation, or C1P/PIP_2 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 PLA_2 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 PLA_2 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 immunity[244] and implicated in several diseases, including thrombosis, cancer, atherosclerosis, asthma, arthritis and rhinitis.[242a,245]

GIVA PLA_2 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 PLA_2 plays a major role in inflammatory diseases. GIVA PLA_2 is now considered a central enzyme for mediating eicosanoid production. GIVA PLA_2 has also been implicated in apoptosis triggered by the hydrolysis product arachidonic acid and its metabolites.[247] The inhibition of arachidonyl-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 PLA_2. This biological function is not specific to GIVA PLA_2, since the lysophospholipid can be potentially generated by all PLA_2 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 PLA_2 is localized in the Golgi complex in epithelial cells, it functions as a Golgi regulatory enzyme instead of inducing cell proliferation.[252] GIVA PLA_2 also regulates the junction protein transports from the Golgi complex to endothelial cell contacts.[253] It was recently shown that GIVA PLA_2 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 PLA_2 regulates the stimulation of NADPH oxidase to produce superoxides.[255] Correlations have been demonstrated between PGE_2 production and GIVA PLA_2 translocation to the nuclear membranes and between superoxide production and GIVA PLA_2 translocation to the plasma membranes.[255–256] While GIVA PLA_2 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 PLA_2.[255–257] Under oxidative stress, down-regulation of GIVA PLA_2 can suppress the NADPH oxidasemediated reactive oxygen species production in astrocytes.[258] GIVA PLA_2 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 PLA_2 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 PLA_2 activity in platelets was not observed in the patient, the mutation sites must be critical for GIVA PLA_2 activity. The patient showed small intestinal ulcerations without COX deficiency. Inherited human GIVA PLA_2 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 PLA_2-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 PLA_2-null mice have been shown to be resistant to ischemia-reperfusion

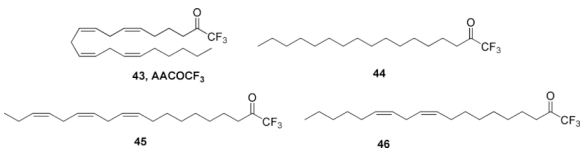
injury,[262] anaphylactic responses,[263] acute respiratory distress syndrome caused by acid or endotoxin,[264] bleomycin-induced pulmonary fibrosis,[265] collagen-induced autoimmune arthritis,[266] experimental allergic encephalomyelitis,[267] fatty liver damage,[268] and autoimmune diabetes.[269] Concerning normal physiology, the loss-of- GIVA PLA_2 causes some defects to the renal concentrating function.[270] The most notable defects caused by the loss-of- GIVA PLA_2 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 PLA_2 have also been associated with cancer pathogenesis.[245a] GIVA PLA_2 activation was shown to mediate estrogen-dependent breast cancer cell growth.[271] Deletion of GIVA PLA_2 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 PLA_2 plays an important organ-specific role in modulating intestinal tumorigenesis. Administering azoxymethane (AOM) triggered the formation of colon tumors in studies of GIVA PLA_2-null mice, showing significant increases in the number of tumors over wild type mice.[273] GIVA PLA_2 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 PLA_2 expression was detected in 18% of Barrett's oesophagus patients and is inversely associated with depth of tumor infiltration.[275] Additionally, the GIVA PLA_2 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 PLA_2 types focused on the recent patent literature.[22f] The GIVA PLA_2 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 PLA_2 was the activated ketone, arachidonoyl trifluoromethyl ketone (**43**).[277] $AACOCF_3$ was shown to be a slow tight binding inhibitor of GIVA PLA_2, 4 orders of magnitude more potent for this enzyme than for $sPLA_2$. ^{19}F and ^{13}C-NMR experiments elucidated the structure of the GIVA PLA_2•$AACOCF_3$ complex and that the mole ratio of $AACOCF_3$ 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 PLA_2 inhibitors.[280]



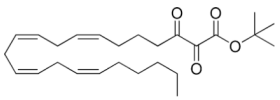
A series of fatty acid trifluoromethyl ketones were analyzed with phospholipid vesicle-, detergent-phospholipid mixed micelle-, and natural membrane-based assays.[281] With few exceptions, the relative inhibitor potencies measured with the three assays were similar (Table 16). Note that some trifluoromethylketones may also inhibit GVIA $iPLA_2$ (see, Section 4.4.1).

$AACOCF_3$ was also able to inhibit the lysophospholipase activity of GIVA PLA_2 at a similar level to PLA_2 activity.[213b] Fatty acid tricarbonyl derivatives, like compound **47**, were found to be inhibitors of GIVA PLA_2.[279] Their potency was approximately the same as that of the corresponding trifluoromethyl ketones.

$AACOCF_3$ 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 PLA_2 inhibitor and it may inhibit other enzymes, for example cycloxygenases.[282] $AACOCF_3$ blocked the production of arachidonic acid and 12-hydroxyeicosatetraenoic acid (12-HETE) in platelets[283] and in human monocytes causing a dose dependent inhibition of GIVA PLA_2 activity and LDL lipid oxidation.[284] Daily treatment of prion-infected cell lines with $AACOCF_3$ for seven days prevented the accumulation of protease-resistant PrP (PrP^{res}) indicating a pivotal role for GIVA PLA_2 in prion disease.[285]

The role of GIVA PLA_2 in allodynia has been studied. Intracerebroventricular injection of $AACOCF_3$ significantly reduced responses to von Frey hair stimulation at 8 h and 1 day after facial carrageenan injection.[286] Intrathecal administration of $AACOCF_3$ dose-dependently prevented thermal hyperalgesia induced by intrapolar carrageenan as well as formalin-induced flinching.[287] Using $AACOCF_3$, Kalyvas and David disclosed that $cPLA_2$ plays an important role in the pathogenesis of experimental autoimmune encephalomyelitis (EA), the animal model of multiple sclerosis.[288] More recently, $AACOCF_3$ has been used in a study reporting that GIVA PLA_2 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 PLA_2 but not of the human $sPLA_2$. The inactivation of the enzyme by some additional alkyl methyl fluorophosphonates was also studied.[281] Like $AACOCF_3$, intrathecal administration of MAFP dose-dependently prevented thermal hyperalgesia induced by intraplantar carrageenan as well as formalin-induced flinching.[290]



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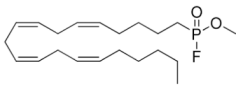
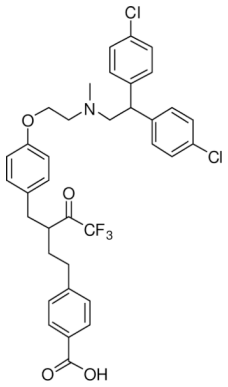


48, MAFP

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 PLA_2 inhibitors for the treatment of inflammatory diseases. These inhibitors presented IC_{50} values in the 1–50 μM range.



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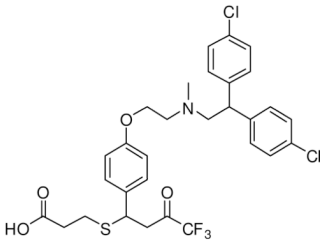
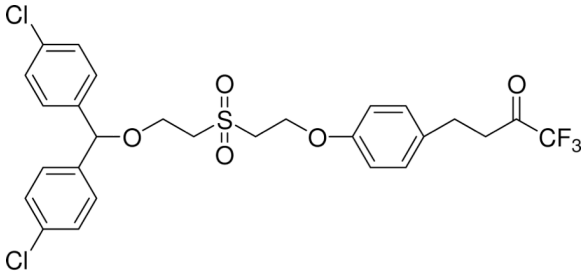


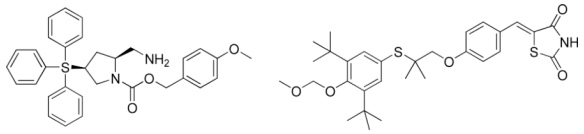
50

BMS-229724 is a tight-binding inhibitor of GIVA PLA_2 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.

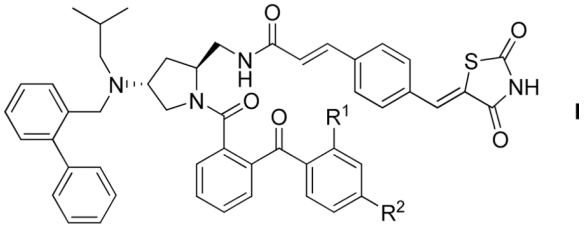


51, BMS-229724

3.4.4 Pyrrolidines—In the search for low molecular weight inhibitors of GIVA PLA_2, 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 PLA_2 by 50%, when present at approximately 0.002 mole fraction in the interface in a number of in vitro assays.[294]



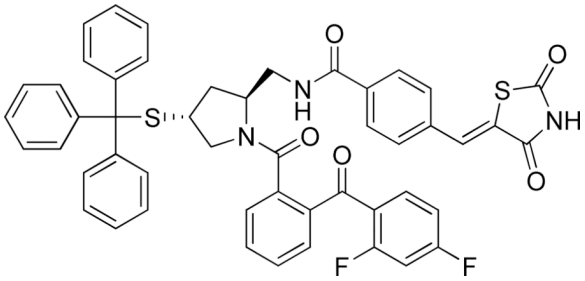
52 **53**



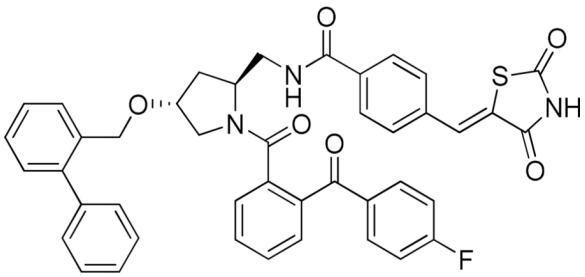
54, R^1=H, R^2=F

55, R^1=R^2=F

In a continuation of the previous studies, pyrrophenone (**56**) proved to be an excellent inhibitor of human GIVA PLA_2 (IC_{50} 4.2 nM).[295,296] Pyrrophenone strongly inhibited arachidonic acid release, prostaglandin E_2, thromboxane B_2 and leukotriene B_4 formation in human whole blood. The magnitudes of PGE_2 and thromboxane B_2 inhibition were the same as those of indomethacine. Pyrrophenone showed reversible inhibition of GIVA PLA_2, not displaying characteristics of slow-binding inhibition. Pyrrophenone potently inhibited LT, PGE_2 and PAF biosynthesis in human neutrophils with IC_{50}s in the range of 1–20 nM.[297] A structurally related inhibitor, pyrroxyphene (**57**), displayed anti-arthritic and anti-bone destructive action in a murine arthritis model.[298]

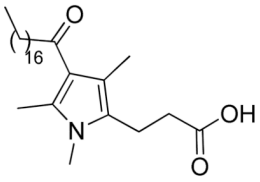


56, Pyrrophenone



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 $AACOCF_3$ (11 μM).[299b]



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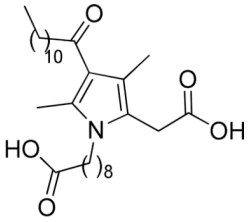
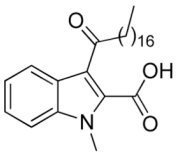


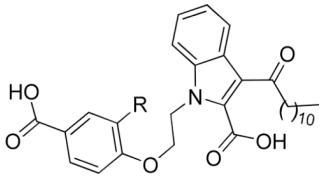
59

To define the structural requirements for improved $cPLA_2$ inhibition, Lehr varied systematically the alkanoic 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 PLA_2 with an IC_{50} value of 8 μM.[300]



60

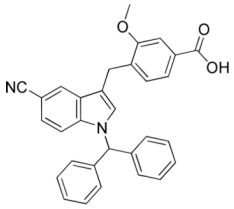


61a, R=H

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 $cPLA_2$-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 PLA_2, 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 PLA_2.[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.



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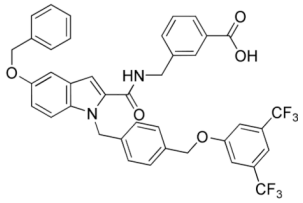
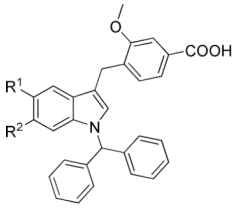


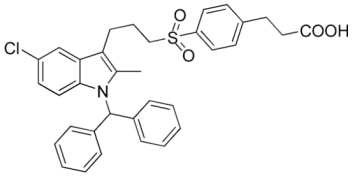
63

Wyeth (which acquired Genetics Institute) has undertaken a tremendous effort aimed toward the discovery of novel GIVA PLA_2 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, R^1=H, R^2=Cl

64b, R^1=Cl, R^2=H



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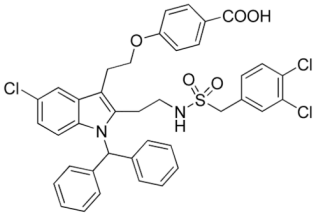
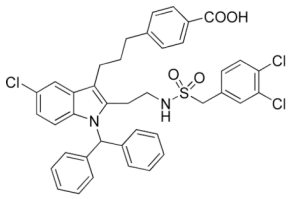
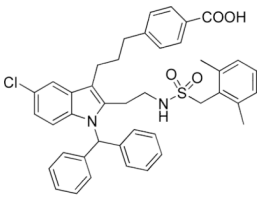


66, Ecopladib

Another study on the importance of the substituent at C3 and the substitution pattern of the phenylmethane sulfonamide region led to efipladib (**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.



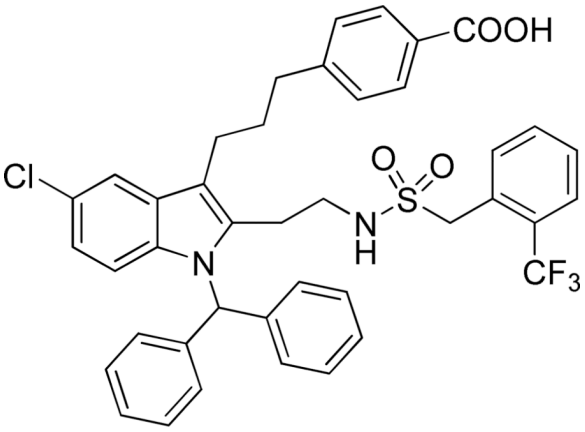
67, Efipladib



68, WAY-196025

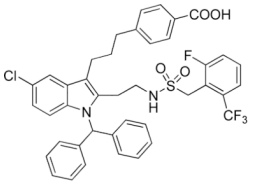
The efficacy of a similar indole inhibitor, giripladib (**69**), in two different mouse models of rheumatoid arthritis has been reported.[308] Giripladib, also known as PLA-695, was

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



69, Giripladib

Further studies aimed at the optimization of in vitro potency and rat pharmacokinetics for oral efficacy[309] and lowering the lipophilicity of the inhibitors.[310] Examples are structures **70** and **71**.



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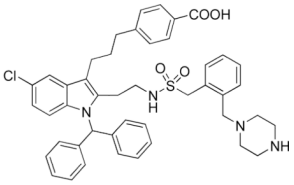
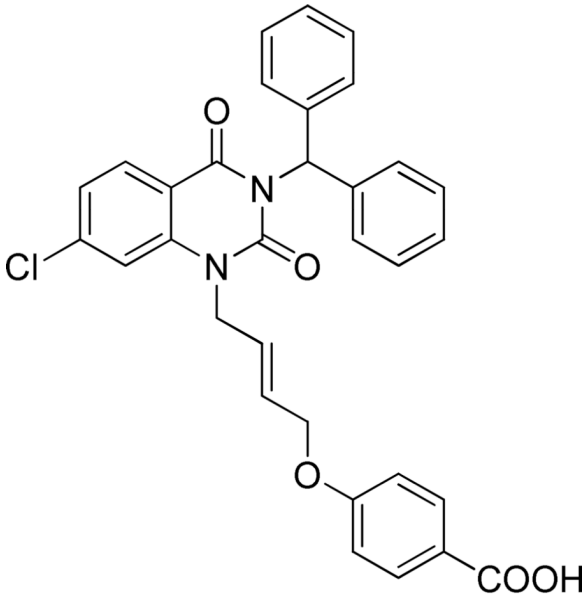


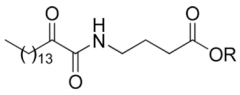
71

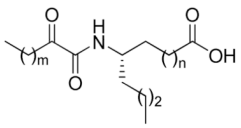
Wyeth has also reported 1,2,4-oxadiazolidin-3,5-diones and 1,3,5-triazin-2,4,6-triones[311] as well as quinazoline-2,4(1H,3H)-dione GIVA PLA_2 inhibitors (for example, compound **72**) with reduced lipophilicity and improved aqueous solubility.[312]



72

3.4.7 2-Oxoamides—In 2002, a novel class of GIVA PLA_2 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 **74a**, m=9, n=1, AX007

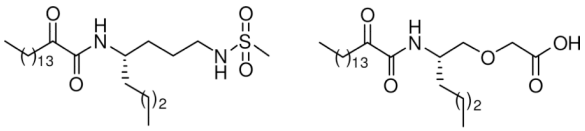
73b, R=OC_2H_5, AX048 **74b**, m=13, n=2, AX109

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 PLA_2, while the corresponding esters may inhibit both GIVA PLA_2 and GVIA PLA_2 (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 affect, being able to block spinal PGE_2 release.[315]

Recently, the location of the 2-oxoamide inhibitor AX007 (**74a**), as well as of pyrrophenone (**56**), in the active site of GIVA PLA_2 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.

