Chapter II

Review of Literature

2.1. Phospholipase A\(_2\)

Phospholipase A\(_2\) (PLA\(_2\), E.C.3.1.1.4) is a small, 120 to 125 amino acid protein which cleaves the sn-2 ester bond in glycerophospholipids, releasing free fatty acids and lysophospholipids (Dennis, 1994). The chief fatty acid formed is Arachidonic Acid. This acid is a precursor of a large family of compounds collectively known as eicosanoids, which include PGs, LTs and TXs. The eicosanoids possess a number of biological activities, among which is their ability to mediate signs and symptoms of inflammatory reactions (Smith, 1992). In addition to eicosanoid biosynthesis, PLA\(_2\) plays another role in inflammation: The lysophospholipid formed is utilized to form platelet activating factor, which is a potent pro inflammatory molecule. (Dennis, 1997 & Snyder, 1995).

PLA\(_2\)s can be broadly classified into two groups based on their molecular masses. The high molecular mass enzymes that are located intercellularly and are calcium independent. Their molecular mass ranges from 40 to 140 KD. (Hazen, Stuppy and Gross, 1990 & Wolf and Gross, 1996). They are located in different cells and tissues, but not in snake venoms. A second group of high molecular PLA\(_2\) (85 to 100 KD) exist intracellularly which is calcium dependent (Leslie, 1991). The low molecular mass PLA\(_2\)s are small sized proteins with molecular mass 13 to 15 KD. They are isolated from platelets, synovial fluid, snake venoms, pancreatic juice etc. They fall into four groups I, II V and X. These groups are closely related and share a common mechanism of cleavage of sn-2 position in phospholipids.
Porcine Pancreatic PLA$_2$ is a 124 amino acid protein. Its crystal structure was first determined by Drenth et al (1973) at 3.0 Å resolution and later by Dijkstra, Drenth and Kalk (1983) at 2.6 Å resolution. Crystallization was done by 0.05 M Tris-maleate Buffer with 5mM CaCl$_2$. Precipiant used was 20% methanol.

The co-crystals of porcine pancreatic PLA$_2$ with various bile salts such as taurocholate were prepared and their structures determined by crystallography by Pan and Bahnson (2007). The bile salt complexes result in partially occluded active site. The ligand displays hydrogen bonding and extensive hydrophobic packing. The active site of this enzyme is made up of several residues: H48, D99, F5, I9, F22, A102 and F106 and the disulphide bridge between C29 and C45. Also, the catalytically essential calcium ion is in this region. A water molecule is located near H48 in the active site (Dijkstra et al., 1981). A second site for calcium ion has been identified in this enzyme, which is close to the N terminus. This calcium ion stabilizes the interaction between the two neighboring molecules in the crystal structure (Slotboom et al., 1978).

### 2.2. Mechanism of PLA$_2$ activity

Calcium ion is essential for PLA$_2$ activity. The primary role of Ca$^{2+}$ is the polarization of carbonyl group of sn-2 ester linkage in electrophilic catalysis. Calcium ion binds to the specific calcium binding loop of PLA$_2$ and makes appropriate conformation of binding site for substrate binding (Yu et al., 1993). The suggested catalytic mechanism of PLA$_2$ is initiated by a H48/D99/calcium complex within the active site. The calcium ion polarizes the sn-2 carbonyl oxygen while coordinating with a catalytic water molecule. H48 improves the nucleophilicity of the catalytic water via a
bridging second water molecule. It has been suggested that two water molecules are necessary to traverse the distance between the catalytic histidine and the ester. The basicity of H48 is thought to be enhanced through hydrogen bonding with D99. An asparagine substitution for H48 maintains wild-type activity, as the amide functional group on asparagine can also function to lower the pKₐ of the bridging water molecule. The rate limiting state is characterized as the degradation of the tetrahedral intermediate composed of a calcium coordinated oxyanion. The role of calcium can also be duplicated by other relatively small cations like cobalt and nickel. PLA₂ can also be characterized as having a channel featuring a hydrophobic wall in which hydrophobic amino acid residues such as F, L, and Y serve to bind the substrate (Verheji, Slothboom and Has, 1981).

