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REVIEW ARTICLE

PHOSPHOLIPASE A2 IN AIRWAY DISEASES: TARGET FOR DRUG DISCOVERY

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ABSTRACT

Importance of the Field:

Inflammatory airway diseases are on the rise world over. There is need for orally active, safe and efficacious anti-inflammatory agent. Existing therapeutic options are plagued by poor efficacy and adverse effects upon long term use. In our search for a novel drug discovery target, we tried to understand phospholipase A2 (PLA_2) class of enzymes.

Phospholipase A_2, a major component of cell membranes, belongs to a family of enzymes that generate arachidonic acid and lysophospholipids from glycerophospholipids. PLA_2 reaction is considered as the first rate-limiting step for the production of several lipid mediators notably arachidonic acid. Arachidonic acid, in turn, is metabolised to prostaglandins and leukotrienes. Importance of arachidonic acid metabolities in inflammation is well established. Inhibitors of prostaglandin and leukotriene biosynthesis have been approved as drugs for arthritis and asthma. Despite intensive research no PLA2 inhibitor has progressed through advanced clinical trials to become a drug.

Area covered in this review: In this review, we shall look into different PLA_2 enzymes and their role in generation of bioactive mediators. Drug discovery effort towards designing PLA_2 inhibitors and the biological data generated thereof. No effort will be made to elaborate in detail biochemistry of PLA_2 isoforms. We shall try to understand why in spite of controlling generation of all eicosanoids, PLA_2 inhibitors did not show efficacy in clinical trials with special reference to respiratory diseases.

What the reader will gain: An understanding of PLA_2 enzymes and their role in generation of bioactive lipid mediators. Interference with PLA_2 activity and its consequence on airway inflammation in experimental animals and in clinical trial will help us for better therapeutics with respect to inflammatory disorcders.

Take home message: PLA_2s are important enzymes of lipid membrane metabolism and plays a major role in etiology and pathology of inflammatory diseases. However, no drug has been approved from this target class as anti-inflammatory therapeutics, thus revisiting each enzyme, and target them selectively may prove a potential to the airway disorders.

Key Words: PLA2, Airway Inflammation, Asthma

INTRODUCTION:

COPD and Asthma is rated as the most severe disease of the pulmonary system, carrying major risks. It has been postulated that the mortality rate will be in the top three by the end of 2020. The annual healthcare expenditure on COPD is estimated as € 1.2 billion in UK and € 12 billion in the US. None of the available treatments have been shown to slow the progression of COPD or suppression of inflammation in small airways or lung parenchyma. However, there are a few new treatments that are undergoing clinical trials that suppress the inflammatory pathways or inflammatory cells, which seems to be a more logical approach for COPD therapy. These therapies include small molecules against adhesion molecules, chemokines, cytokines and enzymes involved in cell signaling.

Asthma is an allergic disease affecting upper respiratory tract. Rapid industrialization, air pollution, increasing population and changing life style are contributing towards increased incidence of bronchial asthma. Although, moderate asthma is well controlled by inhaled corticosteroids, a population of asthma patients does not respond to steroids and need hospitalization. In addition, patient compliance improves with therapy being administered in the form of an oral dosage form. Existing orally active anti asthma therapeutics are either poorly efficacious or suffer from adverse effect upon prolonged use.

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It has become clear that membrane lipids act as a reservoir of important biologically active molecules. Lipases are enzymes that release biologically active molecules from membrane lipids. A key lipase enzyme family consists of phopholipase A_2 (PLA_2). PLA_2 contributes towards release and/or formation of at least three important lipid mediators from membrane - arachidonic acid, platelet activating factor and lysophosphatidic acid (Fig.1). Whereas platelet activating factor and lysophosphatidic acid bind to their respective G-protein coupled receptors (GPCR) arachidonic acid is metabolized to produce pro-inflammatory mediators like leukotrienes and prostaglansdins. Arachidonic acid metabolic pathways have been well studied and its intervention resulted in drugs, like Montelukast, Celecoxib, Zileuton. In this section, we shall look at PLA_2 family of enzymes and evidence that link them to airway inflammation. Effort that has gone into discovery of PLA_2 inhibitor and their development status will be discussed.