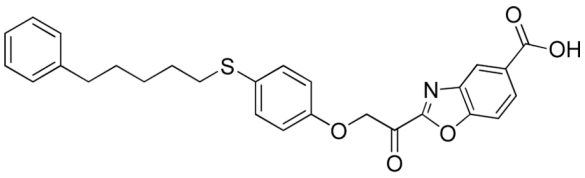
Further studies,[316] revealed that the sulfonamide group constitues a bioisosteric group suitable to replace the carboxyl group in 2-oxoamide inhibitors (for example, compound

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



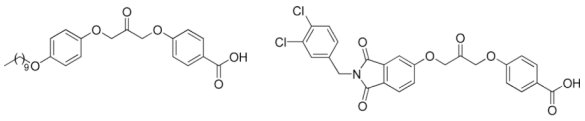
75 **76**

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 PLA_2-mediated arachidonic acid release from HL60 cells.[318]



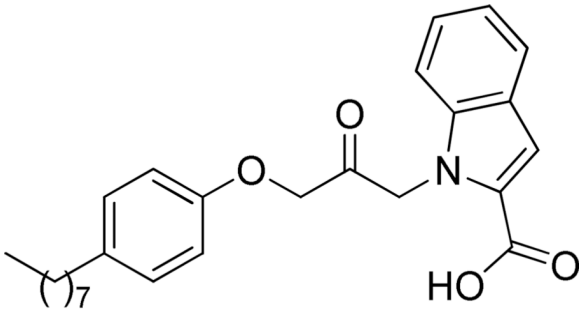
77

In 2002, Conolly et al. presented a series of novel potent inhibitors of GIVA PLA_2 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 μM in a bilayer assay, 0.03 μM in a soluble assay and 2.8 μM 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 μM in a soluble assay and 1.0 μM in a HL60 cell assay).[320]



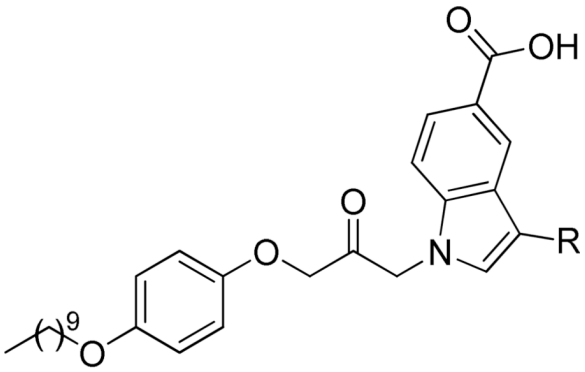
78, AR-C70484XX **79**

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 μM, similar to some of the indole inhibitors Lehr had presented in the past.[300,301a]



80

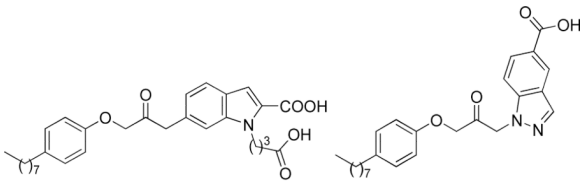
Structure-activity relationship studies on the influence of the position of the carboxylic acid group, the nature of the substituent of the indole ring, the introduction of a second substituent in position 3 of the indole ring and the substitution of the octyl chain by a decyloxy chain led to compounds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



81a, R=H

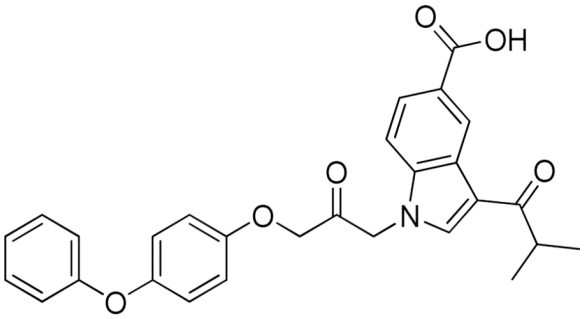
81b, R=$COOCH_3$

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 (IC_{50} 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 $cPLA_2$ and FAAH.



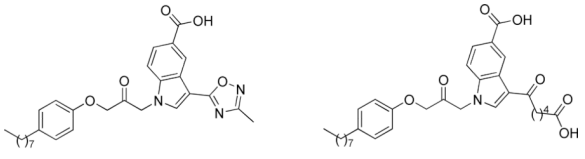
82 83

In an attempt to develop clinically active GIVA PLA_2 inhibitors, a series of structurally related indole-5-carboxylic acids with reduced lipophilicity was synthesized.[326] The $cPLA_2$ inhibition, the thermodynamic solubility and the metabolic stability of the new compounds was evaluated. Compound **84** was the most potent inhibitor (IC_{50} 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 IC_{50} value of 2.1 nM and excellent metabolic stability (81%); however, this compound presented poor aqueous solubility. In addition, compound **86** presented inhibition of $cPLA_2$ with an IC_{50} 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 PLA_2 ($iPLA_2$) from rat brain cytosol and group IB secretory PLA_2 ($sPLA_2$) 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]

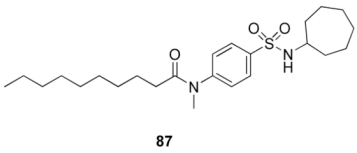


85 86

3.4.9 Other Inhibitors—Annexin V belongs to a family of proteins that interact with phospholipids in a Ca^{2+}-dependent manner. Both recombinant and human placental purified

annexin V inhibited GIVA PLA_2 activity whatever the stimulus used.[328,329] The inhibition of $cPLA_2$ 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 PLA_2 and cytokine release.[331] 9,10-Dihydro-9,10-ethanoanthracene derivatives were also reported to inhibit GIVA PLA_2 without affecting $sPLA_2$ activity.[332] Compound **88** inhibited paw swelling in the carrageenan edema model after i.p. administration with an ED_{50} of 16 mg/kg. A series of pyrimidines, such as compounds **89**, were reported to inhibit the enzyme with IC_{50} values <100 nM.[333]



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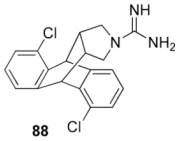

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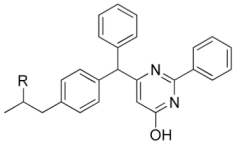
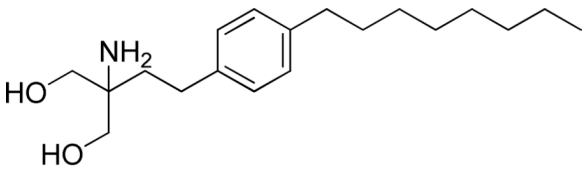


89, R = H or CH_3

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 $cPLA_2$ independently of sphingosine-1-phosphate receptors.[334]



90, FTY720

3.4.10 Summary Status of $cPLA_2$ Inhibitors—A variety of different classes of GIVA $cPLA_2$ 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 $cPLA_2$ *in vitro*, in cells and *in vivo*. Even though this inhibitor does not inhibit $sPLA_2$s, it does inhibit GVIA $iPLA_2$, 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 $cPLA_2$. Shionogi has introduced a series of pyrrolidines that are excellent inhibitors of GIVA $cPLA_2$, 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 $cPLA_2$ inhibitors by Lehr and Genetics Institute, which was later acquired by Wyeth. Ecopladib and later efipladib, WAY-196025 and giripladib are some of these indole inhibitors that have been used in *in vivo* models of inflammation, rheumatoid arthritis, etc. Another important class of

GIVA cPLA$_2$ inhibitors are 2-oxoamides such as AX007 and AX048 that have been shown to bind in the active site of GIVA cPLA$_2$ and to present potent anti-hyperalgesic activity. Finally, another significant and well studied class of GIVA cPLA$_2$ 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 cPLA$_2$ inhibitors to determine the importance of cPLA$_2$ inhibition on inflammatory diseases.

4 Calcium Independent Phospholipase A$_2$ [Group VI iPLA$_2$]

4.1 Groups, Subgroups, Specificity and Mechanism

Group VIA phospholipase A$_2$ is a member of the phospholipase A$_2$ superfamily[13–14] and is characterized by its calcium-independent phospholipase A$_2$ activity. Although the activities of GIVC, GVII, GVIII and lysosomal PLA$_2$s are all independent of calcium, the common name of "calcium independent" PLA$_2$ (iPLA$_2$) applies only to GVI PLA$_2$. The first member of this family, GVIA PLA$_2$, was purified and characterized from macrophages in 1994.[8] To date, the Group VI calcium independent PLA$_2$ (iPLA$_2$) includes six different members: GVIA (iPLA$_2$β; PNPLA9), GVIB (iPLA$_2$γ; PNPLA8), GVIC (iPLA$_2$δ; PNPLA6), GVID (iPLA$_2$ε; PNPLA3), GVIE (iPLA$_2$ζ; PNPLA2), and GVIF (iPLA$_2$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 PLA$_2$ (iPLA$_2$β; PNPLA9)—Calcium-independent PLA$_2$ was so named in order to differentiate it from sPLA$_2$ and cPLA$_2$, whose activities are calcium-dependent. Its gene, located at 22q13.1, expresses GVIA-1 PLA$_2$ 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 Ca^{2+}-independent PLA$_2$ activity referred to a 40-kDa enzyme described as iPLA$_2$[337]. Subsequently, an 85-kDa iPLA$_2$ was purified and well characterized which is now known as GVIA iPLA$_2$ (also iPLA$_2$β)[8]. The same iPLA$_2$ 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 (GXS^{465}XG) located in the catalytic domain (Figure 10).[9] GVIA-1 PLA$_2$ was shown to be active as an oligomer through radiation inactivation studies.[336a] ATP binding protected GVIA-1 PLA$_2$ from cysteine oxidation and prevented loss of activity.[240,336a,340]

The human Group VIA PLA$_2$ gene was found to express multiple splice variants, including GVIA-1, GVIA-2, GVIA-3 PLA$_2$, 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 PLA$_2$ 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 PLA$_2$ activity was reported to be unaffected by ATP, while GVIA-2 PLA$_2$ activity is enhanced by ATP.[337c,341,343] GVIA-2 PLA$_2$ 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 PLA$_2$ by forming hetero-oligomers.[338b]

GVI PLA_2 and GIV PLA_2 both use a serine active site to catalyze the cleavage of the *sn*-2 ester bond, but GVI PLA_2 does not show arachidonic acid specificity in the *sn*-2 position, while GIV PLA_2 does.[240] GVIA PLA_2 also exhibits lysophospholipase activity, transacylase activity and acyl-CoA thioesterase activity.[342,345] It has been suggested that the activity of GVIA PLA_2 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 PLA_2 ($iPLA_2\gamma$; PNPLA8)—The GVIB PLA_2 gene at 7q31 was cloned in 2000 from heart, skeletal muscle,[346] and lymphocyte cDNA[347] and the transcript was detected in heart, placenta, kidney, liver, brain, and skeletal muscle.[346–347] GVIB PLA_2 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 PLA_2.[13] GVIB PLA_2 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 PLA_2 exhibits both PLA_1 and PLA_2 activity and shows low specificity to sn-2 fatty acids for PC hydrolysis.[346,350] Research in GVIB PLA_2-transgenic and GVIB PLA_2-deficient mice show that GVIB PLA_2 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 PLA_2 ($iPLA_2\delta$; PNPLA6)—Group VIC PLA_2 is commonly known as a neuropathy target esterase (NTE), since its discovery in 2002.[352] The human Group VIC PLA_2 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 PLA_2 ($iPLA_2\varepsilon$; PNPLA3)—GVID PLA_2 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 PLA_2 among the GVI PLA_2s.[335] Human GVID PLA_2 has exhibited triacylglycerol hydrolase, transacylase, and calcium-independent PLA_2 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 PLA_2 ($iPLA_2\zeta$; $PNPLA_2$)—$PNPLA_2$ was identified in 2004[358a,362] and was named adipose triglyceride lipase (ATGL), $iPLA_2\zeta$, GVIE PLA_2 and $PNPLA_2$, 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 PLA_2, 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 A_2 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 $PNPLA_2$ 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 PLA_2 ($iPLA_2\eta$; 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 PLA_2 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 PLA_2 activity.[358a]

4.2 Structural Characteristics and Activation Mechanisms

There are currently six forms of the GVI PLA_2. Among them, GVIA is the most well studied and recognized $iPLA_2$. In this section, we will use the long form splice variant GVIA-2 PLA_2 as our main example for discussing the structural characteristics of $iPLA_2$. As described in section 4.1.1, all GVI PLA_2 isoforms contain a patatin-like lipase domain. GVID, E and F PLA_2s are all small enzymes primarily composed of only the patatin-like domain.[335,348] GVIB PLA_2 shows the highest homology to GVIA PLA_2, but does not contain the ankyrin repeats.[335,348] GVIC PLA_2 is a transmembrane protein and also the largest protein in GVI.[352] It shares low similarity with all of the other GVI PLA_2s, except for the patatin-like lipase domain. The 85 kDa human GVIA-2 PLA_2 (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 PLA_2 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 PLA_2 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 PLA_2 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 PLA_2 model to the GIVA PLA_2 crystal structure shows similar folding of the α/β-hydrolase domain to GIVA PLA_2 without the cap region, but the overall sequence homology is low.[202a,344,371] The active site serine of the GVIA PLA_2 lies within a lipase consensus sequence (Gly-X-Ser519-X-Gly) on top of the catalytic domain (Figure 11). Unlike the GIVA PLA_2 active site, which is under the cap region,[202a] the active GVIA PLA_2 site, Ser519, is quite exposed to solvent.[344] This suggests that the cap region contributes to the GIVA PLA_2 AA specificity and that the exposed serine active site results in low substrate specificity. We further found that GVIA PLA_2 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 PLA_2 is also a Ser519/Asp652 catalytic dyad, similar to the dyad in GIVA PLA_2 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 PLA_2 activity and function. We will further discuss GVIA PLA_2 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 PLA_2 has been shown to localize in mitochondria in various cell types.[373] GVIA PLA_2 is the only PLA_2 reported to be regulated by ATP and ADP binding.[240] GVIA PLA_2 can be activated by ATP, but ATP is not a substrate or cofactor for GVIA PLA_2.[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 PLA_2.[240] Research has shown that ATP binding can prevent the dimerization and cysteine oxidation of GVIA PLA_2, enabling it to maintain its activity over time.[340] Because of GVIA PLA_2'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 PLA_2, 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 PLA_2 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 PLA_2s are targets of caspase-3 proteolysis, but only the GVIA enzyme remains active following cleavage.[377] Three forms of the caspase cleaved GVIA PLA_2 have been identified, 70 kDa, 26 kDa and 32 kDa[19a] The preferred cleavage site is $DVTD^{183}$, while $DLFD^{513}$ and $MVVD^{733}$ 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 PLA_2 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 Ca^{2+} 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 Ca^{2+} can respond to intracellular Ca^{2+} through calmodulin. Calmodulin associates with GVIA PLA_2 and was used to purify GVIA PLA_2.[338c] No evidence of Ca^{2+} binding to GVIA PLA_2 has ever been reported, and we have shown membrane penetration by GVIA PLA_2 in the absence of Ca^{2+}.[344] Although GVIA PLA_2 activity is independent of Ca^{2+}, Ca^{2+}-activated calmodulin has been reported to bind to the IQ motif in the tryptic C-terminal 15 kDa region of GVIA PLA_2, inhibiting its activity.[382] The IQ motif is located in residues 694–705 of the catalytic domain of GVIA-1 PLA_2.

4.2.4 Oligomerization—Radiation inactivation studies of murine GVIA PLA_2 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 PLA_2 activity is identified by the truncation mutation.[338b] The splice variant GVI Ankyrin-1 was proposed to be a potential negative regulator of GVIA PLA_2 by blocking oligomerization.[338b] GVIA PLA_2 may also associate with other regulatory proteins to form active complexes.[377] GVIA PLA_2 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 PLA_2 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 PLA_2 has ever been published, and no information is known about the membrane binding surface. Deuterium exchange experiments on GVIA-2 PLA_2 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 PLA_2 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 PLA_2 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 PLA_2 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 PLA_2 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 A_2 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 PLA_2.[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 phylogenic 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 PLA_2 for day to day metabolic functions within the cell. GVIA PLA_2 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 PLA_2 has been involved in both cell growth and cell death in various cell types. On one hand, GVIA PLA_2 promotes cell cycle progression and proliferation by GVIA PLA_2-generated lipid mediators, including free fatty acids, eicosanoids and LPA.[317,384c,d,385,392] On the other hand, GVIA PLA_2 has been shown to be involved in various stimuli-induced apoptosis, including Fas-ligand, thapsigargin, H_2O_2, 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 PLA_2 with diabetes.[394]

Several lines of evidence have shown that GVI PLA_2 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 PLA_2 has also been shown to be activated during ischemia in rabbit myocardium and human coronary artery endothelial cells[396]. Although the GVI PLA_2 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 PLA_2 inhibitor BEL[19e,388].