Figure 2.1. Role of phospholipase A₂ in the production of inflammation mediatory compounds.
2.3. PLA₂ inhibitors

Many natural and synthetic PLA₂ inhibitors have been identified and reported. Some of them are being used as anti-inflammatory drugs. PLA₂ catalyzed hydrolysis of membrane phospholipids results in the stoichiometric production of a free fatty acid, most importantly arachidonic acid, and a lysophospholipid. Both of these phospholipid metabolites serve as precursors for inflammatory mediators such as eicosanoids, or PAF. Since it was initially discovered that non-steroidal anti-inflammatory drugs inhibit PG synthesis, a vast amount of drug development has been performed to selectively inhibit the production of the inflammatory metabolites of arachidonic acid while preserving their protective role. Selective COX 2 inhibitors that act on the inducible, inflammatory COX 2 enzyme, but do not affect the constitutive prostaglandin synthesis in cells that is mediated via COX 1 have been developed. The development of PLA₂ inhibitors as potential anti-inflammatory agents has also been extensively pursued since the release of arachidonic acid from membrane phospholipids by PLA₂ is one of the rate-limiting factors for eicosanoid production. In addition to the production of eicosanoids, PLA₂-catalyzed membrane phospholipid hydrolysis is also the initiating step in the generation of PAF, a potent inflammatory agent. Thus, inhibition of PLA₂ activity should, in theory, be a more effective anti-inflammatory approach. However, developing an inhibitor that would be selective for the production of inflammatory metabolites and not inhibit the beneficial properties of PLA₂ has so far proved to be elusive.

It has been reported that extremely high levels of PLA₂ was observed in the synovial fluid from the inflammatory conditions of joints of arthritic
patients. This suggested that PLA\(_2\) may be responsible for the state of inflammation (Pruzanski, Bogoch and Stefanski, 1991). It is also shown that the architectures of binding sites and the binding mechanisms of inhibitors in both human and snake venom PLA\(_2\)s are similar (Dennis, 1994).

Many PLA\(_2\) inhibitors have been obtained and described from natural and synthetic sources. The crystal structure of the complex formed between PLA\(_2\) and Aristolochic acid has been described (Chandra et al., 2002). The structure contains two crystallographically independent molecules of PLA\(_2\) in the form of an asymmetric centre with a molecule of aristolochic acid bound to one of them specifically. A hydrogen bond is established with D49 of the protein (Figure 2.2).

![Figure 2.2. The complex of PLA\(_2\) (pink) with aristolochic acid (yellow). A hydrogen bond of length 2.42 Å is formed with D49 of PLA\(_2\). (PDB ID-1FVO).](image)

Van der Waal’s interactions are formed with L2, F5, A18, Y22, S23 C45, H48 and F106 residues. The most significant differences introduced by
asymmetric association in the structures of the two molecules pertain to the conformations of their calcium binding loops, beta wings and C terminal regions. The structure is deposited in PDB with ID-1FVO. Aristolochic acid is obtained from a plant *Aristolochia* sps. This plant is used in ayurvedic medicine for the treatment of snakebites.

The complex of PLA₂ with a natural product anisic acid was determined at 1.3 Å resolution (Singh et al., 2006). Anisic acid is a constituent of Aniseed, which is used against inflammation and for treating snakebites in ayurvedic medicine. The X-ray crystal structure showed that anisic acid was located in the hydrophobic channel and fitted well in the binding site of the enzyme, forming a number of hydrophobic interactions with the residues of the channel. The ligand molecule appeared completely buried in the hydrophobic channel. The residues L2, F5, I9, A18, I19 and Y22 were found interacting with the ligand. It also forms hydrogen bond with G30 of the enzyme (Figure 2.3).

