High-Resolution X-ray Crystallography Reveals Precise Binding Interactions between Human Nonpancreatic Secreted Phospholipase A_2 and a Highly Potent Inhibitor (FPL67047XX)

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Phospholipase A_2 (PLA_2; EC 3.1.1.4) catalyzes the *sn*-2 acylhydrolysis of phospholipids liberating free fatty acids, predominantly arachidonic acid, and lysophospholipids. These products can themselves impart biological actions or be further metabolized to form a variety of proinflammatory lipid mediators including prostaglandins, leukotrienes, thromboxanes, or platelet-activating factor.[1] A low molecular weight (14 kDa) human nonpancreatic secretory PLA_2 (hnps-PLA_2) has been purified[2,3] and was found both in inflammatory cells[4,5] and in a variety of inflammatory exudate fluids in soluble form.[6−8] Since hnps-PLA_2 has been associated with the initiation and/or the propagation of inflammatory episodes, its inhibition or antagonism is an attractive approach toward the development of novel anti-inflammatory agents. Although this concept has been disputed,[9] elucidation of its clear role in inflammatory diseases should await clinical evaluation of potent and selective inhibitors of the enzyme.

Using X-ray crystallography as a component of an iterative design protocol has proven to be a successful venture in this[10] and other systems.[11] Three crystal structures of hnps-PLA_2/inhibitor complexes have been reported. One of these contains a transition-state analog (TSA, L-1-*O*-octyl-2-(heptylphosphonyl)-*sn*-glycero-3-phosphoethanolamine),[12] and the second contains an acylamino analog (1-octadecyl-2-acetamido-2-deoxy-*sn*-glycero-3-phosphoethyl methyl sulfide),[13] both of which are inhibitors of moderate potency. The third is a tight-binding inhibitor developed from an initial screening lead which bears no resemblance to naturally occurring substrates.[10] In addition, structural information is available for several other PLA_2s in complex with phospholipid analog inhibitors: a mutant porcine PLA_2 complexed with an acylamino analog, (*R*)-2-(dodecanoylamino)-1-hexanol phosphoglycol,[14] PLA_2s from the cobra venom[15] and bee venom,[16] both complexed with TSA, and the solution structure of the porcine PLA_2 complexed with TSA containing a sulfur substitution of an oxygen atom.[17]

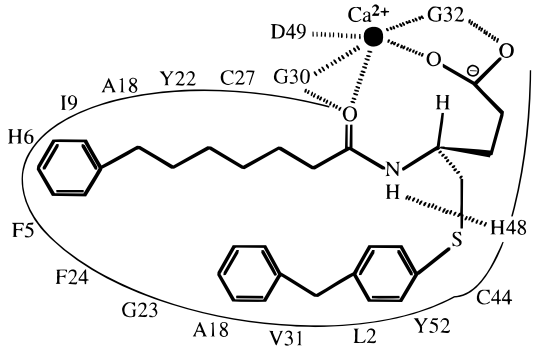

Figure 1. Schematic drawing of the interactions between hnps-PLA_2 and **I**. Amino acids closer than 4.3 Å from the inhibitor atoms are indicated with the one-letter code for amino acid and residue number. Ionic and hydrophilic interactions are indicated by broken lines. The catalytic calcium ion at the active site is labeled.

Recently, a highly potent hnps-PLA_2 inhibitor, FPL67047XX[18] (Figure 1), has been reported that can be classified as an acylamino analog of phospholipids.[19−21] In-house measurement of the IC_{50} value of the inhibitor was 0.013 μM against hnps-PLA_2 under an enzyme assay utilizing [^{3}H]arachidonate-labeled *Escherichia coli* membranes.[22] Under the same conditions, TSA exhibited an IC_{50} of 0.2 μM. We sought to understand the tight-binding mode of the inhibitor, and herein, we report the analysis of the refined 2.0 Å crystal structure of hnps-PLA_2 in complex with the inhibitor. The enzyme/inhibitor complex crystallized as a complex of six protomers in the asymmetric unit. The hexameric structure was determined by the molecular replacement method using the monomeric model of the uninhibited hnps-PLA_2.[12]

The inhibitor, referred as **I** hereafter, is an extensively modified form of the phospholipids and emerged from a rational drug design effort[23] based initially on the structure of hnps-PLA_2 complexed with a phosphonate analog.[12] Notable differences between **I** and naturally occurring substrates include the three phenyl rings, one carboxylate group, an amide group, and one sulfur atom. The crystal structure reveals how each of these components contributes to the tight binding of the inhibitor.