Several lines of evidence also show the significant role of GVIA PLA_2 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 contraction[400] and Barth syndrome with cardinal disorder characteristics. GVIA PLA_2 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 PLA_2 activity with several diseases including neurodegeneration with brain iron accumulation (NBIA) disorders,[402] infantile neuroaxonal dystrophy,[403] and memory loss.[404] GVIA PLA_2 also plays an important role in Wallerian degeneration and axon regeneration in nerve injury in mouse models.[165,405]

4.3.2 Diabetes—GVIA PLA_2 has been related to diabetes due to its participation in β-cell apoptosis, superoxide production and glucose-induced RhoA/Rho kinase activation. GVIA PLA_2 genetically-modified mice and various cellular studies suggest that GVIA PLA_2 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 PLA_2 enhanced superoxide generation in neutrophils and siRNA knockdown inhibited superoxide generation.[407] GVIA PLA_2-deficient cell and inhibitor studies have found that GVIA PLA_2 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 PLA_2 localizes in mitochondria, maintains the integrity of the mitochondrial membrane and prevents the rupture of mitochondria and the release of cytochorme c under oxidative stress.[373] Recent research suggests that GVIA PLA_2 participates in cardiolipin remodeling in cardiomyocytes.[390a] The other isoform, GVIB PLA_2, shows alterations to hippocampal cardiolipin content in transgenic and deficient mice[412] and a role in cardiac phospholipid homeostasis and mitochondrial function.[349,351] This enzyme may be responsible for cardiolipin remodeling in mitochondria.[351] GVIA PLA_2 is also responsible for cardiolipin deacylation and monolyso cardiolipin accumulation in Barth syndrome.[390b] Genetic inactivation of GVIA PLA_2 can suppress the phenotype of tafazzin knockout drosophila.[390b] GVIA PLA_2 inhibition is a possible treatment for Barth syndrome patients.

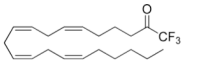
4.3.4 NBIA/Neuroaxonal Dystrophy—Recent clinical studies have shown that GVIA PLA_2 mutations are associated with “neurodegeneration with brain iron accumulation” (NBIA) disorders and infantile neuroaxonal dystrophy.[389b,402,413] Neither the active site, Ser-519, nor the potential active site, Asp-652, was found to be mutated in the more than 40 mutation sites occurred in patients. These GVIA PLA_2 mutants may retain different level of activities, except for some truncation mutants. Double mutations of GVIA PLA_2 tend to

correlate to earlier age of disease onset, suggesting that GVIA PLA_2 activity is critical in development.[389b,402]

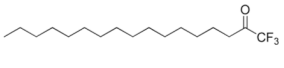
4.4 Chemical Inhibitors and Therapeutic Intervention

In comparison to the secreted and cytosolic Ca^{2+}-dependent PLA_2 enzymes, the research on inhibitors of GVI PLA_2 is much more limited. However, a summary of GVIA PLA_2 inhibitors is included in two review articles.[22c,22f]

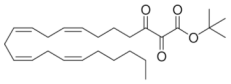
4.4.1 Fatty Acid Trifluoromethyl Ketones and Tricarbonyls—In 1995, the inhibition of macrophage Ca^{2+}-independent PLA_2 by $AACOCF_3$ (**43**) and palmitoyl trifluoromethyl ketone (**44**) were reported.[414] In contrast to the case with GIVA PLA_2, for GVIA PLA_2 the saturated derivative was found to be 4-fold more potent than $AACOCF_3$ (Table 22). Fatty acyl tricarbonyls **47** and **91** also inhibited GVIA PLA_2 but appeared to be much poorer inhibitors than the corresponding trifluoromethyl ketones.[279]



43, $AACOCF_3$



44, $PACOCF_3$



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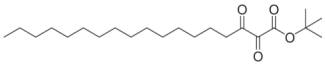

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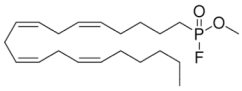
4.4.2. Methyl fluorophosphonates—MAFP (**48**) was found to irreversibly inhibit GVIA PLA_2.[415] In addition, the saturated fluorophosphonates **92** and **93** inhibited GVIA PLA_2 showing similar potencies and considerably higher potency than that of MAFP.



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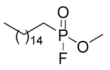

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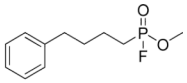
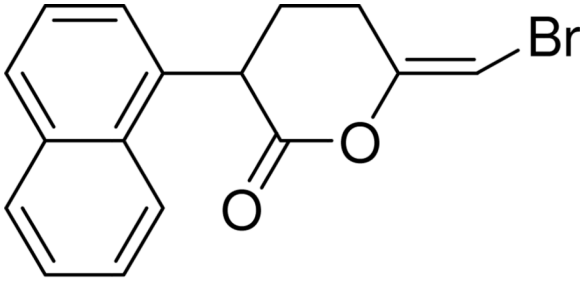


93

4.4.3. Bromoenol lactone—



94, BEL

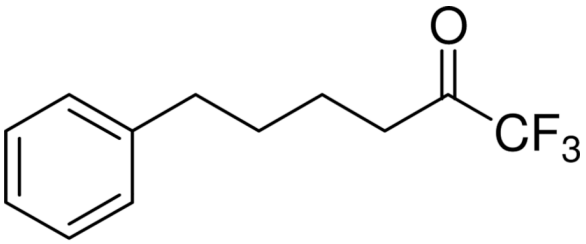
Bromoenol lactone (**94**, BEL) is an irreversible, covalent inhibitor of GVIA PLA_2,[414,376b] inhibiting the enzyme at concentrations far lower than those required to inhibit $sPLA_2$ or $cPLA_2$ family members. As a result, BEL is commonly used to selectively inhibit GVIA PLA_2 in cellular systems. However, although BEL can distinguish GVIA PLA_2 among other PLA_2s, 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 $iPLA_2$ 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 PLA_2.[418] Using BEL it has been suggested that PGE_2 generation upon stimulation may be partially mediated by $iPLA_2$ in addition to $sPLA_2$.[419] In a number of studies, Turk et al. showed that GVIA PLA_2 ($iPLA_2\beta$) 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 PGE_2 production in 3T6 fibroplast 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 $iPLA_2\beta$ on chemotherapeutic-induced death and phospholipid profiles in renal cells was studied.[393c] Inhibition of $iPLA_2$ 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 $iPLA_2$ in prostate cancer cell signaling. Most recently, it was shown that $iPLA_2$ inhibition by BEL activated p38 MAPK signaling pathways during cytostasis in prostate cancer cells.[423]

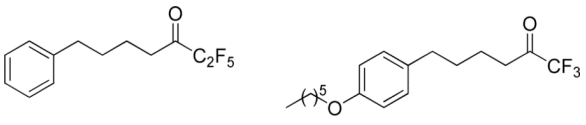
Interestingly, in a number of studies it has been demonstrated that (*R*)- and (*S*)-enantiomers of BEL present different inhibitory properties.[424] GVIA PLA_2 ($iPLA_2\beta$) and GVIB PLA_2 ($iPLA_2\gamma$) have been found to be selectively inhibited by (*S*)- and (*R*)-enantiomers of BEL, respectively.[424a,b] Turk et al. have reported that BEL inactivates GVIA PLA_2 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 PLA_2 in a mixed-micelle assay.[269] Trifluoromethyl ketone **95** was found to be a potent inhibitor of GVIA PLA_2 (Table 22), 10-fold more potent in comparison to GIVA PLA_2. In 2008, a series of polyfluoro ketones for the selective inhibition of GVIA PLA_2 was synthesized.[405b] Such a task is very challenging, because both the intracellular GVIA PLA_2 and GIVA PLA_2 share the same catalytic mechanism and cross-reactivity is expected for inhibitors targeting the active site serine.



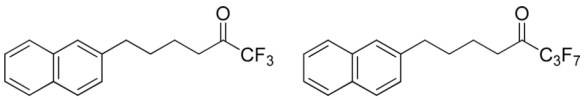
95

It was found that the pentafluoroethyl ketone functionality favored selective inhibition of GVIA PLA_2. FKGK11 (**96**) was found to be a selective GVIA PLA_2 inhibitor, while the trifluoromethyl ketone FKGK2 (**97**) can be considered as a pan-inhibitor inhibiting GVIA PLA_2, GIVA PLA_2, and even GV $sPLA_2$ (Table 23).



96 **97**

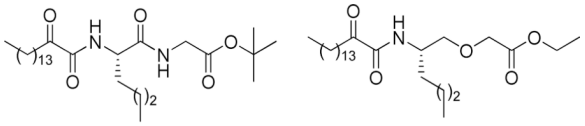
In a continuation of SAR studies on polyfluoro ketones and using an improved assay for GVIA PLA_2, compound FKGK18 (**98**) was identified as the most potent GVIA PLA_2 inhibitor yet reported (Table 24).[405c] Being 195 and >455 times more potent for GVIA PLA_2 than for GIVA PLA_2 and GV $sPLA_2$, respectively, makes it a valuable tool to explore the role of GVIA PLA_2 in cells and in vivo models. Heptafluoro derivative **99** also presented interesting inhibition of GVIA PLA_2, inhibiting GIVA PLA_2 and GV $sPLA_2$ at least 90 times less potently (Table 24).



98 **99**

Selective PLA_2 inhibitors contributed to the clarification of the role of each PLA_2 class in neurological disorders.[405a,426] Using the selective GVIA PLA_2 inhibitor FKGK11, the selective GIVA PLA_2 AX059 and the pan-inhibitor FKGK2, the role of the various classes of PLA_2 in EAE, the animal model of multiple sclerosis, was studied.[426] The results suggested that GIVA PLA_2 plays a role in the onset of the disease, while GVIA PLA_2 plays a key-role both on the onset and the progression of the disease. Thus, it seems that GVIA PLA_2 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 PLA_2 inhibitors, 2-oxoamides based on esters of amino acids may inhibit both GVIA PLA_2 and GIVA PLA_2. Recently, two 2-oxoamides based on esters of dipeptides or pseudodipeptides (**100** and **101**) were reported to preferentially inhibit GVIA PLA_2 (Table 25).[317]



100 101

4.4.6 Summary Status of iPLA$_2$ Inhibitors—Research on GVIA iPLA$_2$ 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 iPLA$_2$ plays a significant role in a number of medical conditions. As with GIVA cPLA$_2$, the first series of potent reversible GVIA iPLA$_2$ inhibitors were trifluoromethyl ketones of fatty acids. Bromoenol lactone (BEL) is the most important irreversible inhibitor of GVIA iPLA$_2$ and it has been used in a number of *in vitro*, *ex vivo* and *in vivo* studies to elucidate the role of GVIA iPLA$_2$. One should note that even though BEL is selective against GVIA iPLA$_2$ when compared to the other PLA$_2$ 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 iPLA$_2$ are polyfluoroketones bearing an aromatic ring and a small aliphatic chain as a spacer between the two funcional groups. The most potent and selective polyfluoroketone inhibitor, namely FKGK11, has been used to show the important role that GVIA iPLA$_2$ plays in EAE, the animal model of multiple sclerosis. Most recently, it has been reported that a combination therapy of GVIA iPLA$_2$ 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 iPLA$_2$ emerges and efforts to acquire its crystal structure advance, it seems essential to persist in developing potent and selective GVIA iPLA$_2$ inhibitors.

5. PAF Acetylhydrolases (GVII and GVIII PAF-AH PLA$_2$s)

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 PLA$_2$s, 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 PLA$_2$ 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 plasma[429]. Therefore, the enzyme is also known as plasma PAF-AH (pPAF-AH) or lipoprotein-associated PLA$_2$ (Lp-PLA$_2$). GVIIB PLA$_2$, also referred to as PAF-AH II, is an intracellular protein with a molecular weight of 40 kDa that has an N-terminal myristoylation site and which shares 41% amino acid sequence identity with GVIIA PLA$_2$.[430] GVIII PLA$_2$ is a brain intracellular heterotrimeric protein complex that is also referred to as PAF-AH Ib.[431] GVIII PLA$_2$ 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 PLA$_2$, 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 PLA$_2$s.

PAF acetylhydrolases are calcium independent PLA$_2$s. These enzymes all feature the same catalytic triad, Ser/His/Asp.[20a] The secreted GVIIA Lp-PLA$_2$ shows broad substrate specificity. In addition to PAF, GVIIA Lp-PLA$_2$ can also hydrolyze phosphatidylcholines with short chain *sn-2* residues.[433] Lp-PLA$_2$ 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-PLA_2. Several studies indicate that Lp-PLA_2 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-PLA_2 inhibitor on the PC compositions of human LDL has shown that oxidized PCs are recognized as substrates by Lp-PLA_2 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-PLA_2 and that truncated oxidized PCs are major substrates.[434b] In a recent study, both Lp-PLA_2 and the intracellular GVIIB PAF-AH II have been shown to have the ability to release F_2-isoprostanes from esterified phospholipids, though at a much slower rate than they hydrolyze PAF or POVPC.[435] Interestingly, both GVII PLA_2s show about a 200- to 1000-fold higher affinity for esterified F_2-isoprostanes than for PAF and POVPC.[435] GVIIB PAF-AH II shows very similar substrate specificity to the plasma form of Lp-PLA_2.[436] Both forms of GVII PLA_2 display PLA_1 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 PLA_2s, GVIII PLA_2 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 PLA_2 (Lp-PLA_2)—Lp-PLA_2 is a 45 kDa, Ca^{2+}-independent PLA_2 that contains a *GXSXG* motif, which is a characteristic fingerprint for neutral lipases and serine esterases.[429] The first 20 residues comprise a hydrophobic signal peptide[12]. 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-PLA_2 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-PLA_2 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-PLA_2/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-PLA_2.[441] In fact, in humans who are apoB-100 deficient, Lp-PLA_2 was found to be entirely associated with HDL[442]. A recent study has identified a domain (residues 367–370) that mediates Lp-PLA_2/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-PLA_2 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-PLA_2 would bind to its substrate(s) from the lipid membrane phase, as is the case for all classic membrane-associating PLA_2 enzymes. A recent study by Pande[445] has shown that the lipid composition of membrane vesicles affects Lp-PLA_2 activity and that membrane binding of Lp-PLA_2 increases enzyme activity, which suggests that Lp-PLA_2 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-PLA_2 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-PLA_2 in LDL/HDL lipoproteins have been suggested to affect Lp-PLA_2 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-PLA_2 under the circumstance that excess enzyme is present.[449] This suggests that the Lp-PLA_2 interaction with HDL could be different for the same enzyme interacting with LDL and the enzyme associated with HDL may form a catalyticly unactive conformation. In order to access the substrate from the lipid membrane, Lp-PLA_2 would have to either undergo a conformational change or dissociate from the HDL.

Additionally, Lp-PLA_2 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-PLA_2 is mainly associated with the LDL(−) subfraction.[451] Lipoprotein(a) [Lp(a)] also accounts for Lp-PLA_2 activity to a small extent.[20d] In addition to lipoproteins, Lp-PLA_2 may also associate with microparticles in human plasma.[452] Furthermore, different lipoprotein carriers of Lp-PLA_2 may result in different roles of the enzyme in atherosclerosis.[447]

5.2.2 GVIII PLA_2 (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 GTPases[431]. 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-PLA$_2$ in Atherosclerosis—Lp-PLA$_2$ 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-PLA$_2$ is considered a very important enzyme for atherosclerotic progression. However, since its discovery, the role of Lp-PLA$_2$ 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-PLA$_2$ could act as a potent anti-atherogenic enzyme. However, growing evidence has shown that Lp-PLA$_2$ may also play a pro-atherosclerotic role. This is because Lp-PLA$_2$ 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-PLA$_2$.

Lp-PLA$_2$ was initially considered to be an anti-inflammatory enzyme. Recombinant Lp-PLA$_2$ 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 activity[457] and Lp-PLA$_2$ deficiency is shown to be an independent risk factor for cardiovascular disease and stroke[458]. 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-PLA$_2$. Lp-PLA$_2$-treated mildly oxidized low density lipoprotein (MM-LDL) lost the ability to induce endothelial cells to bind to monocytes.[459] Lp-PLA$_2$ 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-PLA$_2$ and modulation of Lp-PLA$_2$ activity could lead to anti-atherogenic effects in the vessel wall.[455] Transgenic apoE deficient mice expressing human Lp-PLA$_2$ have shown increased Lp-PLA$_2$ 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-PLA$_2$ inhibits injury-induced neointima formation and reduces spontaneous atherosclerosis in the absence of mechanical injury.[461] In nonhyperlipidemic rabbits the expression of Lp-PLA$_2$ reduces oxLDL accumulation in arteries and exerts anti-inflammatory, antithrombotic, and antiproliferative effects.[462] All together, the potential anti-atherosclerotic role of Lp-PLA$_2$ may be attributed to hydrolysis of the oxidized phospholipids and therefore a reduction in the accumulation of oxidized lipoproteins. However, Lp-PLA$_2$ 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-PLA_2 is associated with HDL and only 5% with LDL.[463] In fact, humans are the only mammals where Lp-PLA_2 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-PLA_2 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-PLA_2 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-PLA_2 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-PLA_2 activity is associated with increased levels of lyso-PC, oxidized LDL and inflammation.[463] Thus increased Lp-PLA_2 levels further accelerate atherosclerosis in the hypercholesterolemic minipig model.[463] A recent study has shown that the ratio of Lp-PLA_2 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-PLA_2 activity, the risk for CVD is increased.[456] More evidence suggesting a pro-atherogenic role for Lp-PLA_2 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-PLA_2 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-PLA_2 and hence reduce the development of advance coronary atherosclerosis in diabetic and hypercholesterolemic swine.[469] Thus Lp-PLA_2 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-PLA_2.[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-PLA_2 and Neonatal Necrotizing Enterocolitis—Several studies have indicated that Lp-PLA_2 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-PLA_2 levels.[477] A neonatal rat model treated with human recombinant Lp-PLA_2 shows a reduced incidence of NEC compared with controls.[478] Enteric Lp-PLA_2 administration resulted in significant intestinal Lp-PLA_2 activity but no serum Lp-PLA_2 activity.[478] Lp-PLA_2 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-PLA_2 may play a protective role in the development of NEC and exogenous Lp-PLA_2 supplementation may help to reduce the incidence of NEC in premature infants.