Phospholipase A_2:

Phospholipase A_2 has been studied in detail by several groups [1-7]. These enzymes cleave glycero-phospholipids (Fig. 2), which are esters of long chain fatty acids with glycerol. One of the hydroxyl groups of glycerol is usually coupled with phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol or phosphatidyl ethanolamine. As shown in fig. 2, R1 and R2 in glycerolipids represent long chain fatty acids. Usually R1 is a saturated fatty acid while R2 is an unsaturated fatty acid. R3 can be choline, ethanolamine, serine or inositol. PLA_2 enzymes cleave a phospholipid at R2 position, also called sn-2 position, releasing a fatty acid, usually arachidonic acid, and creating a lysophospholipid.

Broadly, as shown in Table 1, PLA_2 enzymes can be grouped into cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$) and calcium independent PLA_2 ($iPLA_2$). Classification is based on molecular weight, calcium requirement, structural features, substrate specificity and functional role. It is important to mention that in this article no effort will be made to understand in detail classification of different PLA_2 subtypes. Foucs of this article will remain on PLA_2 subtypes around which experimental animal data have been generated using gene knockout / knock in technology or using pharmacological tools. We shall also explore molecules that have moved into clinical development from focused PLA_2 inhibitor programs.

Cytosolic Phospholipase A_2:

Cytosolic phospholipase A_2 ($cPLA_2$) can be divided into several subgroups. For the purpose of this article, most of the discussion will be around $cPLA_2$ – α subtype. However, it is important to know that several additional subtypes of $cPLA_2$ exists, namely - β, γ, δ, ε and ζ (reviewed in [6-8]. $cPLA_2$ β and γ isoforms have about 30% homology with α subtype. Exact functional significance of and $cPLA_2$β and $cPLA_2$γ is not known.

Cytosolic PLA_2 is a 85 KDa protein. The enzyme has a calcium binding site (CALB) at amino terminal end and a phosphorylation site. Calcium dependent phospholipid binding site (CALB) binds calcium and promotes translocation of the enzyme to membrane. For enzyme activity, cPLA2 needs phosphorylation at active site serine residue (Ser 505). A wide variety of different agents including cytokines, growth factors, hormones, mitogens, calcium ionophore etc. can promote gene expression as well as activation of $cPLA_2$ by promoting phosphorylation and migration to membrane. Cytosolic PLA_2 is present in many different cell types with the exception of mature lymphocytes [2, 3]. In response to stimulus induced elevation of intracellular calcium level, $cPLA_2$ moves from cytosol to perinuclear and endoplasmic reticular membranes where enzymes for arachidonic acid metabolism are located. $cPLA_2$ is a phospholipase that is specific for arachidonic acid containing phospholipid namely phosphatidyl inositol [3, 4, 7].

Secretory Phospholipase A_2:

Secretory PLA_2 ($sPLA_2$) family has several different members. Namely, phospholipase A2 – I, IIa, V and X. Biological role of these enzymes have been relatively well understood. However, there are several other members in $sPLA_2$family (Table 2), for which biology is not as clear as well as a few of them are present in species other than humans.

Secretory phospholipases are 13 – 15 KDa proteins. These proteins have 6 – 8 disulphide bridges that give rigidity to their tertiary structure and protect these proteins from proteolysis in circulation. Secretory phospholipase A_2 enzymes need millimolar calcium for activation. The secretory phospholipase enzymes have an N-terminal signal peptide to facilitate secretion **[5]**. Secretory phospholipase A2 enzymes are distributed in different cell types **[9, 6]**). Message of type IIA, type V and type X $sPLA_2$ enzymes have been reported in lung epithelial cells. In addition, inflammatory cells like neutrophils express type V and type X $sPLA_2$. Type IIa is secreted by alveolar macrophages as well as by eosinophils. Type X is expressed mainly in immune cells. Unlike cytosolic phospholipase A_2, secretory phospholipases are not selective for arachidonic acid containing lipids.