The three phenyl rings are novel substitutions for parts of the *sn*-1 and *sn*-2 *n*-alkyl chains on the glycerol backbone that are found in many substrate or transition-state analog inhibitors of the enzyme known to date.[21,24,25] The phenyl rings participate in extensive aromatic/aliphatic or aromatic/aromatic interactions with the nonpolar amino acids lining the hydrophobic channel of the enzyme (Figure 2). They also interact intramolecularly with each other to form an "aromatic core", and notably the *sn*-2 phenyl ring is approximately perpendicular to the two phenyl rings on the *sn*-1 chain (Figure 2). Although bulky, both the *sn*-1 and *sn*-2 chains of **I** are accommodated within the hydrophobic channel in an extended conformation without causing any noticeable conformational change of the enzyme except for the side chain rotation of His-6 due to its displacement by the *sn*-2 terminal phenyl ring (Figure 2). The root-mean-squares deviation of all atoms comprising the 17 amino acids lining the hydrophobic pocket

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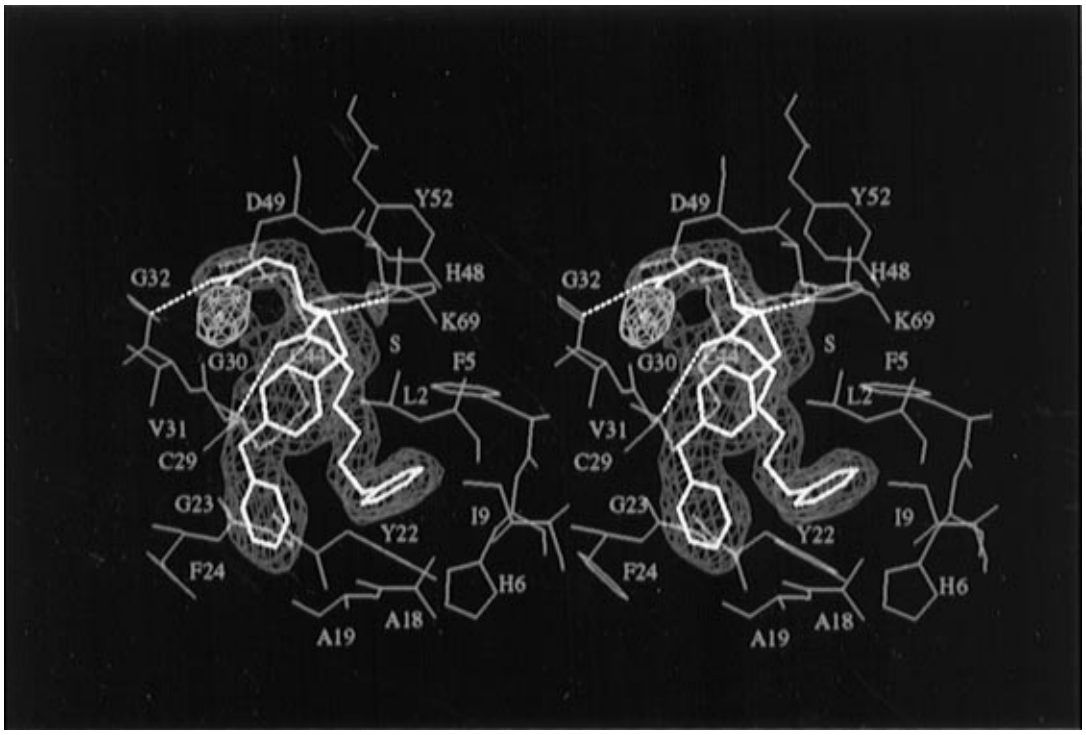

Figure 2. Stereodiagram of the $2F_o - F_c$ electron density for the bound inhibitor (blue) and the catalytic calcium ion (purple). The electron density map was calculated for the final model of the hnps-PLA_2/inhibitor complex at 2.0 Å resolution. The contour level is 1.2σ. The calcium ion density was deliberately reduced for clarity. The bound inhibitor is in yellow, and the protein atoms are in green. Labeled are amino acid residues in contact with the inhibitor (<4.3 Å). The sulfur atom of the inhibitor is labeled as "S". The hydrogen bonds are shown as dotted lines.

(Figure 1, excluding His-6) of this structure and that of the uninhibited enzyme[12] is only 0.29 Å. The minimal perturbation of enzyme conformation upon binding by **I** indicates that the three phenyl rings are precisely tailored replacements for the *n*-alkyl chains and effectively improve van der Waals interactions with the enzyme.