In addtion to atherosclerosis and neonatal necrotizing enterocolitis, Lp-PLA_2 may also be related to severe anaphylaxis. The PAF hydrolysis activity of Lp-PLA_2 was found to be significantly lower in patients with fatal anaphylactic reaction to peanuts than control groups and Lp-PLA_2 enzymatic activity was inversely correlated with the severity of the anaphylactic response.[480]

5.3.3 GVIIB PLA_2 (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 F_2-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 F_2-isoprostane esterified phospholipids *in vitro*.[435] Therefore, the accumulation of F_2-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 $F_{2\alpha}$ 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 PLA_2s *in vivo*.

5.3.4 GVIII PLA_2 (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 α_1 and α_2, LIS1 interacts with a number of other proteins, such as cytoplasmic dynein, tubulin and NudE. Both PAF-AH Ib catalytic subunits α_1 and α_2 and LIS1 are expressed at high levels in the brain and testis.[485] Haplo 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 α_1, α_2 knockout mice or the α_1/α_2 double knockout mice exhibit brain abnormalities.[485,487] α_1 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.

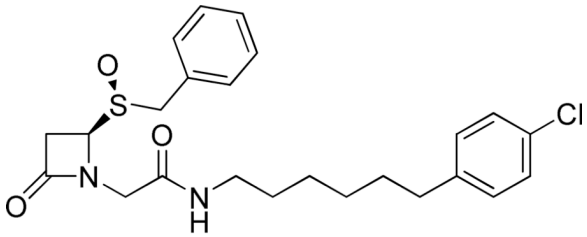
In addition to interacting with LIS1, the PAF-AH Ib catalytic αsubunits are shown to interact with the Reelin very low density lipoprotein receptor (VLDLR), binding to the C terminal of VLDLR, but not with the apolipoprotein E receptor 2.[488] The binding of α subunits to VLDLR is very specific and requires 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-tublin.[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 membranes[489]. Both PLA_2 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-PLA_2 has been reported, one of them, darapladib, has reached the most advanced step of clinical trials (Phase III). The recent developments on Lp-PLA_2 inhibitors are summarized in a number of review articles.[22e–i]

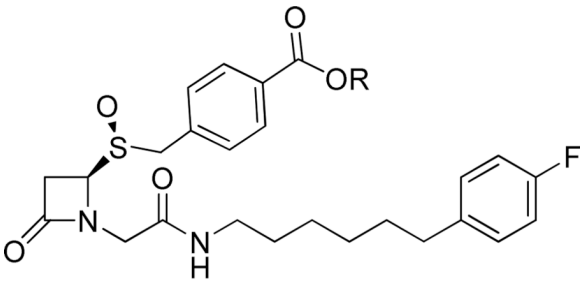
5.4.1. Azetidinones—In 1998, SmithKline Beecham presented a novel series of monocyclic β-lactams (or azetidinones) as inhibitors of Lp-PLA_2.[490] Even though these compounds presented only modest inhibition, they gave way to the further investigation on Lp-PLA_2 inhibitors and to the first potent azetidinone inhibitor of Lp-PLA_2, 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 μM.[491]



102, SB-222657

Another β-lactam, SB-245713 (**103a**), also presented inhibitory potency with an IC_{50} 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 hereditable hyperlipidaemic rabbits (WHHL rabbits).[468,492]

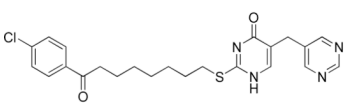
Histological analysis of aortic segments showed a decrease in both lesion cross-sectional area and thickness, particularly in segments with the most complex, raised plaque. These results supported the theory that Lp-PLA_2 plays a significant role in the development of atherosclerotic plaque and that Lp-PLA_2 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.



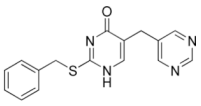
103a, R=H, SB-245713

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-PLA_2. For example, compounds **104** and **105** inhibited the enzyme with IC_{50} values of 54 nM and 1.1 μM, respectively.[493]



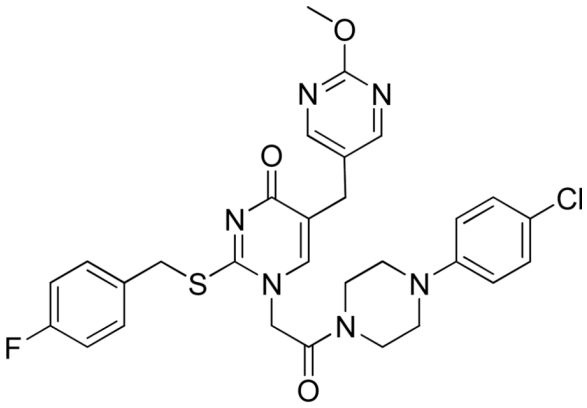
104



105

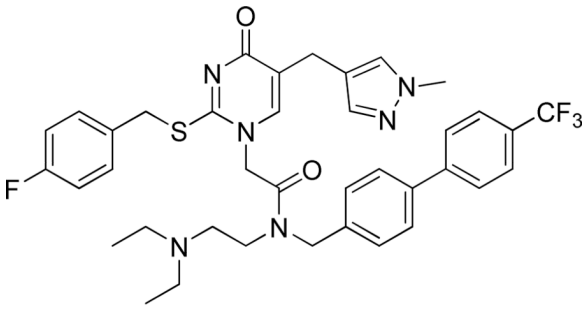
In a series of communications, various modifications were undertaken in those first pyrimidone structures in order to find the most potent inhibitor that would at the same time present excellent in vivo activity and oral bioavailability.[494] Based on this series, novel 1-((amidolinked)-alkyl)-pyrimidones were designed as nanomolar inhibitors of human Lp-PLA_2. 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 IC_{50} 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-(biphenylmethylamidoalkyl)-pyrimidones that were highly potent inhibitors of Lp-PLA_2 and showed excellent activity in the WHHL rabbit.[494d]



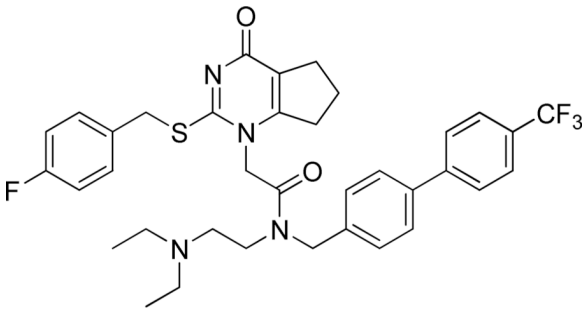
107

Based on this series, they discovered a more potent, orally active inhibitor of Lp-PLA_2, SB-435495 (**108**) with an IC_{50} 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 IC_{50} 0.25 nM against rhLp-PLA_2, showed prolonged inhibition of plasma Lp-PLA_2 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-PLA_2. Furthermore, the presence of SB-480848 during the copper catalyzed oxidation of human LDL prevented the production of lysoPC (IC_{50} 4 ± 3 nM). Additional in vivo studies showed that SB-480848 had good oral bioavailability, but also presented excellent inhibition of Lp-PLA_2 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-PLA_2 and risk of stable coronary artery disease (CAD) and thus further support to the hypothesis that Lp-PLA_2 may be considered as a novel risk marker for CAD.[464f] The effect of darapladib on plasma Lp-PLA_2 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-PLA_2 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-PLA_2 inhibition with darapladib prevented necrotic core expansion, a key determinant of plaque vulnerability. In another study, selective inhibition of Lp-PLA_2 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-PLA_2 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 A_2 [Group XV $LPLA_2$]

6.1 Groups, Subgroups, Specificity and Mechanism

GXV PLA_2 was first identified in the soluble fraction of Madin-Darby canine kidney (MDCK) cells[501] and was subsequently purified from bovine brain.[502] The genes encoding

this enzyme were also identified in mouse, rat, cow and human (Table 28).[503] GXV $LPLA_2$ has high protein sequence identity to a human lecithin: cholesterol acyltransferase-like lysophospholipase (LLPL).[504] However GXV $LPLA_2$s do not show significant lecithin cholesterol acyltransferase or lysophospholipase activity under acidic or neutral conditions.[504] Instead, the protein possesses Ca^{2+} independent PLA_2 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 (PLA_2 activity).[504–505] Thus, ACS activity may be related to PLA_2 activity. GXV PLA_2 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 PLA_2 ($LPLA_2$). In addition, $LPLA_2$ also shows PLA_1 activity.

GXV $LPLA_2$ is a water-soluble glycoprotein with a molecular mass of 45 kDa. GXV $LPLA_2$ has a signal sequence cleavage site and several N-linked glycosylation sites.[504] Although the enzymatic activity of GXV $LPLA_2$ does not require divalent cations, such as Ca^{2+} and Mg^{2+}, milimolar Ca^{2+} or Mg^{2+} does enhance the activity [502]. $LPLA_2$ has shown specificity toward phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and also has positional specificity. $LPLA_2$ is able to hydrolyze the acyl chain at both the *sn*-1 and *sn*-2 positions of PC or PE. $LPLA_2$ 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. $LPLA_2$ 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 $LPLA_2$. In addition, $LPLA_2$ 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-steraroyl-*sn*-glycero-3-phosphocholine (OSPC) and 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (POPC) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (OPPC) as substrates, $LPLA_2$ favors the oleoyl group at the *sn*-2 position of SOPC and POPC or the *sn*-1 position of OSPC and OPPC.[506]

$LPLA_2$ contains a *GXSXG* motif, which is a characteristic fingerprint of neutral lipases and serine esterases. $LPLA_2$ 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 $LPLA_2$ and lecithin cholesterol acyltransferase (LCAT)[507]. A site-directed mutagensis 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 $LPLA_2$ 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 $LPLA_2$ 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]

$LPLA_2$ 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 $LPLA_2$ and the negatively charged lipid, and the interaction could promote the association of $LPLA_2$ with lipid vesicles. $LPLA_2$ operates using the same interfacial mechanism as the classic membrane-associating PLA_2s where substrate exclusively originates from the lipid vesicle. Adding NaCl or increasing the pH can

markedly reduce the increase in $LPLA_2$ activity due to negatively charged lipids.[505] The increased Na^+ concentration could destroy the electrostatic interaction between $LPLA_2$ and the lipid-water interfacial surface.

6.2 Biological Functions and Disease Implications

$LPLA_2$ plays an important role in lysosomal phospholipid degradation. $LPLA_2$ knockout mice ($LPLA_2^{-/-}$) 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 $LPLA_2$ protein from HEK293 cells was added to $LPLA_2^{-/-}$ mice alveolar macrophages. The uptake of exogenous $LPLA_2$ significantly decreased phospholipid accumulation.[509] In contrast, the catalytically inactive $LPLA_2$-protein treated $LPLA_2^{-/-}$ mice alveolar macrophages do not show a decrease in phospholipid accumulation, which suggests that $LPLA_2$ enzymatic activity is responsible for the reduction in phospholipid.[509]

$LPLA_2$ may be involved in surfactant phospholipid catabolism in alveolar macrophages. $LPLA_2$ 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 $LPLA_2$ activity than wild type mice.[503] One year old $LPLA_2^{-/-}$ mice showed marked splenomegaly, foam cell formation, and increased lung surfactant phospholipid levels.[508] Thus, $LPLA_2$ may be a major enzyme that is responsible for pulmonary surfactant phospholipid degradation.

Several lines of evidence suggest that $LPLA_2$ is involved in phospholipidosis. In addition to the accumulation of phospholipid, $LPLA_2$ knockout mice show the formation of foam cells with lamellar inclusion bodies, which is a hallmark of cellular phospholipidosis.[508] $LPLA_2$ 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 $LPLA_2$-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 $LPLA_2$ activity.

In apolipoprotein E (apoE) knockout mice, $LPLA_2$ was found in foam cells in severe atherosclerotic lesions.[511] $LPLA_2$ 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 $LPLA_2$ may play an important role in atherogenesis.

7. Adipose-Specific Phospholipase A_2 [GXVI AdPLA]

7.1 Groups, Subgroups, Specificity and Mechanism

Adipose-specific phospholipase A_2 (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 A_2 activity[15a,513–514] and was designated as GXVI PLA_2.[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 A_1 and A_2 activities toward PC and PE, but lacks acyltransferase activity.[15a,514] Its PLA_1 activity is higher than its PLA_2 activity.[514] The 18kDa AdPLA is distinct from $iPLA_2$ and $cPLA_2$ in size. The *GXSXG* or *GXSGS* motif in the active sites of $iPLA_2$ and $cPLA_2$ 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 $sPLA_2$, AdPLA does not contain the highly disulfide bonded features or calcium dependency of $sPLA_2$s. Note that other members of the LRAT family, such as the calcium-independent N-acyltransferase (iNAT), also exhibit phospholipase A_1/A_2 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 A_2 Activities

The phospholipase A_2 superfamily had earlier been categorized into three major types, $sPLA_2$, $cPLA_2$ and $iPLA_2$.[13–14] The diversity and growing number of enzymes, whose activity is independent of Ca^{2+} in addition to GVI $iPLA_2$, including the GVII and GVIII PAF-AHs and GXV lysosomal PLA_2, have complicated the classification and there are still some enzymes that display PLA_2 functionality or demonstrate homologous sequences to current PLA_2 enzymes that are not included in the naming system. We will discuss particularly the Otoconin-90-containing homology domain to $sPLA_2$, the phospholipase B1 containing homology domain to $cPLA_2$, the patatin domain containing proteins containing a homology domain to $iPLA_2$ and peridoxin-6, which is also an acidic lysosomal phospholipase (Table 29).

8.1 Otoconin-90 ($sPLA_2$ homology)

$sPLA_2$ 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 PLA_2 domain. Otoconin-90/95 (OC90), also named as PLA_2-like (PLA_2L), contains a similar domain to Groups I, II, V, and X $sPLA_2$s. The human otoconin-90 was identified with 493 amino acids.[516] The partial murine otoconin-90 containing 453 residues and two $sPLA_2$ 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, PLA_2 activity has not yet been demonstrated in this protein and it has not been categorized as a PLA_2.

8.2 Phospholipase B (cPLA$_2$ homolog)

$cPLA_2$ was initially characterized by the cytosolic property and calcium dependent activity. The current members of the $cPLA_2$ type contain a C2 domain, except for the GIVC PLA_2, which has high homology in the catalytic domain.[14] Phospholipase B (PLB) was mentioned in association with the PLA_2 family in our previous review.[13] Because its PLA_2 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 PLA_2 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 $cPLA_2$ 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 (iPLA$_2$ homolog)

$iPLA_2$ is currently defined as a calcium independent PLA_2. However, there are more and more groups of PLA_2 having calcium independent activity. Now, $iPLA_2$ is referred to as the GVI PLA_2. In GVI PLA_2, 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 PLA_2s are included in 6 of them.[14,335a] The PLA_2 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 PLA_1 and PLA_2 activity.[525] Patatins have the lipase consensus sequence (Gly-Thr-Ser-Thr-Gly) as does the GVIA PLA_2. Its crystal structure was determined and showed the same catalytic dyad as $cPLA_2$.[371] The sequence alignment and structure modeling showed that patatin has high homology to $iPLA_2$.[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 neucleotide 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 aiPLA$_2$ (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 activity[528] and phospholipase A_2 activity.[529] It was shown to be a lysosomal protein and has an optimal Ca^{2+}-independent PLA_2 activity at pH 4.0,[529–530] and also named as acidic

calcium-independent PLA_2 (aiPLA$_2$) in 1997. It is structurally and functionally different from the GXV LPLA$_2$. 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 A_2 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 PLA$_2$s, 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 PLA_2 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 PLA$_2$s due to the isolation, characterization, cloning, and general availability of the pure human recombinant enzymes. With time the specialized role of each type of PLA_2 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 PLA$_2$s 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 sPLA$_2$, the oldest type of PLA_2 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 sPLA$_2$ inhibitors, much effort has been devoted to the discovery of inhibitors for cPLA$_2$. It has to be noted that for both sPLA$_2$ and cPLA$_2$ many structurally different classes of synthetic inhibitors have been reported. However, although cPLA$_2$ 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. Unfornately, this trial (http://clinicaltrials.gov/, Identifier: NCT00396955) was terminated because of an "imbalance of gastrointestinal and lipase effects".

Although Lp-PLA$_2$ is the most recently recognized enzyme among the major PLA_2 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). iPLA$_2$ has received little attention up to now as a therapeutic target. However, recent studies on animal models have demonstrated the importance of this PLA_2 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 PLA_2 inhibitors will become useful in clinical practice for cardiovascular diseases. In addition, we anticipate that the continuing research efforts on

cPLA$_2$ and iPLA$_2$ 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 PLA$_2$ groups is of paramount importance in the effort to develop PLA$_2$ inhibitors as phamaceutical agents. Selective inhibitors for the major groups that have been especially useful for *in vitro* mechanistic studies have been reported, for example pyrrophenone for cPLA$_2$, varespladib for sPLA$_2$, pentafluoroketone FKGK11 for iPLA$_2$. However, inhibitors able to selectively inhibit the various sPLA$_2$ groups and subgroups (IIA, V, X) and the various cPLA$_2$ and iPLA$_2$ subgroups are still needed for use in animal and human studies.

Of course, the availability of the pure, cloned human PLA$_2$s 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 A$_2$ superfamily!

Acknowledgments

We would like to thank the National Institutes of Health RO1-GM20501(EAD) for support of our work on phospholipase A$_2$ 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 A_2 as well as on signal transduction, inflammation, lipid metabolism, eicosanoid action and lipidomics.