Calcium Independent PLA_2:

Three different enzymes come under the category of calcium independent PLA2. Namely, calcium-indepdendent PLA_2 (subtypes β and γ), acidic calcium independent PLA_2 (aiPLA2), lysosomal PLA_2 ($LPLA_2$) and PAF acetylhydrolase (PAF-AH). However, these differ in their molecular weight, substrate specificity, functional activity etc, as shown in table 1 [10, 6, 7].

Group VI PLA2 family can be divided into gVIPLA2 into gPLA2VIA and gPLA2VIB categories [10, 7]. gviPLA2A belongs to chromosome 22q13.1 whereas gviPLA2B belongs to chromosome 7q31. GviPLA2A undergoes post translational modification to give five splice variants gviPLA2A1, 2, 3, 4 and 5. GviPLA$_{2A1}$, A$_2$ and B have consensus GXSXG sequence with an active site serine residue. However, gviPLA$_{2A4}$ and 5 do not have any consensus sequence and do not exhibit any enzymatic activity. At the N terminal region, PLA$_{2VIA}$ enzyme family has 7 – 8 ankyrin repeats, whereas PLA$_{2VIB}$ does not. All enzymes have ATP binding sites. GviPLA$_2$ members do not need calcium for enzyme activity. Infact, it has been shown that depletion of calcium activates PLA$_2$ activity. Calmodulin binding reduces enzyme activity whereas protein kinase C regulates enzyme activity positively.

Group gviPLA2A$_2$ and gviPLA$_{2B}$ enzymes are also known as iPLA$_2$ β and γ, respectively. Calcium independent PLA$_{2\gamma}$ protein expression is seen in mouse and rat heart and human platelets. PLA$_{2\beta}$ and PLA$_{2\gamma}$, have been implicated in arachidonic acid release, monocyte activation and chemotaxis [11, 12, 13]. Additional members of gVIPLA$_2$ family have been reported such as – gVIPLA$_{2C}$ (PLA $_{2\delta}$), gVIPLA$_{2D}$ (PLA $_{2\varepsilon}$), gVIPLA$_{2E}$ (PLA$_{2\zeta}$) and gVIPLA$_{2F}$ (PLA $_{2\eta}$) [7]. Other subunits of iPLA$_2$, namely δ, ε, ζ, , η isotypes have role in triacyl glycerol hydrolysis and acylcoA independent transacylation [14].

Remaining calcium independent phospholipase A$_2$ can be grouped into those that regulate lung surfactant catabolism and those that regulate hydrolysis of platelet activating factor. Lysosomal phospholipase A$_2$ (LPLA$_2$) and acidic calcium independent PLA$_2$ (aiPLA$_2$) are two enzymes that regulate lung surfactant metabolism. Both enzymes need acid optima of 4 for activity and a catalytic serine residue for activity. LPLA$_2$ is present in alveolar macrophage where as aiPLA2 is presented in alveolar eptithelium. LPLA$_2$ acts on phosphatidyl choline and phosphatidyl ethanolamine, whereas aiPLA$_2$ acts on dipalmitoyl phosphatidyl choline. In addition to phospholipase A$_2$ activity, LPLA$_2$ also has phospholipase A$_1$ activity and ceramide O acyl transferase activity. AiPLA$_2$ has glutathione dependent peroxidase activity [6].
PAFAH enzyme can be grouped into type VII and type VIII PLA$_2$. Type VII enzyme includes a circulating PAFAH or lipoprotein associated PLA2 (LpPLA$_2$) and a liver enzyme (PAFAH$_2$). Type VIII enzymes are is intracellular and can be grouped into type I and type II PAFAH. Type VIII PAFAH is strictly specific for PAF alone. Type VII enzymes recognize upto 6 carbon length acid at Sn-2 position [15].