The *sn*-1 chain of **I** contains one sulfur atom (Figure 2) which is a replacement for the oxygen atom in substrates or other weak hnps-PLA_2 inhibitors. No polar atom within 4.3 Å of the sulfur atom is found indicating that it is a suitable hydrophobic replacement for the more polar oxygen atom. A contact is made with the edge of the aromatic ring of Phe-5.

The carboxylate functionality of **I** substitutes for the *sn*-3 phosphate group which exists in both substrates and in most of other hnps-PLA_2 inhibitors.[10,21,25−27] A problem with many inhibitors of the enzyme studied to date is their lack of cell permeability. This is most likely due to the highly charged or zwitterionic nature of the phosphate head groups. It was anticipated that the carboxylate substitution would attenuate this effect. The inhibitory activity of **I** in the monocyte leukotriene C_4 release assay was measured according to the previously described protocol.[28] **I** exhibited an IC_{50} of 0.35 μM in this cellular system. Though we have not prepared phosphate analogs of **I**, we prepared and tested several other *sn*-3 phosphate-containing substrate analogs including TSA. They exhibited no activity at concentrations up to 50 μM, indicating that those compounds can hardly reach the inner leaflet of the cell membrane. In a topical model of TPA-mouse ear inflammation, **I** also exhibited activity; inhibition of ear thickness was dose dependent with 50% inhibition at 300 μg/ear (personal communication). Thus, the carboxylate group appears to be an effective substitution for enhancing the cell permeability of the inhibitor. Our crystal structure shows that the carboxylate group of **I** chelates the catalytic calcium ion (Figure 3), like the phosphate groups in other hnps-PLA_2 inhibitors. The refined structure shows that the carboxylate group fits nicely into the coordination shell. The chelating oxygen atoms of Asp-49,[29] Gly-30, Gly-32, and amide carbonyl of **I** are on a nearly flat equatorial plane. The angle between the calcium ion and the two axially coordinating oxygen atoms of **I** and His-28 is 177° (the average of six protomers). In comparison, the corresponding angle observed in the structures of hnps-PLA_2/TSA[12] and the mutant porcine PLA_2[14] is about 164°. This discrepancy may be due to the difference between the carboxyl group of **I** and the *sn*-3 phosphate group of other inhibitors in the interactions with the neighboring protein atoms. The structures of hnps-PLA_2/TSA[12] and the mutant porcine PLA_2[14] show a hydrogen bond between the chelating oxygen atom of *sn*-3 phosphate and Gly-32 which is not observed in our structure, and the remaining nonbridging phosphate oxygen exhibits an ionic and/or hydrogen-bonding interaction with Lys-69 (Tyr-69 in the porcine enzyme). In contrast, the nonchelating carboxylate oxygen atom of **I** does not interact with Lys-69 but makes a hydrogen bond with Gly-32.

The *sn*-2 amide of **I** was employed as a mimic for the ester functionality. The amide oxygen is involved in the chelation of the catalytic calcium ion and in a hydrogen bond with Gly-30. The amide hydrogen partakes in a hydrogen bond with the imidazole nitrogen of the catalytic His-48 (Figure 2), and this interaction has been proposed to contribute 1.5 kcal/mol toward binding.[30] Followed by the amide group is a stretch of a six-carbon *n*-alkyl chain whose conformation was determined