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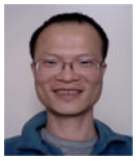


Yuan-Hao Hsu received his BS from National Chung-Shin University in 1994 and his MS from National Taiwan University in 1996. He moved across the Pacific Ocean to the University of California, Riverside and received an MS in 2001 and a PhD in 2006, both in Biochemistry, with Dr. Jolinda A. Traugh. He 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 A_2 ($cPLA_2$) and calcium independent phospholipase A_2 ($iPLA_2$).

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 A_2s. 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.

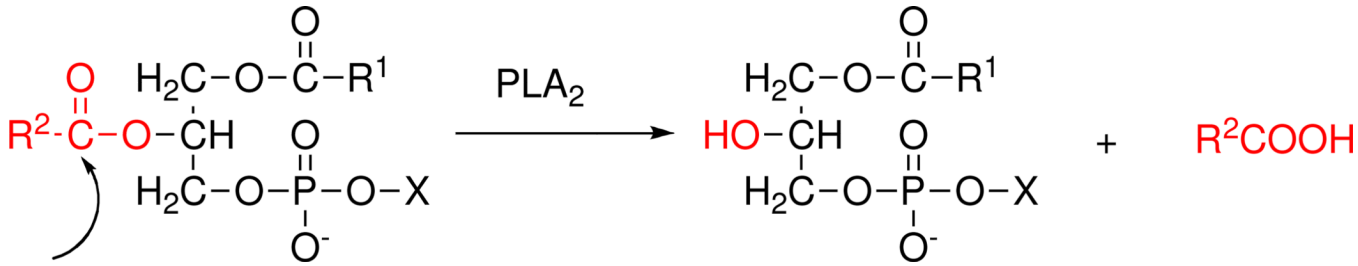
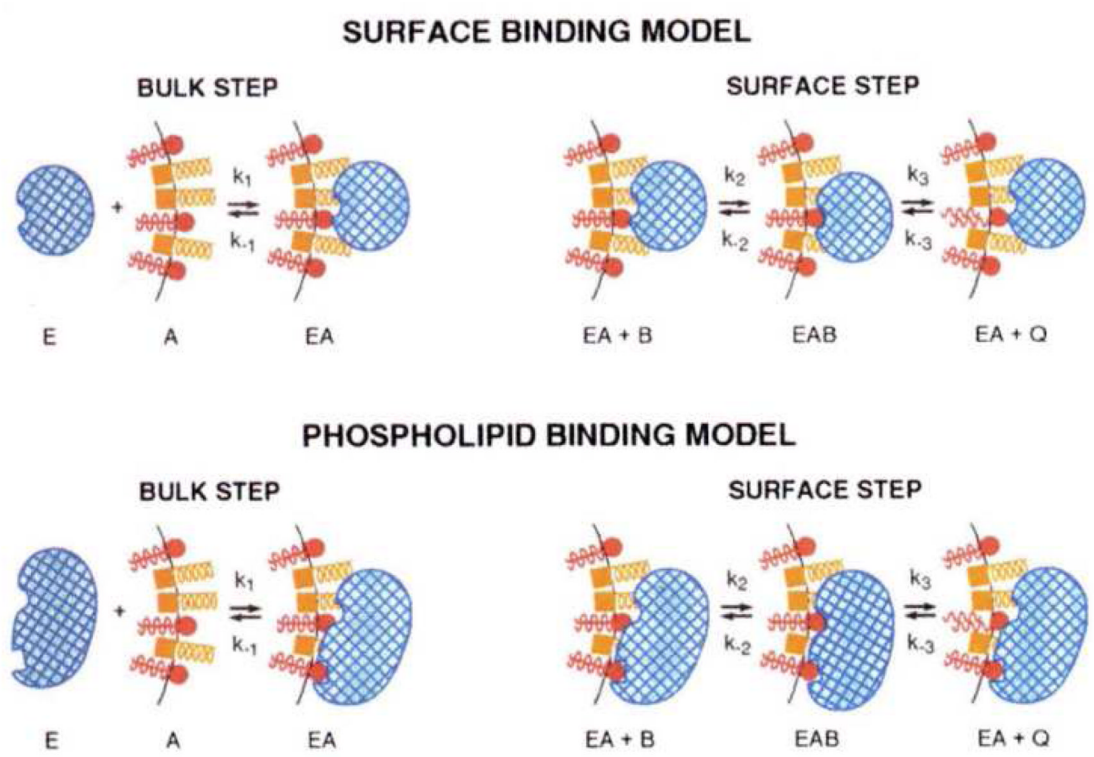


Figure 1.
The specific reaction catalyzed by phospholipase A2 at the sn-2 position of the glycerol backbone is shown. X, any of a number of polar headgroups; R1, fatty acids, or alkyl, or alkenyl groups and R2, fatty acids or acyl moieties.



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 A_2-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]

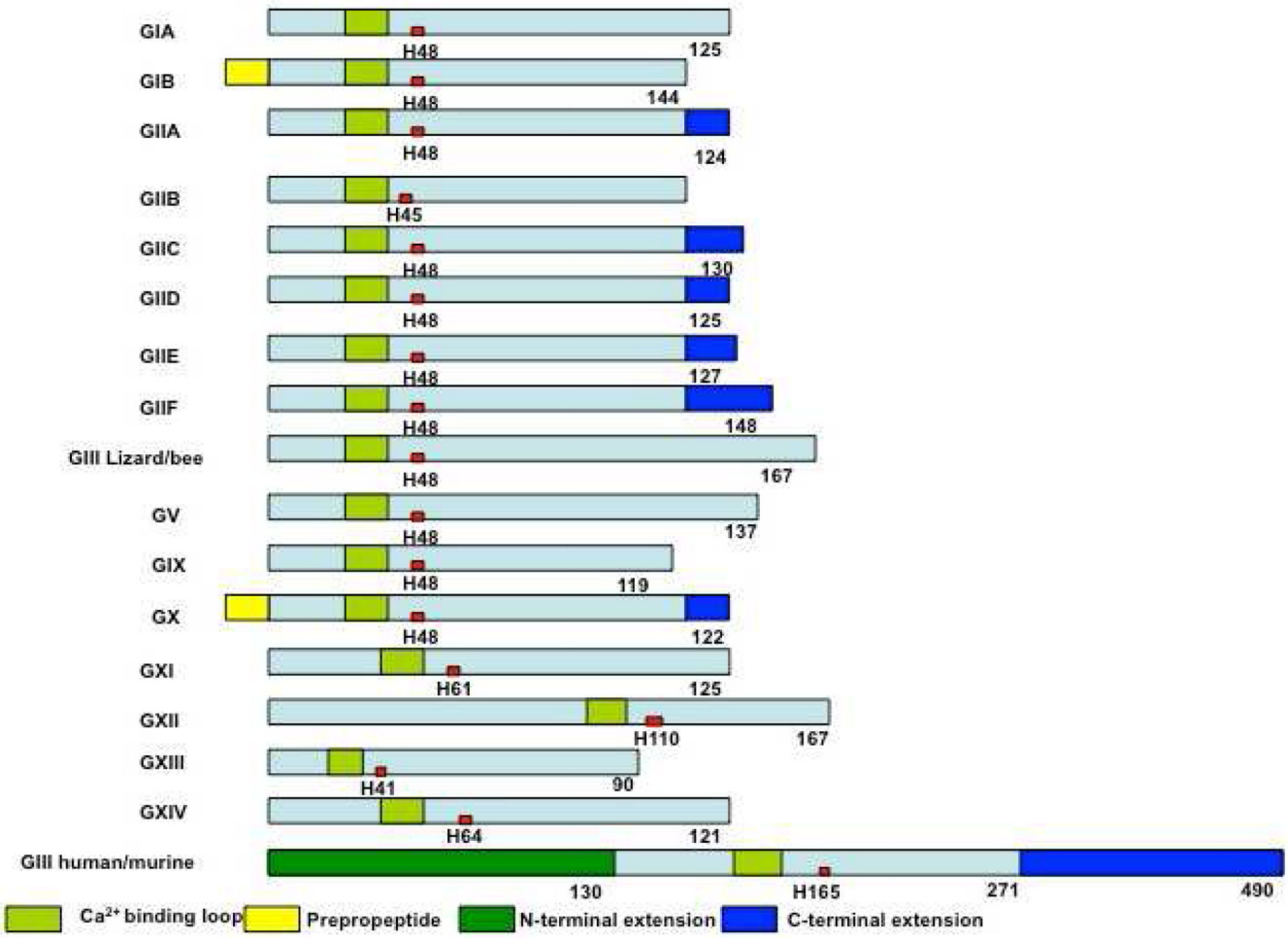


Figure 3.

Schematic presentation of secreted PLA_2s. Calcium binding loops, active sites (red squares), N and C-terminal extension residues are shown.

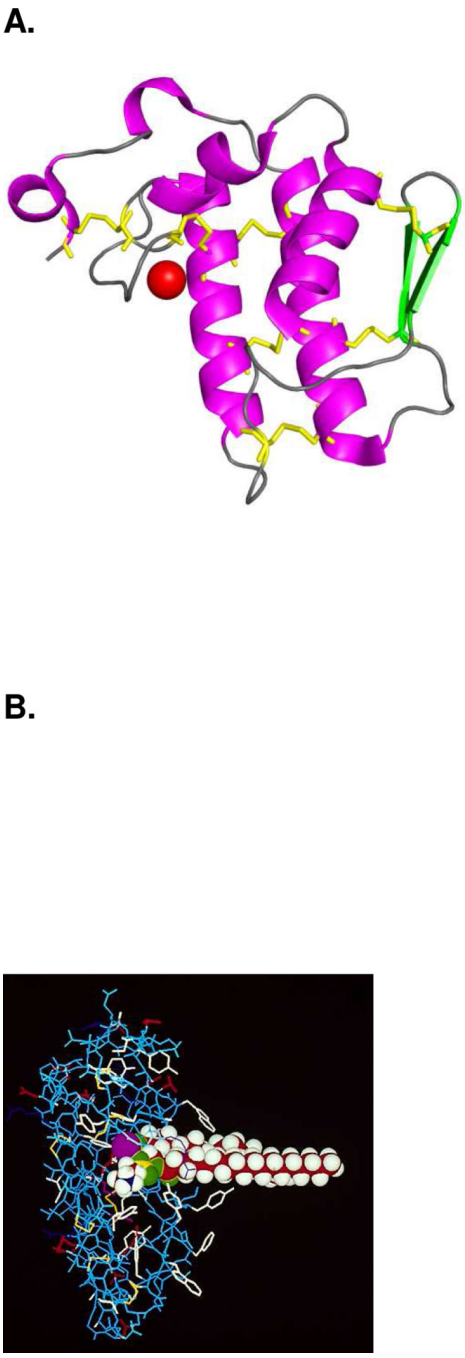


Figure 4.

A. Overall structure of Group IA sPLA$_2$. 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 sPLA$_2$ 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 Ca^{2+} are shown in purple. Ca^{2+} 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]

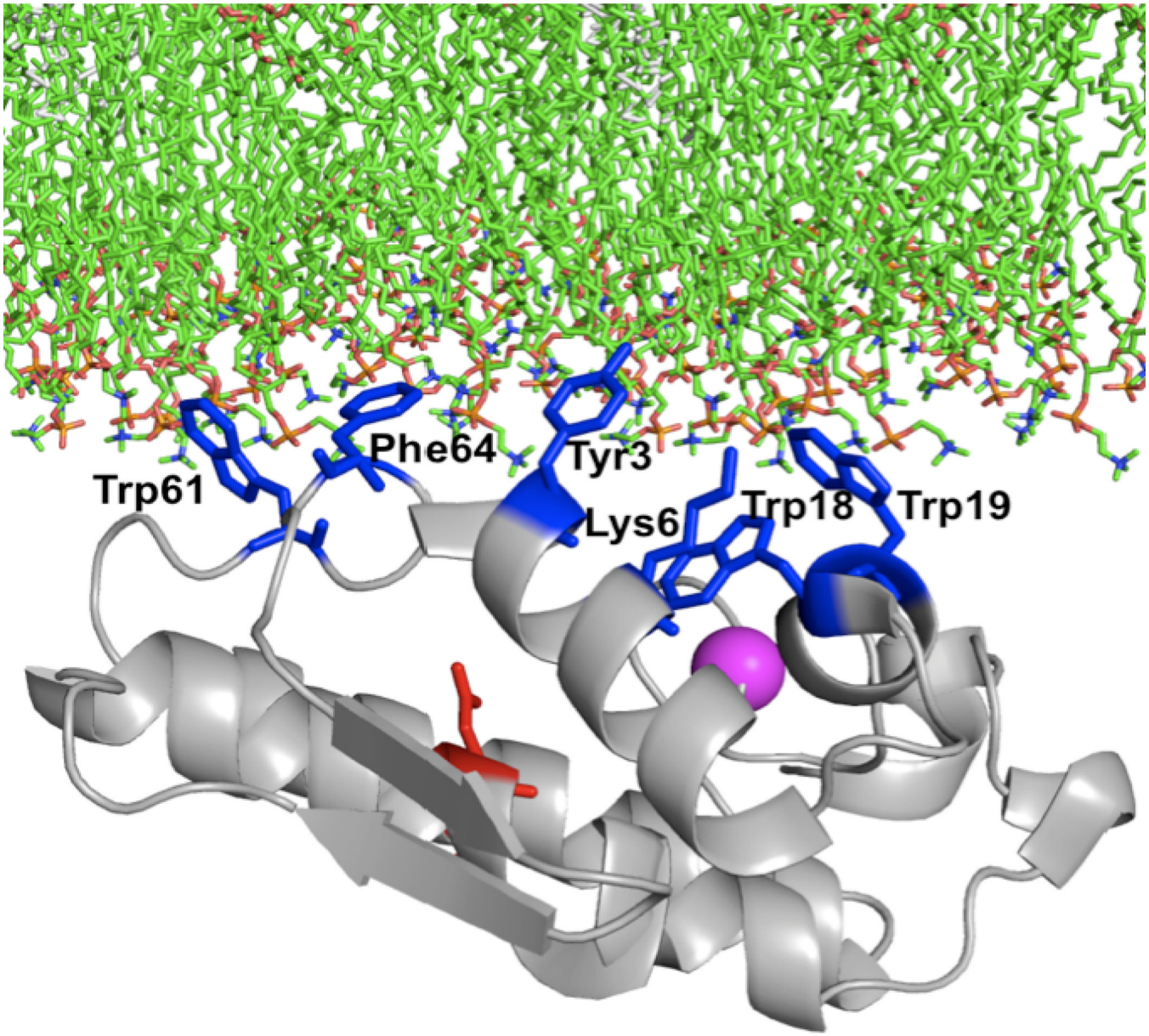


Figure 5.
Model of the lipid surface binding of the Group IA sPLA$_2$ is shown with residues on the interfacial binding surface Tyr-3, Trp-19, Trp-61, and Phe-64 shown in blue stick form. Adapted from Burke et al.[32]

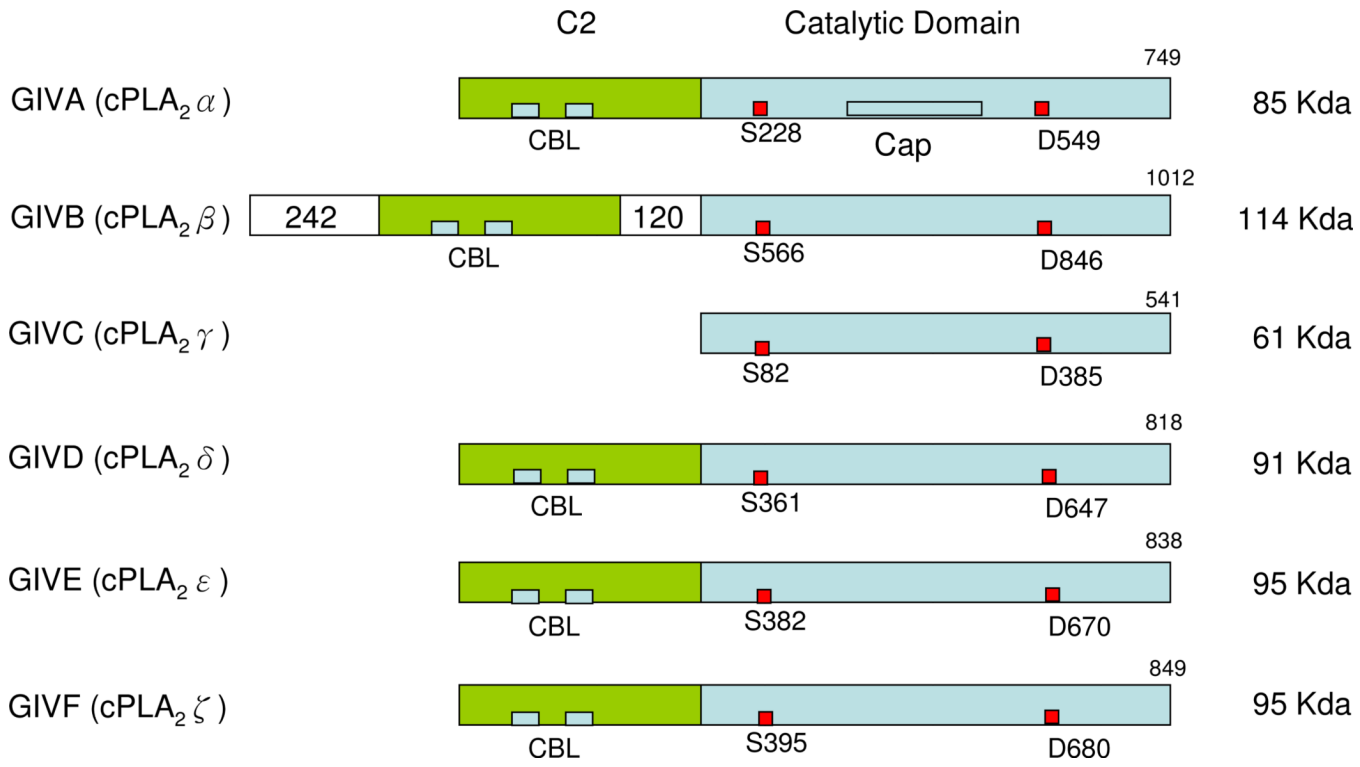


Figure 6.
Schematic presentation of Group IV cPLA$_2$s. Calcium binding loops (CBL), active sites (red squares), and the 242 residue- and 120 residue-inserts of GIVB PLA$_2$ are shown.