Role in inflammation:

It is well established that arachidonic acid metabolites play important role in the pathophysiology of inflammatory diseases like bronchial asthma. Since cPLA$_2$ is key to release of arachidonic acid from phospholipids, it is implicit that an effective inhibitor of cPLA$_2$ may be effective in treating asthma along with other inflammation associated diseases..

It has been shown using different tools that interference with phospholipase expression reduces production of leukotrienes and prostaglandins. Using selective inhibitors, several groups have shown [16-18] that blocking cPLA$_2$ results in attenuation of production of arachidonic acid, prostaglandins and leukotrienes. Similar studies have been done using gene knockout animals. Peritoneal macrophages from cPLA$_2$ gene knockout mouse are deficient in producing prostaglandin and leukotrienes upon challenge with calcium ionophore [19].

In experimental animal models of asthma, acute respiratory distress syndrome and bleomycin induced lung fibrosis a role of cPLA$_2$ has been ~~was~~ demonstrated. Uozumi *et al* (1997) investigated the effect of cPLA$_2$ gene knockout in mice on allergen induced airway response and airway inflammation [19]. Mice were made allergic to ovalbumin by repeated exposure. Wild type mice developed lung resistance following ovalbumin exposure that peaked in 2 min. The peak lung resistance value in cPLA$_2$ gene deleted animals was lower and recovery to baseline was much faster compared to wild type animals. Compared to allergic wild type animals, development of airway hyperresponsiveness to methacholine was much less pronounced, almost comparable to nonallergic animals, in gene deleted animals. Upon histopathological examination, allergic wild type animals exhibited narrowing of airway and thickening of alveolar septum. These features were absent in cPLA$_2$ (-/-) mice. Data suggested that in mouse allergy model, cPLA$_2$ plays an important role in development of allergen induced bronchoconstriction, airway hyperresponsiveness and airway inflammation (Table 3).

Findings of gene knockout study were confirmed using small molecule inhibitors of cPLA$_2$. In a model where mouse were made allergic to ovalbumin by repeated exposure, administration of an inhibitor by oral route attenuated allergen induced anaphylactic response and nonspecific hyperreactivity to methacholine [16]. In a different study using allergic balbc mouse, Choi et al. (2005) demonstrated biphasic airway reactivity – early response and late response, to ovalbumin [20]. The authors observed that expression of cPLA$_2$ peaked around 3 hours post allergen challenge. Blocking PLA$_2$ expression using TNF antibody, attenuated late phase airway reactivity and eosinophil influx. In a different animal species, Myou *et al* (2001) demonstrated that allergic airway inflammation and airway hyperresponsiveness can be blocked by cPLA$_2$ inhibition in guinea pig [21].

LPS induced airway inflammation is used as a model of acute respiratory distress syndrome. In two

different studies, Nagase *et al* (2000 and 2002) investigated the role of $cPLA_2$ in acute respiratory distress syndrome [22, 23]. Respiratory distress was induced by administration of intravenous LPS / zymosan to mice. LPS exposure increased lung resistance, plasma carbon dioxide concentration. Protein level in bronchoalveolar lavage fluid increased indicating increased vascular permeability. Neutrophil count and myeloperoxidase activity in lavage fluid increased following LPS challenge. Markers of arachidonic acid metabolism, namely thromboxane A_2, cysteinyl leukotrienes and leukotriene B4 increased in lavage fluid. In $cPLA_2$ (-/-) mice, increase in lung resistance was much less pronounced following LPS challenge. Absence of cPLA2 gene protected animals from LPS induced mortality. Most physiological, biochemical, histopathological and inflammatory abnormalities induced following LPS administration to wild type mice, were very close to normal in gene deleted mice [22]. Administration of $cPLA_2$ inhibitor, Arachidonyl trifluromethyl ketone, at a dose of 20 mg/kg by intraperitoneal route reversed in a statistically significant manner most of the abnormalities in lung function, lung inflammation at cellular and molecular level [23].