![Figure 2.3. The complex of PLA₂ (pink) with Anisic acid (yellow). No hydrogen bond is formed. (PDB ID-1TGM).](image)
Diclofenac is used in the treatment of rheumatoid arthritis, spondylitis, gout, sprains etc. It is a non selective inhibitor of cyclo-oxygenase enzymes. Its complex with PLA₂ at 2.7 Å resolution was determined (Singh et al., 2004). As in the case of other complexes, diclofenac was located in the hydrophobic channel and fitted well in the binding site of the enzyme. The binding was stabilized by a hydrogen bond established between H48 and an oxygen atom of the carboxylic group of diclofenac (Figure 2.4). It established hydrophobic interactions with L2, F5, I9 and F106 of the protein. The diclofenac molecule underwent slight changes in conformation upon binding with the protein. The overall structure of PLA₂ does not get perturbed much upon binding to diclofenac. The notable conformational changes occur in the calcium binding loop, C-terminal region and some surface loops.

Figure 2.4. The complex of PLA₂ (pink) with diclofenac (yellow). A hydrogen bond is formed of length3.03 Å with H48 of PLA₂. (PDB ID-2B17).
The complex of PLA$_2$ with eugenol has been studied (Kumar et al., 2007). Eugenol is the active constituent of cloves. It forms a highly stable complex with three hydrogen bonds with H48, D49 and C45 of the protein (Figure 2.5). van der Waal's interactions are established with F5, Y22, Y28, G30, C45 and H48 of the protein. This explains the use of eugenol as an anti-inflammatory and pain relieving drug in traditional as well as modern medicine.

Figure 2.5. The complex of PLA$_2$ (pink) with Eugenol (yellow). Three hydrogen bonds are established with C45, H48 and D49. (PDB ID-2QU9).

The complex of PLA$_2$ with oxyphenbutazone was prepared and its structure was solved at 1.6 Å (Singh et al., 2004). It was observed that oxyphenbutazone fits well in the binding site of the enzyme (Figure 2.6). A large number of hydrophobic interactions between the enzyme and ligand are observed. Y52 and K69 were observed to be interacting particularly
with the ligand. Other residues in the hydrophobic channel such as L3, F5, M8, I9 and A18 also interact with oxyphenbutazone. Being a flexible molecule, the conformation of the ligand changes markedly upon binding with the enzyme. The inherent flexibility and strongly hydrophobic nature of oxyphenbutazone are its favorable properties for interaction with the enzyme. But it does not form hydrogen bonds with the protein. However, its binding to active site suggests that the anti-inflammatory activity of this drug may be due to its PLA$_2$ inhibitory property.

![Figure 2.6. The complex of PLA$_2$ (pink) with oxyphenbutazone (yellow). No hydrogen bonds are formed. The interaction is purely hydrophobic. (PDB ID-1Q7A)](image)

The complex of PLA$_2$ with an antihypertensive drug, atenolol has been reported (Kumar et al., 2007a). This drug molecule does not form hydrogen bonds with the protein, but forms extensive van der Waal’s interactions with L2, F5, I19, H48, Y52 and K69 (Figure 2.7).
Besides the binary complexes, in which a PLA$_2$ molecule is associated with only one ligand, some ternary complexes in which a PLA$_2$ molecule is associated with two different ligands are known. A well known complex is with ajmaline and anisic acid. In this complex, anisic acid establishes a hydrogen bond of length 2.8 Å with K69 of the protein (Figure 2.8). This molecule forms hydrophobic interactions with L2, I19 and K69 of the protein. On the other hand, Ajmaline does not form hydrogen bonds, but enters into hydrophobic interaction with Y52, P56, D49 and G53 of the protein (Kumar et al., 2007b).
Figure 2.8. The ternary complex of PLA$_2$ (pink) formed with Ajmaline (yellow) and anisic acid (blue). Ajmaline does not form hydrogen bonds. Anisic acid forms one hydrogen bond of length 2.8 Å with K69 of the protein. (PDB ID- 2QUE).