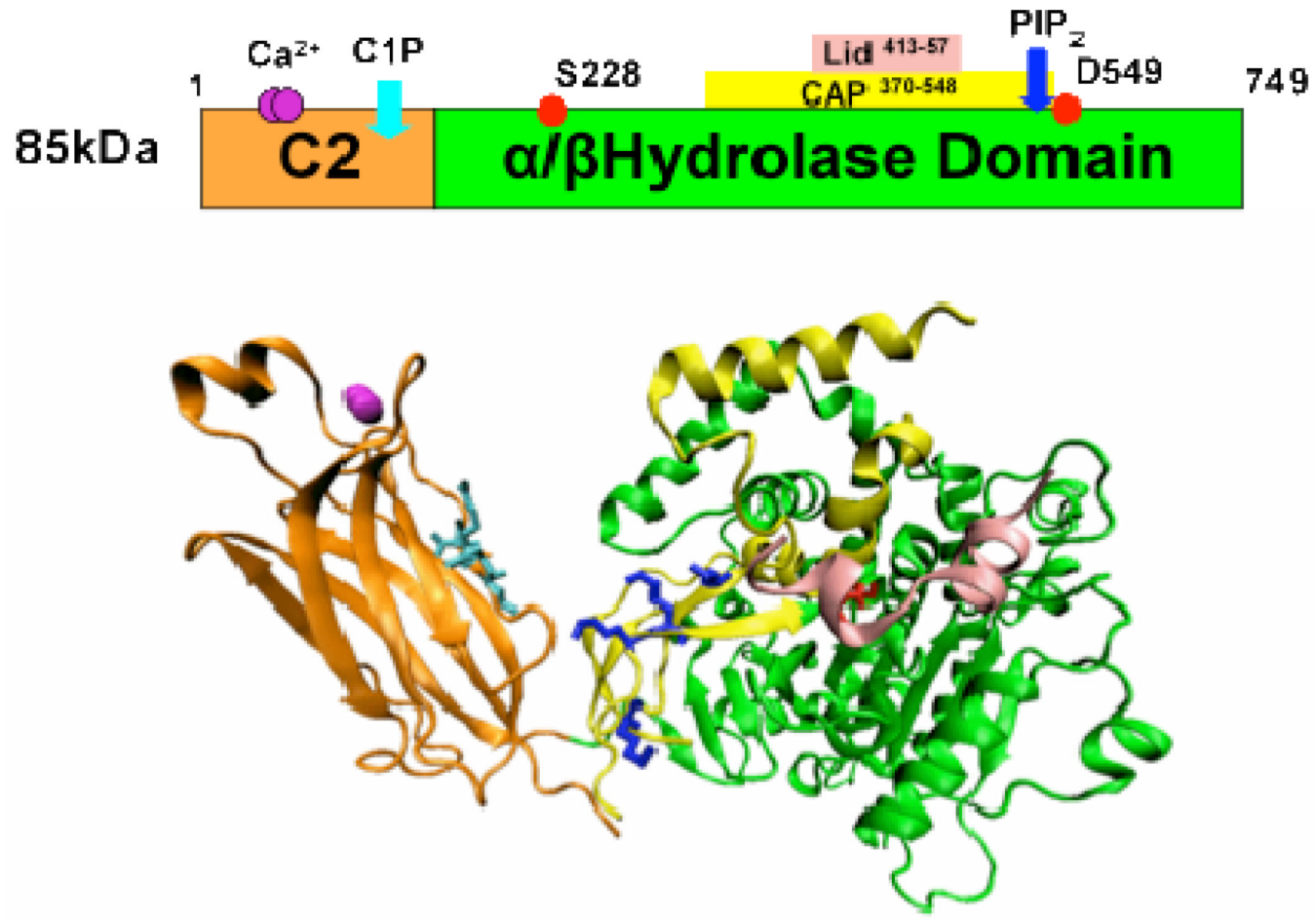


Figure 7.
Features of the Group IVA cPLA$_2$ crystal structure. The C2 domain is in orange, the α/β hydrolase domain is in green, the cap region is yellow, and the lid is in pink. The C1P binding site is in magenta, the PIP$_2$ binding site is in blue and the active site residues are in red. Adapted from Ref.[218]

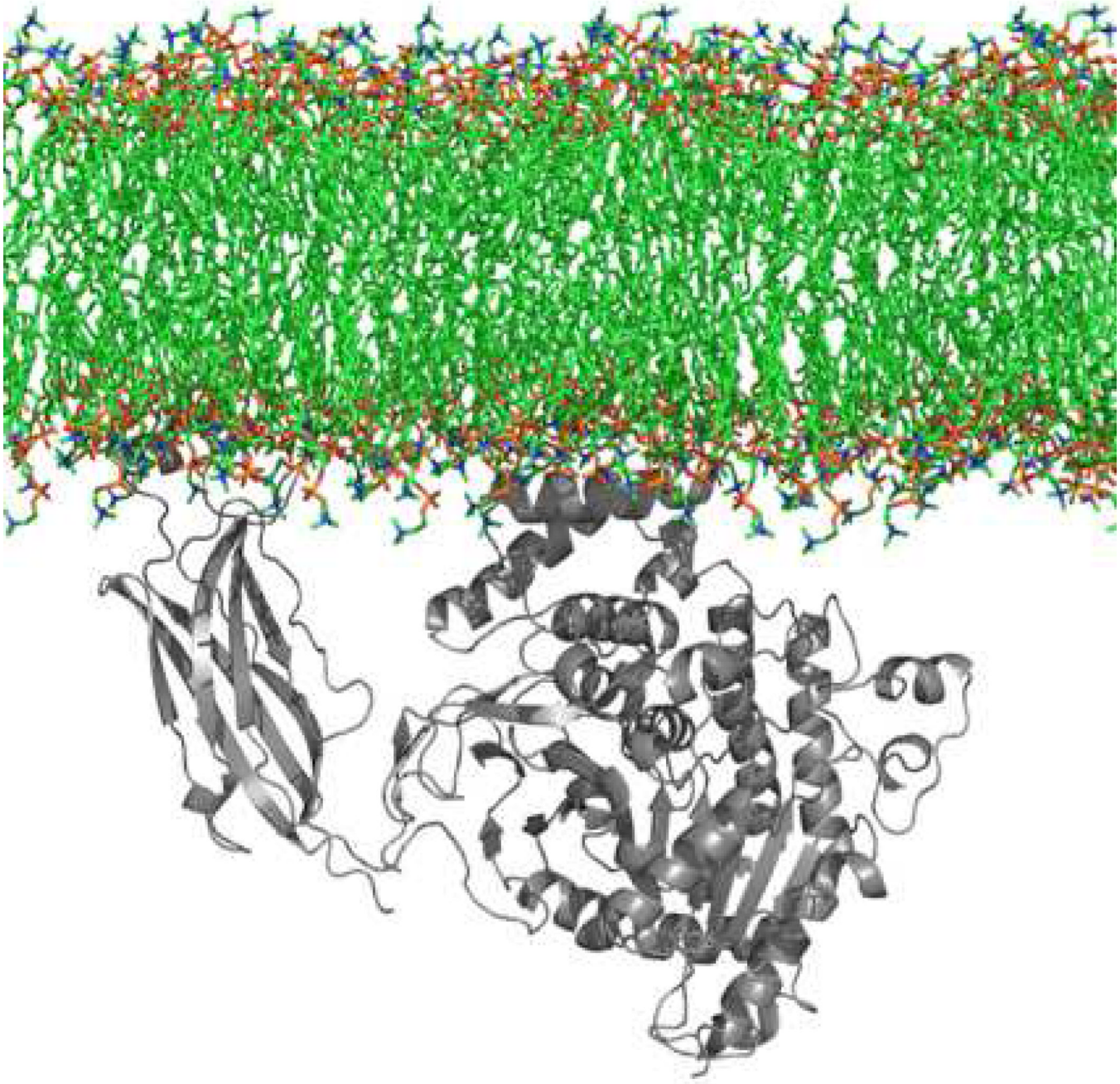


Figure 8.
Model of the lipid-binding surface of Group IVA cPLA$_2$. Residues interacting with the lipid membrane are based on the experiments of hydrogen/deuterium exchange in the presence of phospholipid vesicles. Adapted from Burke et al.[222]

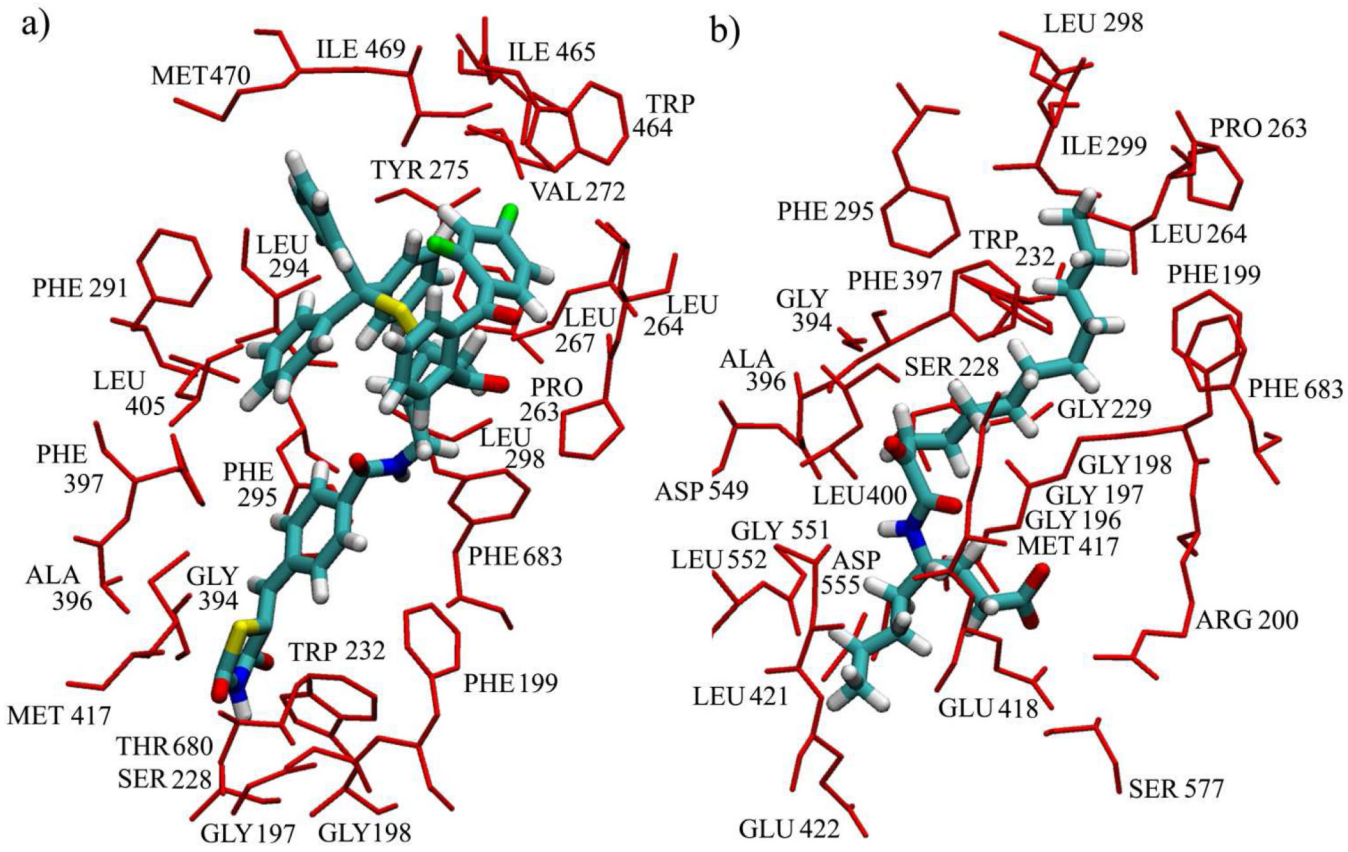


Figure 9.

A. Group IVA cPLA$_2$ residues involved in binding pyrrophenone. **B**. Group IVA cPLA$_2$ 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]

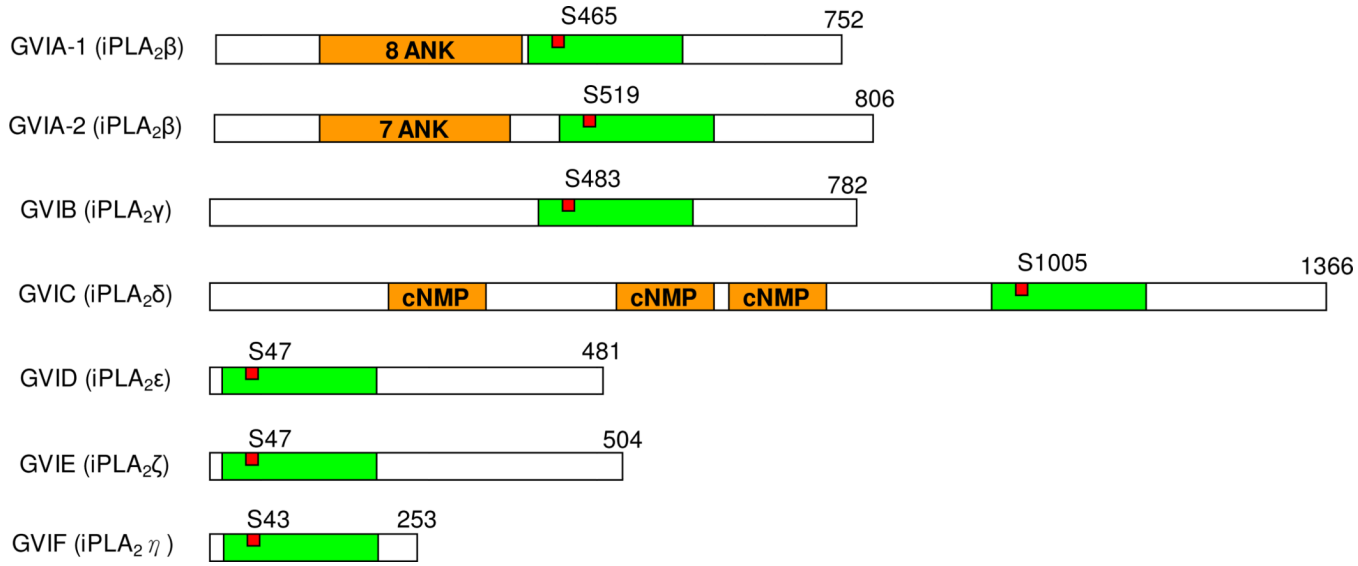


Figure 10.

Schematic presentation of Group VI iPLA$_2$. Ankyrin repeats (ANK), nucleotide phosphate binding domain (cNMP), active site residues in red squares, and patatin-like lipase domains are indicated in green.