To explore the role of cPLA2 in initiation and propagation of lung fibrosis, Nagase *et al* (2002) used bleomycin induced lung fibrosis model in $cPLA_2$ gene knock out mouse. Bleomycin was administered by intratracheal route [23]. Fourteen days after bleomycin administration, mice were monitored for lung elastance, lung inflammation, lung fibrosis, arachidonic acid breakdown products in bronchoalveolar lavage fluid and lung hyrdroxyproline content. Bleomycin increased lung elastance and upon histopathology of lung there was evidence of collagen deposition and neutrophil infiltration. There was increase in protein and neutrophils in bronchoalveolar lavage fluid along with prostaglandins and leukotrienes. Most of the changes observed in wild type mice following bleomycin treatment were attenuated in $cPLA_2$ gene knockout mice exposed to bleomycin. On many parameters, $cPLA_2$ (-/-) mice were very similar to wild type mice not treated with bleomycin.

In addition to airway inflammation, studies have shown that mice lacking phospholipase A_2 gene exhibit resistance to collagen induced arthritis and experimental autoimmune encephalomyelitis [24].

Secretory Phospholipase A_2 and Airway Inflammation:

Secretory phospholipases are normally undetectable in healthy tissue. Exposure to inflammatory stimuli induces expression $sPLA_2$. Using transfected cell lines, purified / recombinant $sPLA_2$ and selective inhibitors, it has become apparent that $sPLA_2$ promote arachidonic acid metabolism, chemokine and cytokine production, lung surfactant catabolism [6].

Inflammatory Mediator Release:

Group IB PLA_2 and group X PLA_2 are capable of inducing production of cytokines in macrophages. It is suggested that this activity is mediated through activation of specific cell surface receptors. Type IIA enzyme is secreted by alveolar macrophages, whereas type X expression goes up in alveolar epithelium and interstitial tissue below epithelium. Addition of secretory PLA_2 (type IB and IIA) to airway epithelial cells result in generation of arachidonic acid [6]. Transfection of lung epithelial cells with secretory PLA_2 genes (Type V, X) results in secretion of arachidonic acid and prostaglandin production. Group V PLA_2 release LTB4 from neutrophils.

Studies have shown that $sIIPLA_2$ inhibitors like SB 203347, BMS 181162, Variablin inhibit A23187 induced release of arachidonic acid, PAF and leukotriene B4 from human neutrophil [25-27]. In another study, Snyder *et al* (1999) reported $sIIPLA_2$ inhibit release of thromboxane A_2 from guinea pig lung bronchoalveolar lavage cell [28]. This can be inhibited by LY315920. Using inhibitors of phospholipase A_2, it has been shown that PLA_2 induced arachidonic acid release and PLA_2 induced contractile response of guinea pig lung pleural strips can be blocked by inhibitor [28].

Secretory phospholipase A_2 enzymes play role in airway inflammatory disease by (i) promoting release of arachidonic acid metabolites from inflammatory cells, (ii) promoting bronchoconstriction, (iii) recruiting inflammatory cells to the airway (v) participating in airway remodeling, (vi) promoting lung surfactant breakdown (Table. 4).

Efficacy in Airway Inflammation Model:

Experimental animals exposed to antigens, allergens, lipopolysaccharides or humans suffering from inflammatory diseases of airway like asthma [29, 30], acute respiratory distress syndrome etc, and expression of secretory PLA_2 increases and many of these enzymes are detected in the bronchoalveolar lavage fluid [6].

Expression of group V $sPLA_2$ was observed in lung sections of mice made allergic to ovalbumin. Allergic mice exposed to inhaled $gVsPLA_2$, exhibited bronchoconstriction. Anti $gVsPLA_2$ antibody blocked expression $sPLA_2$, $sPLA_2$ induced bronchoconstriction, airway hyperresponsiveness to methacholine and eosinophilia [29]. In the same study it was observed that $gVsPLA_2$ gene knockout mice did not exhibit allergen induced bronchoconstriction and eosinophilic inflammation. Henderson *et al* (2007) created a mouse model of asthma where animals were made allergic by repeated exposure to ovalbumin as shown in table 5. By day 29, animals exhibited circulating levels of IgE, airway reactivity to methacholine, and increase in eosinophils in airway, and mucus glycoproteins, cytokines like IL4, IL5, IL13, and arachidonic acid breakdown products in the lavage fluid.