Another ternary complex of PLA$_2$ with two synthetic compounds, indomethacin and nimesulide has been reported (Kumar et al., 2007c). Indomethacin forms a hydrogen bond of length 3.13 Å with K69 of the protein (Figure 2.9). Hydrophobic contacts are established with D49, T52, P56 and K 69. Nimesulide does not form hydrogen bonds. It forms hydrophobic interactions with L2, A18, I19 and K69 of PLA$_2$. 

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Figure 2.9. The ternary complex of PLA₂ (pink) with Indomethacin (blue) and Nimesulide (yellow). A hydrogen bond of length 3.13 Å is formed between indomethacin and K69 of PLA₂. (PDB ID- 2OTH).

2.4. Features of PLA₂ inhibitors

1. All the reported PLA₂ inhibitors are low molecular mass compounds, and are therefore suitable to be developed into drugs.
2. All of them possess a large hydrophobic surface such as benzene rings or other heterocyclic rings which are capable of entering into hydrophobic interactions with the active site residues of PLA₂, which reside in a hydrophobic channel.
3. Many of the inhibitors possess groups which form hydrogen bonds with the inner residues of the active site of the protein.
4. Many of the naturally occurring PLA₂ inhibitors are derived from plants exhibiting anti-inflammatory activity.
5. The amino acids commonly involved in hydrogen bonding are H48 and D49.

6. The three dimensional structure of the enzyme is altered only very slightly due to ligand binding. In the case of flexible ligands, the structure of the ligand undergoes appreciable changes upon binding.

<table>
<thead>
<tr>
<th>Complex of PLA&lt;sub&gt;2&lt;/sub&gt; ligand with PDB ID</th>
<th>H-Bond interactions</th>
<th>Vander Waal’s interactions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aristolochic acid (1FVO)</td>
<td>D49</td>
<td>L2, F5, A18, Y22, S23, C45, H48, F106.</td>
</tr>
<tr>
<td>Anisic acid (102E)</td>
<td>G-30</td>
<td>L2, F5, I9, A18, Y22.</td>
</tr>
<tr>
<td>Oxyphenylbutazone (1Q7A)</td>
<td>Nil</td>
<td>L2, F5, M8, I9, A18.</td>
</tr>
<tr>
<td>Diclofenac (2B17)</td>
<td>Nil</td>
<td>L2, F5, I9, F106.</td>
</tr>
<tr>
<td>Atenolol (2OTF)</td>
<td>Nil</td>
<td>L2, F5, I19, H48, K69.</td>
</tr>
<tr>
<td>Ajmaline - Anisic acid (2QUE)</td>
<td>K69 with Anisic acid</td>
<td>D49, Y52, G53, P56</td>
</tr>
<tr>
<td>Indomethacin-Nimesulide (2OTH)</td>
<td>K69 with Indomethacin</td>
<td>L2, A18, I19, D49, T52, P54, K69</td>
</tr>
</tbody>
</table>

Table 2.1. Interactions of various natural and synthetic compounds with PLA<sub>2</sub>.
2.5. Cyclo-oxygenase Inhibitors:

Crystal structures of several COX 1 or COX 2 inhibitors have been published in the PDB. They are small molecules which are used or can be developed into anti inflammatory drugs.

Crystal structure of a commonly used anti inflammatory drug diclofenac bound to the active site of COX 2 from mouse was determined at 2.90 Å (Kiefer et al., 2003). The ligand molecule establishes two hydrogen bonds with Y385 and S530 of the protein. Numerous van der Waal’s contacts are also established with V349, A527, S530, Y 385, M527, G526 and W387 of the protein. The structure is available in PDB with ID 1PXX (Figure 2.10).

![Figure 2.10. Crystal structure of the complex of diclofenac and COX 1. (PDB ID 1PXX).](image)
The structure of sheep COX 1 complexed with indomethacin alpha ethyl ethanolamide has been reported at 2.85 Å (Harman et al., 2007). The ligand establishes three hydrogen bonds with E524, R120 and Y355 of the protein. A number of van der Waal’s contacts are formed with S353, L352, F518, I523, V349, A527, S530 G256, W387 and R120. The structure is available in the PDB with ID 2OYE. (Figure 2.11).