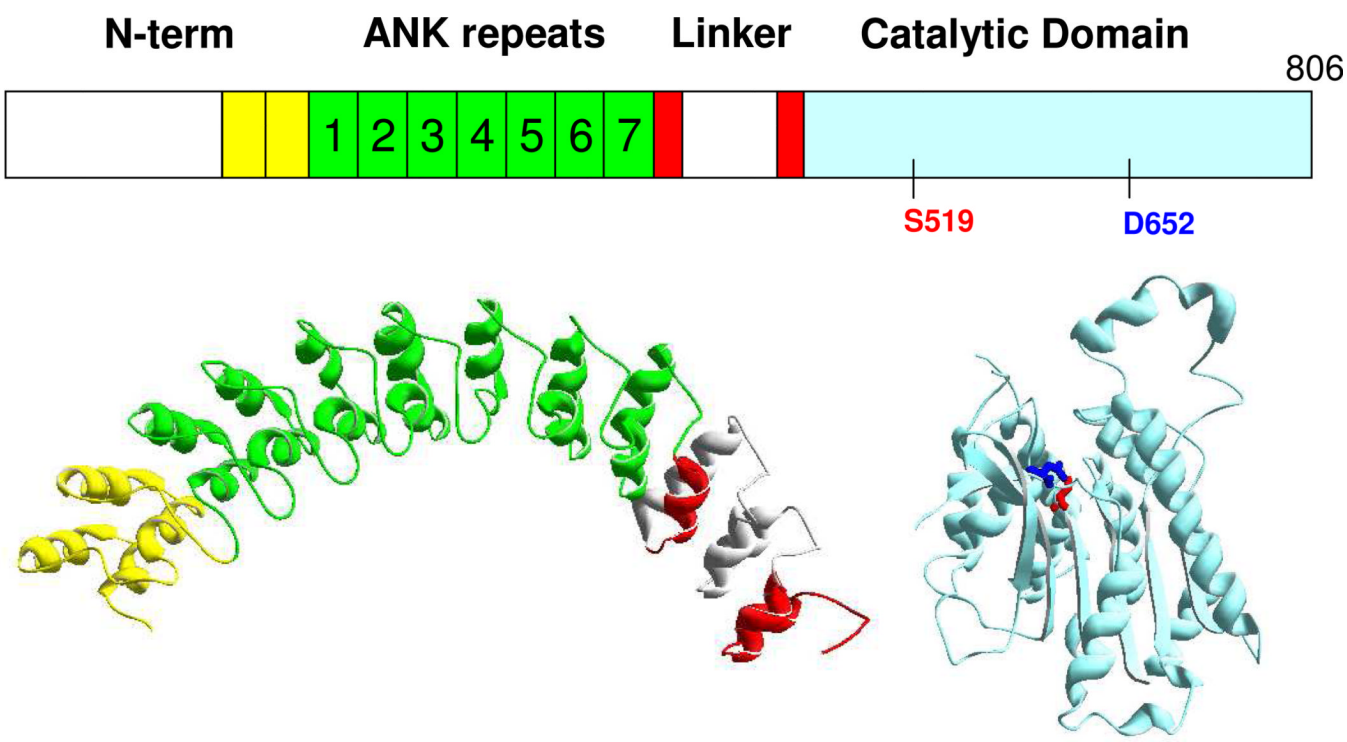


Figure 11.
Features of Group VIA PLA_2 homology models. The domains and binding sites are differentiated by colors. Adapted from Ref.[344]

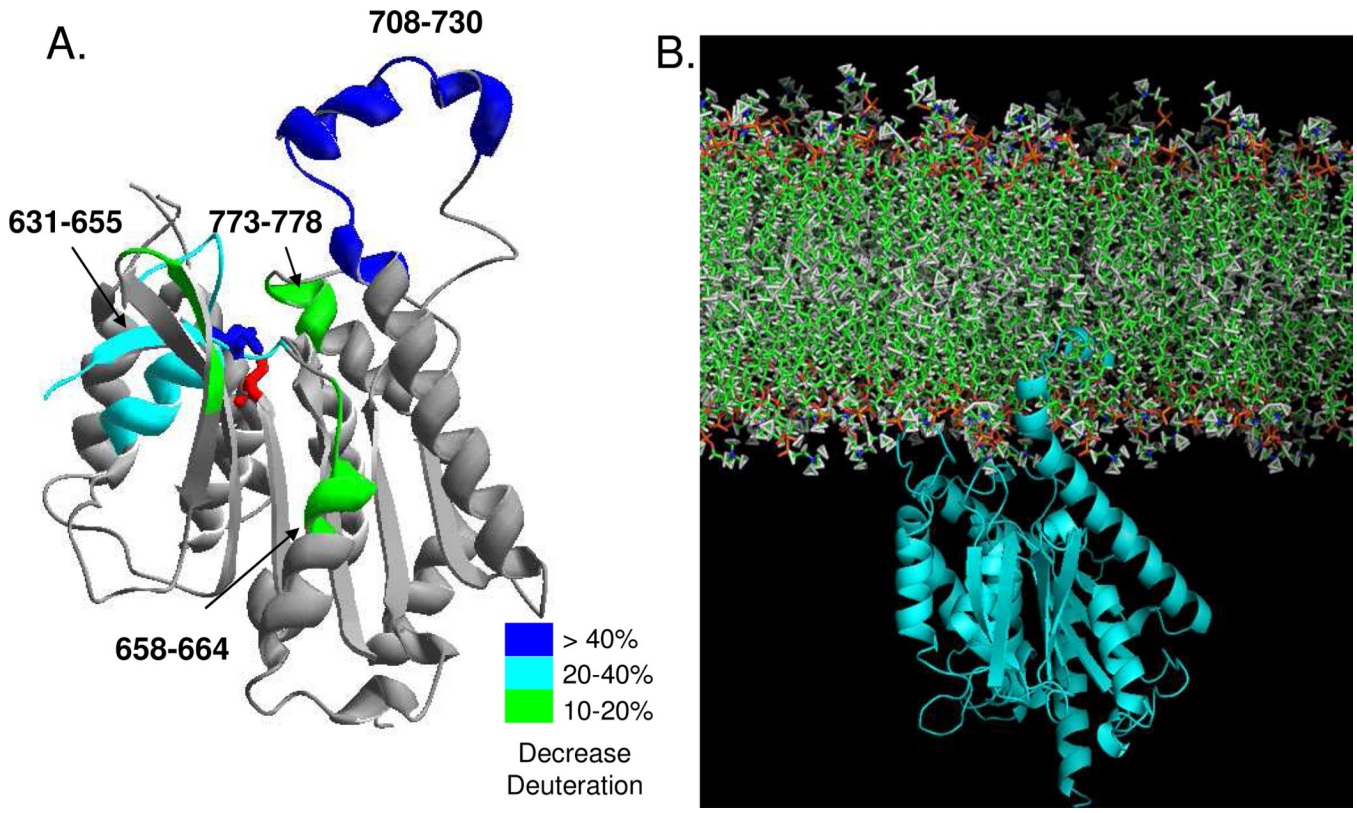


Figure 12.
Phospholipid binding of the Group VIA iPLA$_2$. A. Phospholipid membrane binding effects on H/D exchange of the Group VIA iPLA$_2$ mapped onto the catalytic domain model. B. Model of the lipid-binding surface of the Group VIA iPLA$_2$ based on interactions with lipid membrane. Adapted from Ref.[344]

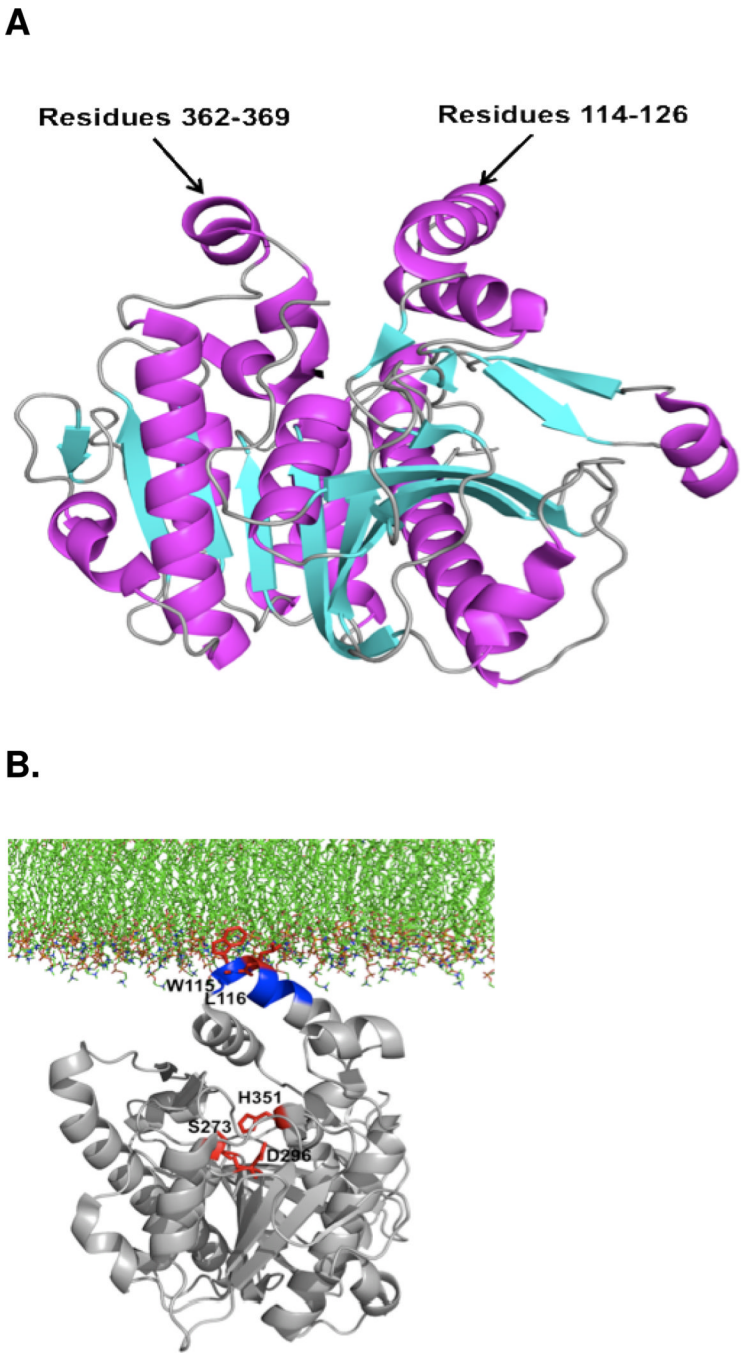


Figure 13.

A. The α/β hydrolase fold of the Group VIIA PLA_2 (PAF-AH/Lp-PLA_2) crystal structure (PDB entry:3D59). Helixes 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-PLA_2 association with the DMPC lipid membrane surface. The Lp-PLA_2 region implicated for Lp-PLA_2/liposome association (residues 113–120), is shown in blue and the proposed key residues for Lp-PLA_2/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]

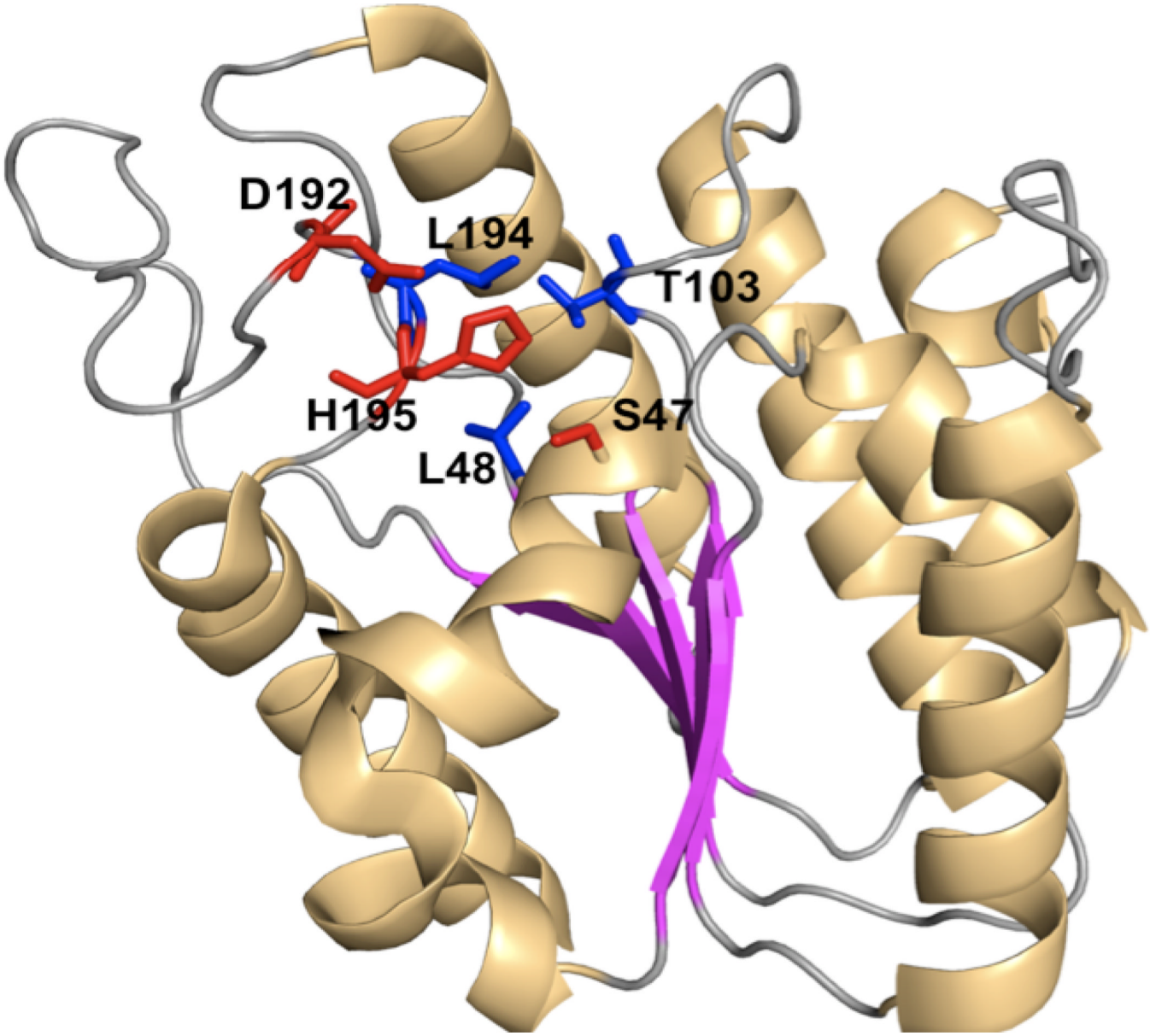


Figure 14.

The crystal structure of Group VIII PLA_2 (PAF-AH IB) α_1 subunit (PDB entry 1WAB). Helixes 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 1

The Phospholipase A_2 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 2

Secreted Phospholipase A_2s (sPLA_2)

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]

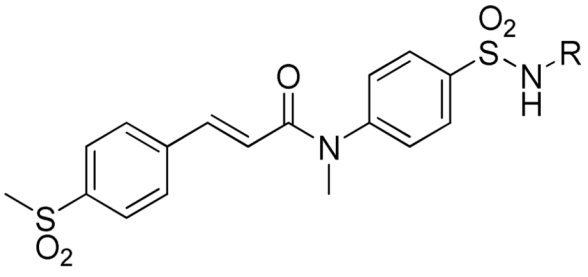
Table 3

Group IIA PLA_2 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 4

Intracellular Membrane Bound PLA_2 Inhibition by Benzenesulfonamides



12a,b

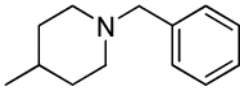
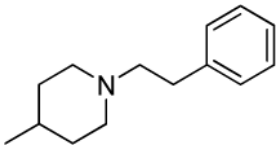
Compound	R	IC_{30} (μM)
12a		0.028 ± 0.012
12b		0.009 ± 0.004

Table 5

Group IB and Group IIA PLA_2 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

[a] $X_I(50)$ is the molar fraction of an inhibitor in the lipid layer that is required to reduce the activity of the enzyme in half.

Table 6

Group IB PLA_2 Inhibition by Acylamino Phospholipid Analogues

Compound	IC_{50} (μM)
16a	1.4
16b	0.23
16c	4.5

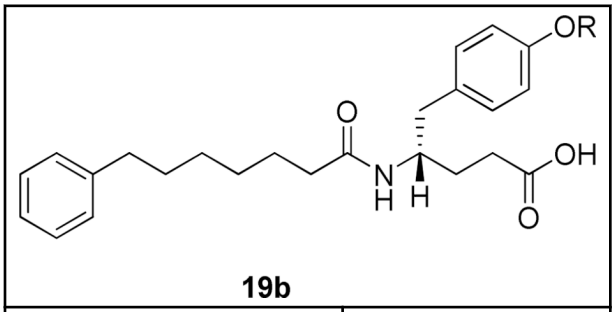
Table 7

Group IB and Group IIA PLA_2 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 8

Group IIA PLA_2 Inhibition by Amides Derived from D-tyrosine

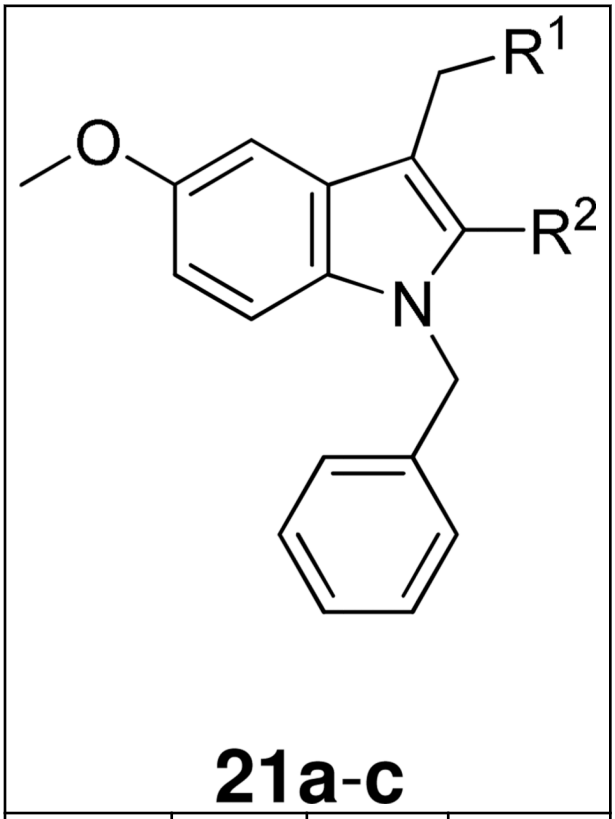


19b

R	IC_{50} (μM)
benzyl-	0.029
2-picolyl-	0.214
cyclopentylmethyl-	0.057
1-napthylmethyl-	0.019
2-napthylmethyl-	0.039
cinnamyl-	0.116
iso-butyl	0.170
n-heptyl	0.086
H	2.57