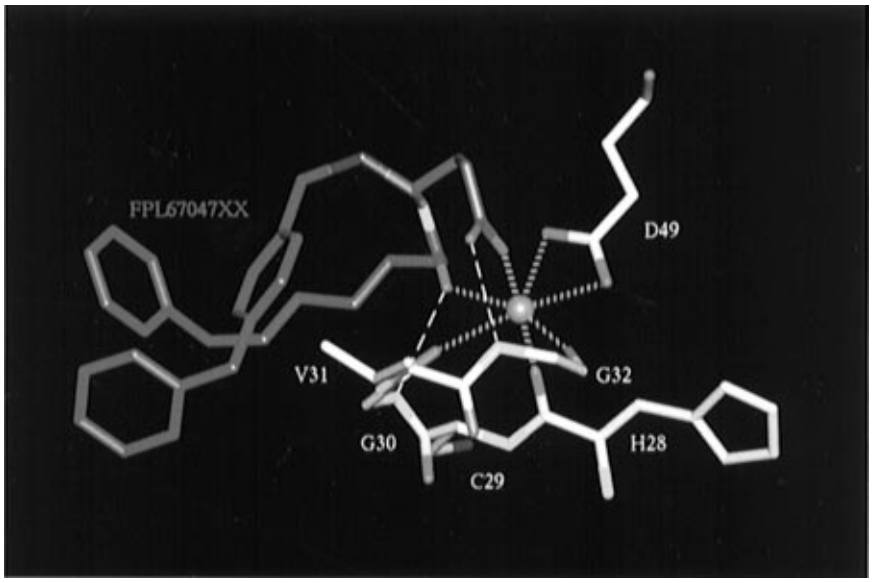

Figure 3. Coordination shell of the catalytic calcium ion. White dotted lines indicate the hydrogen bonds between **I** and the protein atoms. Oxygen, nitrogen, and sulfur atoms are indicated by red, yellow, and blue, respectively. The calcium ion is in purple.

unambiguously due to the high-resolution data. The amide plane and the *n*-alkyl chain make about a 94° angle. Similarly, in the mutant porcine structure,[14] the *sn*-2 acyl chain of the bound inhibitor has a kink of ~90°. The same array of interactions was found in the structures of PLA_2s in complex with an amide inhibitor.[13,14]

In total, **I** engages in close contacts (<4.3 Å) with 96 atoms among 18 different amino acids within the binding site (Figure 1), resulting in the exclusion of 366 $Å^2$ in solvent accessible surface. All three water molecules found in the hydrophobic channel of the uninhibited enzyme[12] are excluded upon binding of **I**. The channel is optimally occupied by **I** as indicated by zero or near zero solvent accessibility of all the residues located inside of the channel (Phe-5, Ile-9, Ala-18, Tyr-22, Cys-44, 0 $Å^2$; Gly-30, Gly-23, His-48, 2 $Å^2$; Tyr-52, 5 $Å^2$). An optimal packing of the hydrophobic channel appears to be a key to the success in designing high-affinity hnps-PLA_2 inhibitors. This is because the presence of the phenyl rings is the main difference between the structure of **I** and the other weak inhibitors. The indole inhibitor mentioned earlier[10] contains a bulky indole ring instead and also binds to the enzyme tightly. In addition to the more favorable van der Waals interactions, less conformational freedom of the bulky groups compared to the *n*-alkyl chains will result in less entropy loss in the inhibitor binding to the enzyme.

It was shown that the binding affinity of **I** for the wild-type porcine pancreatic PLA_2 is as high as for hnps-PLA_2.[18] When the structures of the complex and the porcine pancreatic PLA_2 were superimposed, a geometric fit and energetic complementarity between **I** and the hydrophobic channel of the porcine enzyme were achived with van der Waals distance violations caused by three residues, Asn-23, Leu-31, and Tyr-69. However, the side chains of those residues are explosed to the bulk solvent and can be rotated out to avoid the steric clashes. Probably, **I** would bind to the porcine enzyme in a very similar conformation as it binds to hnps-PLA_2, while the three residues should undergo conformational changes of their side chains.

Since the precise binding mode of **I** has been elucidated, the next step of iterative design strategy can now be undertaken. There appears to be unoccupied space about the region resided by the carboxylate moiety. This is because a bulky and negatively charged tetrazole functionality was shown to be an effective substitution for the *sn*-3 phosphate in another series of inhibitors (unpublished result). The same study has shown that neutral groups such as alcohols and ketones are not effective replacements for the negatively charged phosphate. It has been demonstrated that there is additional binding affinity to be gained in a region normally occupied by the *sn*-3 chain of phosphate-containing inhibitors.[27] Thus, the replacement of the tetrazole with another acidic heterocycle which allows additional substitution on the ring may lead to the occupation of this binding region. The structure of the complex also suggests a way of improving aqueous solubility of **I**. The *sn*-1 phenyl rings of **I** are partly exposed to the bulk solvent (Figure 2), and thus a heterocyclic ring or a more hydrophilic aromatic ring as a substitution for the phenyl rings may result in the improvement of water solubility without sacrificing the binding affinity.

In summary, we have reported the X-ray crystal structure of hnps-PLA_2 in complex with a highly potent inhibitor. The structure reveals an optimum fit of the bulky hydrophobic tails within the active site with minimal perturbation of enzyme conformation relative to the uninhibited state. The carboxylic acid function of **I** is a successful bioisosteric replacement of the *sn*-3 phosphate group. The coordinates of the complex have been deposited in the Brookhaven Protein Data Bank (code 1kvo).

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Supporting Information Available: Experimental procedures for the enzyme purification, crystallization, and structure determination (4 pages). Ordering information is given on any current masthead page.

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