![Figure 2.11. Crystal structure of the complex of a derivative of indomethacin with COX 1. (PDB ID 2OYE).](image)

The crystal structure of sheep COX 1 complexed with an anti inflammatory drug celecoxib was determined at 2.75 Å (Rimon et al., 2009). Three hydrogen bonds are established between the protein and the ligand. The residues Q192, L352 and W518 made these bonds. A large number of van der Waal’s contacts are established with the residues H590, S516, L352,
S353, I523, F518, Y355, V349, A527 and M522. The structure is deposited with the PDB ID 3KK6 (Figure 2.12.).

![Crystal structure of COX 1 in complex with celecoxib. (PDB ID 3KK6).](image)

**Figure 2.12.** Crystal structure of COX 1 in complex with celecoxib. (PDB ID 3KK6).

### 2.6. *Cardiospermum halicacabum*

Balloon vine, (*Cardiospermum halicacabum*) of Sapindaceae family is a herbaceous climber found throughout the plains of India and elsewhere. The plant is a dioecious, hairy climbing vine with clusters of white flowers framed by finely dissected delicate foliage. The blooms are followed by attractive balloon-like seedpods that have inspired the common name “Balloon vine”. The pods are filled with black seeds, with a heart shaped white mark which earned the scientific name “*Cardiospermum*”.

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Balloonvine is extensively used in Ayurvedic system for the treatment of rheumatism, lumbago, fever, snakebites etc. (Nadkarni, 1976). In Africa, balloon vine is used to treat snakebites, nervous diseases, lumbago, rheumatism etc. (Venkatesh Babu and Krishnakumari, 2006). Leaves of this plant are mixed with castor oil and administered internally for lumbago and rheumatism (Kirthikar, Basu and Ann, 1969). Kumaran and Karunakaran (2006) investigated the antioxidant activity of the methanolic extract of balloon vine by employing various methods established in vitro systems. The multiple antioxidant activity was evident, as it possessed reducing power, superoxide scavenging ability, NO scavenging activity and ferrous ion chelating potency. The antipyretic activity was evaluated by Asha and Pushpangadan (1999). Different extracts of this plant showed positive antipyretic activity in rats with yeast induced pyrogenesis. Ethanolic extract was most active. It was reported by Khunkitti, Fujimaki and Aoki (2000) that the extracts of this plant has a mild but definite microfilaridical action against *Brugia pahangi*.

The antihyperglycaemic effect of *Cardiospermum halicacabum* in streptozotocin induced diabetic rat models was investigated (Veeramani, Ganesan and Vishwanathan, 2008). It was observed that alcoholic extract of this plant had antihyperglycaemic activity and provided evidence for its traditional use in the control of diabetes. The rats showed a significant decrease in glycosalated haemoglobin levels, which might be due to antihyperglycaemic effect of *Cardiospermum halicacabum*. The effect was weaker than the standard glibenclamide.

The anti-inflammatory effect of *Cardiospermum halicacabum* was investigated in mouse macrophage cell lines RAW 264.7. It was revealed that the ethanol extract of the whole plant inhibited dose dependently the
mRNA expression of COX 2, TNF-α iNOS and COX 2 protein expression. But the extract did not affect the expression of mRNA of COX 1 (Sheeba and Asha, 2009).

The ethanolic extract of balloon vine inhibited gastric ulcers in rats. The administration of the extract also resulted in an increase in the level of gastric glutathione and a decrease in alkaline phosphatase activity. The extract also displayed potent in vitro hydroxyl radical scavenging effect and inhibition of lipid peroxidase activity (Sheeba and Asha, 2006).