Table 9

Group IIA PLA_2 Inhibition by Indoles Using the Chromogenic Assay

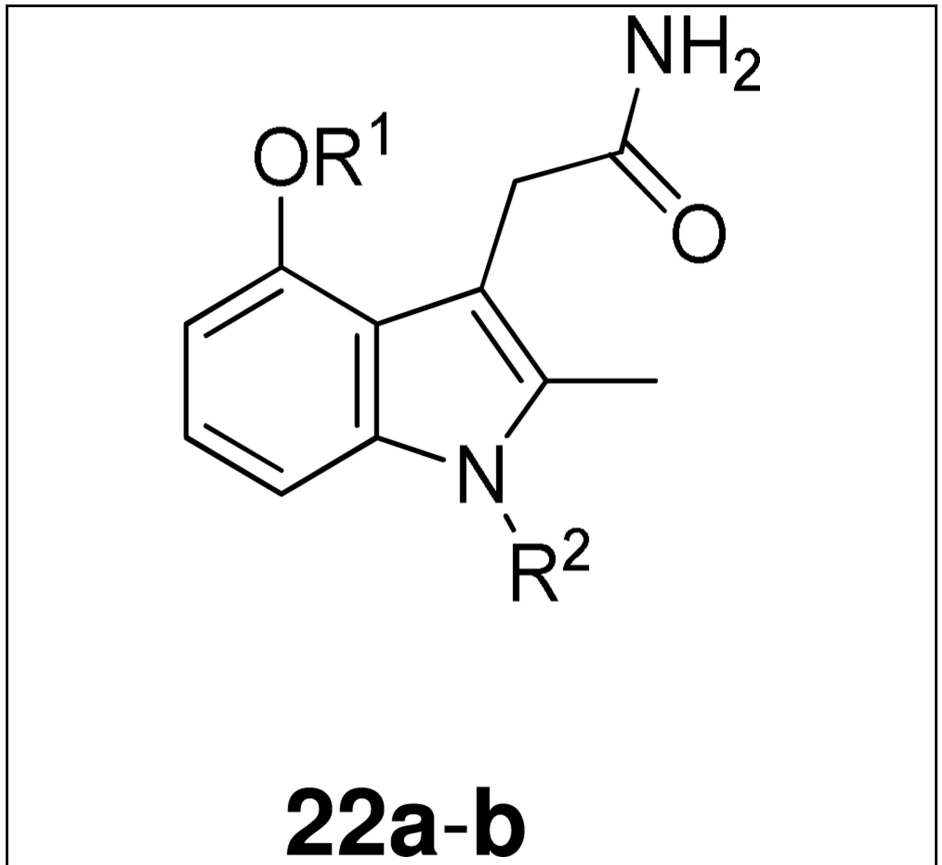


21a-c

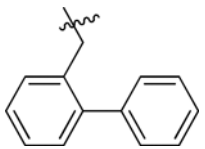
Compound	R^1	R^2	IC_{50} (μM)
21a	COOH	CH_3	13.6 ± 4.2
21b	$CONH_2$	CH_3	0.84 ± 0.17
21c	$CONH_2$	CH_2CH_3	0.26 ± 0.11

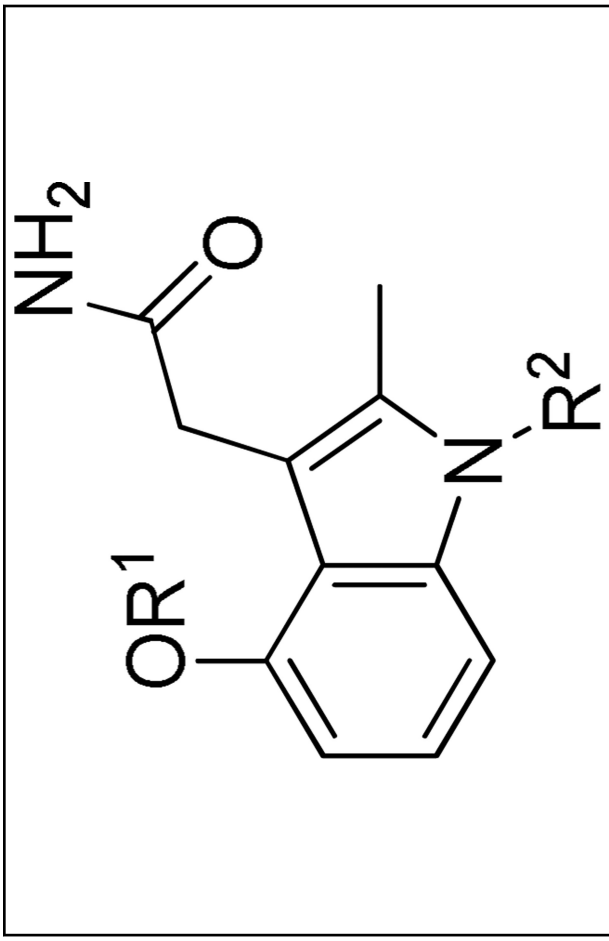
Table 10

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)		
			hGIIA PLA_2	hGIB PLA_2	PLA_2 pGIB PLA_2
22a	CH_2COONa		0.010 ± 0.001	4.09	0.014



22a-b

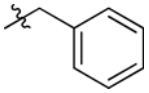
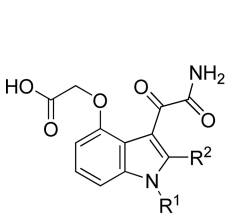
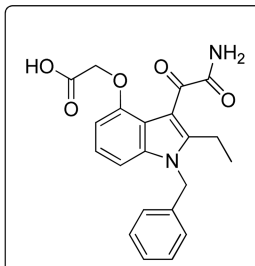
Compound	R^1	R^2	IC_{50} (μM)			
			hGIIA PLA_2	hGIB PLA_2	PLA_2 pGIB PLA_2
22b	CH_2COOH		0.052 ± 0.010	1.4	0.15

Table 11

Comparison of GIIA and Group IB PLA_2 Inhibition by Varespladib and Analogues Using the Chromogenic Assay



23a-g

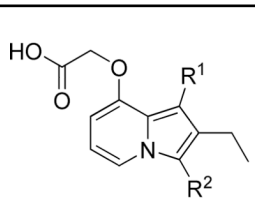


24, LY315920
or **Varespladib**

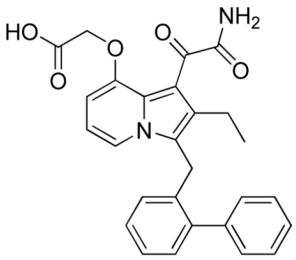
Compound	R^1	R^2	IC_{50} (μM)		
			hGIIA PLA_2	hGIB PLA_2	pGIB PLA_2
23a	$C_6H_5CH_2$	CH_3	0.011 ± 0.004	0.761	0.015
23b	2-(C_6H_5)$C_6H_4CH_2$	CH_3	0.006 ± 0.001	0.364	0.097
23c	3-(C_6H_5)$C_6H_4CH_2$	CH_3	0.009 ± 0.001	0.57	0.007
23d	1-naphthylCH_2	CH_3	0.009 ± 0.004	1.2	
23e	n-C_8H_{17}	CH_3	0.008 ± 0.003	0.78	
23f	2-($C_6H_5CH_2$)$C_6H_4CH_2$	CH_2CH_3	0.004 ± 0.001	0.062	
23g	3-Cl$C_6H_4CH_2$	CH_2CH_3	0.007 ± 0.002	0.390	0.003
24	$C_6H_5CH_2$	CH_2CH_3	0.009 ± 0.001	0.228	0.048

Table 12

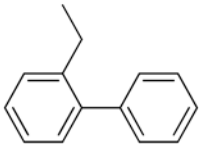
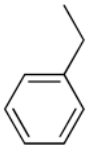
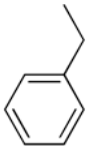
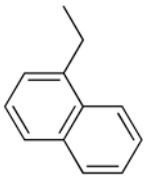
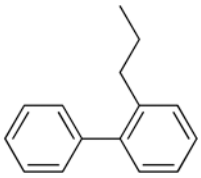
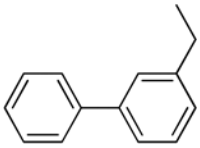
GIIA PLA_2 Inhibition by Indoxam and Analogues Using the Chromogenic Assay and a Deoxycholate Assay

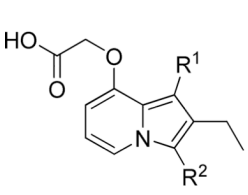


26a-f

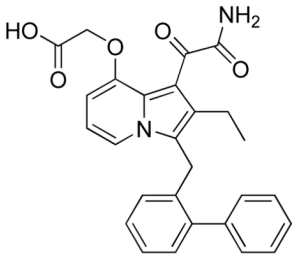


27, **Indoxam**

Compound	R^1	R^2	IC_{50} (μM)	
			chromogenic assay	PC/DOC assay
26a	CH_2CONH_2		0.014	0.014
26b	CH_2CONH_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



26a-f



27, **Indoxam**

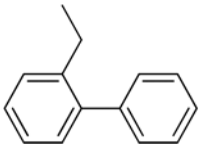
Compound	R^1	R^2	IC_{50} (μM)	
			chromogenic assay	PC/DOC assay
27 – Indoxam	$COCONH_2$		0.006	0.003

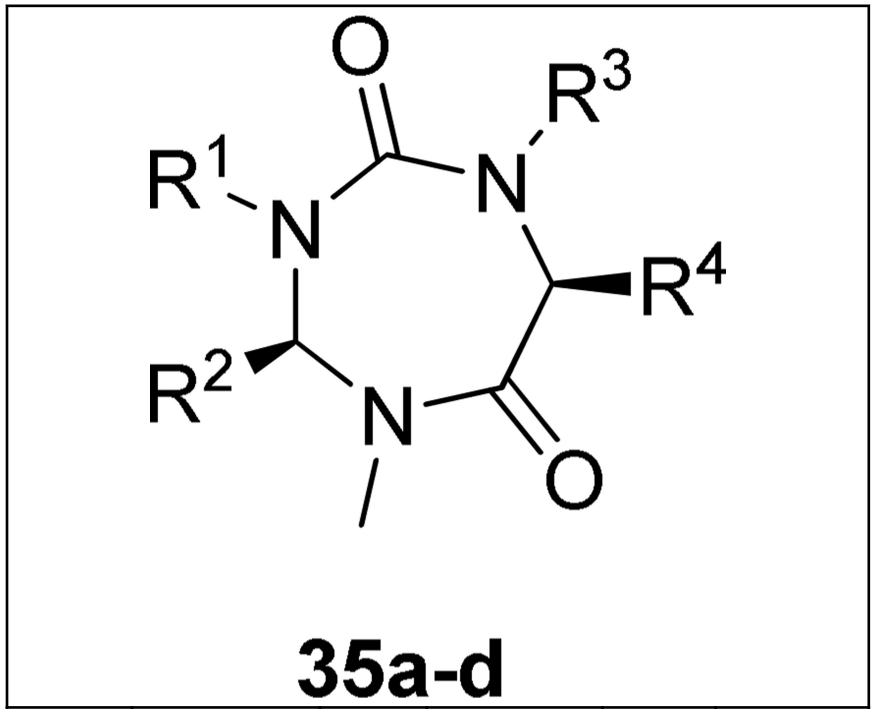
Table 13

Group IB and Group IIA PLA_2 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 PLA_2 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	$CH_2CH{=}CHCOOH$	H	H	$CH_2C_6H_5$	12	10
35b	$CH_2COOC(CH_3)_3$	H	H	$CH(CH_3)_2$	12	11
35c	H	$CH_2C_6H_5$	H	$CH_2C_6H_5$	11	13
35d	$CH_2COOC(CH_3)_3$	H	$CH_2COOC(CH_3)_3$	$CH_2C_6H_5$	11	11

Table 15

Group GIV Cytosolic Phospholipase A_2s (cPLA$_2$)

Subgroup	Initial/common sources	Residues /Molecular Mass	Domain	Activation Factor	Substrate	Activity	Post Translational Modification	Human chromosome	Swiss-Prot
GIVA (cPLA$_2$α)	Human macrophage–like U937 cells/ platelets/RAW 264.7/rat kidney, Ubiquitous	749/ 85 Kda	C2 α/ β hydrolase Cap	Ca^{2+} PIP_2 C1P Phosphor y-lation	PC, PE, PI High *sn*-2AA specificity	PLA_2, PLA_1, Lyso-PLA transacylase	Phosphorylation	1q25	P47712
GIVB (cPLA$_2$β)	Human pancreas/ liver/heart/brain, Ubiquitous	1012 /100−114 Kda	JmjC insert C2 α/ β hydrolase	Ca^{2+}	PC, PE No *sn*-2 specificity	PLA_1, PLA_2, Lyso-PLA transacylase		15q11.2 - q21.3	AAD32135
GIVC (cPLA$_2$γ)	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	AAC32823
GIVD (cPLA$_2$δ)	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$_2$ε)	Murine heart /skeletal 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$_2$ζ)	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 16

Group IVA PLA_2 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 17

Group IVA PLA_2 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 18

Group IVA PLA_2 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 PLA_2 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 20

Group IVA PLA_2 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

Table 21

Group VI Calcium Independent Phospholipase A_2s ($iPLA_2$)

Subgroup	Alternative name	Sources	Residues /Molecular Mass	Domain	Alt Splice	Activity	Post Translational Modification	Human chromosome	Swiss-Prot
GVIA	$iPLA_2\beta$ PNPLA9	Human/ Murine	750/85 kDa 806/87 kDa	7–8 Ankyrin Repeats α/ β hydrolase (Patatin like Lipase)	5	PLA_2, Lyso-PLA transacylase acyl-CoA thioesterase		22q13.1	O60733
GVIB	$iPLA_2\gamma$ PNPLA8	Human/ Murine	782/90 kDa	α/ β hydrolase (Patatin like Lipase)	2	PLA1, PLA_2, Lyso-PLA transacylase		7q31	Q9NP80
GVIC	$iPLA_2\delta$ PNPLA6 NTE	Human/ Murine	1366/146 kDa	3 cNMP α/ β hydrolase (Patatin like Lipase)	3	PLA_2 Lyso-PLA	Phosphorylation (5)	19p13.3-13.2	Q8IY17
GVID	$iPLA_2\varepsilon$ PNPLA3 ADPN	Human	481/52 kDa	α/ β hydrolase (Patatin like Lipase)	2	PLA_2, TG hydrolase, transacylase		22q13.31	Q9NST1
GVIE	$iPLA_2\zeta$ PNPLA2 ATGL	Human	504/55 kDa	α/ β hydrolase (Patatin like Lipase)	2	PLA_2 TG hydrolase, transacylase	Phosphorylation (1)	11p15.5	Q96AD5
GVIF	$iPLA_2\eta$ PNPLA4 GS2	Human	253/27 kDa	α/ β hydrolase (Patatin like Lipase)		PLA_2, TG hydrolase, retinylester hydrolase acylglycerol and retinol transacylase	Phosphorylation (1)	xp22.3	P41247

Table 22

Group VIA PLA_2 Inhibition by Fatty Acyl Trifluoromethyl Ketones

Compound	IC_{50} (μM)	$X_I(50)$
43	15	0.028
44	3.8	0.0075
95		0.0043

Table 23

Comparison of Group VIA, Group IVA and Group V PLA_2 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 24

Comparison of Group VIA, Group IVA and Group V PLA_2 Inhibition by Polyfluoroalkyl Ketones[a]

Compound	GVIA PLA_2 $X_I(50)$	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 25

Comparison of Group VIA, Group IVA and Group V PLA_2 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.

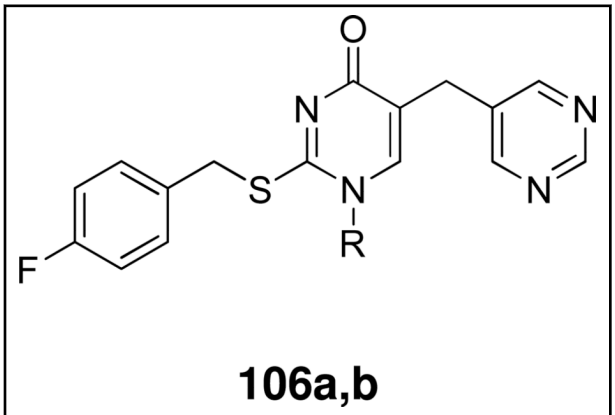
Table 26

Group VII and Group VIII Phospholipase A_2s (PAF-AH)

Group	Source	Molecular mass (kDa)	Features	Alternate names
VIIA	Human, murine, porcine, bovine	45	Secreted α/βhydrolase N-linked glycosylation	Lipoprotein-associated PLA_2 (Lp-PLA_2) Plasma PAF-AH (pPAF-AH)
VIIB	Human bovine	40	Intracellular, myristoylated, α/βhydrolase	PAF-AH II
VIIIA	Human	26	Intracellular, Homodimer or heterodimer with GVIIIB associates with regulatory β subnunit	PAF-AH Ib (α_1 subunit)
VIIIB	Human	26	Intracellular, Homodimer or heterodimer with GVIIIA associates with regulatory β subnunit	PAF-AH Ib (α_2 subunit)

Table 27

Group VIIA PLA_2 Inhibition by Pyrimidones



106a,b

Compound	R	IC_{50} (nM)
106a	(*E*)-$(CH_2)_3CONH(CH_2)_8CH{=}CHC_8H_{17}$	0.7
106b	(*Z*)-$(CH_2)_3CONH(CH_2)_8CH{=}CHC_8H_{17}$	0.4

Table 28

Group XV Lysosomal Phospholipase A_2 ($LPLA_2$)

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

Summery of Human Proteins Not Traditionally Categorized as Phospholipase A_2s, but which Express Phospholipase A_2 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_2L	$sPLA_2$	Protein (mouse)	493/53	Predicted		$sPLA_2$-like (2)	Q02509
$cPLA_2$	PLB1	Phospholipase B1		Protein	1458/163	Verified	Lyso-lecithin acylhydrolas e	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	Q7Z6Z6
	PNPLA1			Transcript	532/57			Patatin-Like	Q8N8W4
$aiPLA_2$	Peroxiredoxin-6	1-Cys peroxiredoxin $aiPLA_2$		Protein	224/25	Verified	peroxiredoxi n activity	Thioredoxin domain	P30041