The larvicidal activity of aqueous and ethanolic extracts of *Cardiospermum halicacabum* against *Strongyloides stercoralis* was investigated (Boonmars *et al*., 2005). They reported immobilization of larvae within 72 hours of exposure. The essential oil from the seeds produced an immediate fall in the blood pressure in anaesthetized dogs. A water soluble fraction obtained from the ethanolic extract of this plant caused a prolonged hypotensive action in dogs (Modi and Deshmankar, 1972).

An alkaloid fraction of the plant obtained from its seeds showed in vitro antibacterial action against some pathogenic organisms, transient hypotension and cardiac inhibition in dogs. It blocked the spasmogenic action of acetylcholine, histamine and 5-Hydroxytryptamine on guinea pig ileum and dog tracheal chain, exhibited typical tonic effect on rat uterus and a biphasic effect on frog rectus abdominalis muscle (Shukla, Modi and Deshmankar, 1973).

The petroleum ether extract of the seeds have a strong insect repelling property, as tested on the insect *Tribolium castaneum* (Khan *et al*., 1983). Aqueous extract of the fruits of this plant showed remarkable anti phage activity towards several bacteriophages in *E. coli* and *Pseudomonas aeruginosa* (Deliteous *et al*., 1972).
Balloon vine contains proanthocyanidin, apigenin and stigma sterol (Dass, 1966). Hopkins, Ewing and Christom (1968) reported the isolation of a volatile ester, Methyl 4, 4 dimethoxy 3- (methoxymethyl) butyrate from the seeds of this plant. The seeds also contain luteolin. (Tifikar and Mustaq 1993). Rao and Gunasekhar (1987) reported pinitol and the glucouronides of apigenin, luteolin and chrysoeriol from the leaves. The leaves contain saponin, tannins and traces of alkaloids (Gopalakrishnan, Dananjayan and Kameshwaran, 1976), such as berberine (Lewis and Lewis, 2003). The leaves contain flavones such as apigenin, acacetin, 7-O-Methyl apigenin, phenolic acids such as vanillic acid, syringic acid, melitotic acid, p-coumaric acid and ferulic acid (Daniel, 2006). Ethanol extract of this plant contains phlobatannin and phlobaphene (Desai and Sethna, 1954). The seeds of balloon vine contain 33% fatty acids, out of which 11-eicosenoic Acid is the major component. Other components include oleic acid, arachidic acid, linolenic acid, palmitic acid and stearic acid (Chisolm and Hopkins, 1958). Seed chemistry studies of Cardiospermum halicacabum indicate a high protein content as high as 35% of dry weight, which is higher than that in popular legumes. The high protein content has resulted in its utilization in cattle feeds (Prakash, Jain and Mishra, 1988). The seeds contain some toxic cyanogenic compounds chiefly cardiospermin (Conn, 1980) and therefore must be properly processed before using as a source of nutrition. Phytochemical studies revealed the presence of sterols and flavonoids (Khanna, Javed and Khan, 1990). The most common use of balloon vine in medicine is for the treatment of Rheumatoid Arthritis. Ethanolic extract of this plant may be useful as an anti-inflammatory preparation. The development of edema induced by carragnenan in rat paws was significantly reduced in rats fed with the extract. The inflammatory properties of the
extract were also evident in cotton pellet granuloma assay, where cotton pellets were implanted subcutaneously and inflammation evaluated. In general, there was a reduction in the lipid peroxide content and PLA$_2$ activity in the exudates of cotton pellet granuloma (Sadique et al., 1987 & Gopalakrishnan, Dananjayan and Kameshwaran, 1976). It was concluded that a reduction in PLA$_2$ activity may down regulate the prostaglandin biosynthesis, thus reducing inflammation. Conclusive information of the active ingredients in this plant is not known. It appears that anti-inflammatory properties of this plant may be the major effect that causes a reduction in the symptoms associated with rheumatism, wounds and swellings as observed in its use in traditional medicine (Raghupathy et al., 2007).

Hence the present investigation was conducted to identify the active constituent responsible for PLA$_2$ inhibition and hence, the anti inflammatory activity of this plant.