Upon continuation of ovalbumin challenge by day 76, mice develop goblet cell hyperplasia, subepithelial collagen deposition, increased smooth muscle mass and airway wall thickening. In $sXPLA_2$ gene deleted mice, airway reactivity to methacholine, airway inflammation markers, prostanoid breakdown products were blunted in a statistically significant manner compared to allergic wild type mice, and were similar to naïve non allergic mice. Similarly, on parameters of airway remodeling, collagen deposition, goblet cell hyperplasia and mucus occlusion of airway, mice without $cPLA_2$ and non allergic mice were very similar and statistically significantly different from allergic mice. Secretory PLA_2 X deficient mice exhibit resistance to ovalbumin induced airway inflammation, smooth muscle hyperplasia and subepithelial fibrosis compared to wild type control.

Secretory phospholipases A_2 IB, stimulate mucus secretion, induce airway inflammation, and produce secretory hyperresponsiveness to neutrophil elastase in ferret trachea [32].

Effect on Lung Surfactant Metabolism – *in vitro* and *in vivo:*

Secretory PLA_2 are capable of hydrolyzing phospholipid component of surface active agents in the lung. Group IB, V and X hydrolyze phosphatidyl-choline whereas group IIA hydrolyze phosphatidyl-glycerol [9]. Arbibe *et al* (1998) demonstrated induction of $gIIsPLA_2$ in guinea pig lung following LPS exposure [33]. This was followed by increase in the level of fatty acid and lysophosphatidyl choline. Inhibitor of $sPLA_2$, Ly 311727 inhibited LPS response. Similar response was observed, when recombinant guinea pig $sPLA_2$ was administered intratracheally to guinea pig.

Ohtsuki *et al* (2006) reported that $gVsPLA_2$ overexpressing mice exhibit abnormality in lung architecture and die immediately after birth [8]. These mice exhibit thickened aleveolar wall and narrow airway. On the other hand $gXsPLA_2$ overexpression does not affect lung architecture.

Secretory phospholipases are synthesized and secreted by one inflammatory cell and to act on a different cell type. These enzymes can act on extracellular as well as intracellular surfaces of cells to release arachidonic acid. It has been shown that, depending upon cell type involved, secretory PLA_2 enzymes (type IIA, and V) can release arachidonic acid in a cytosolic PLA_2 dependent and independent manner [6]. It is not clear if there exists cells surface receptors to which these proteins bind.

Calcium Independent PLA_2 and Airway Inflammation:

Calcium independent PLA2 have been ascribed a house keeping role [4, 6, 7, 10, 34]. Lot of evidence exists in support of a role of $iPLA_2$ in cells and tissues other than airway, for generation of arachidonic acid and prostaglandin generation. These responses are sensitive to inhibition by antisense oligonucleotide of $gviPLA_{2b}$ as well as by small molecule inhibitors like arachidonyl fluoromethyl ketone and bromoenol lactone [12]. In cardiac tissue PLA_{2b} activation has been linked to ventricular arrhythmias [4, 10]. Evidence of a role of $gviPLA_2$ in airway is lacking. Recently, it has been shown that in small airway epithelial cells, $gviPLA_{2g}$ plays a role in arachidonic acid prostaglandin and platelet activating factor generation. This response can be antagonized by PLA_2 inhibitor R-BEL [11]. Using monocytin cell line Tay and Melendez (2004) have demonstrated that $gviPLA_{2b}$ plays a role in monocyte activation [12]. Mishra *et al* (2008) have further extended this observation when they have shown that $gviPLA_{2b}$ plays a role in MCP-1 induced monocyte chemotaxis [13]. Inhibition of $gviPLA_{2b}$ using antisense oligonucleotide prevented monocyte migration to the site of inflammation in thiogylcolate induced peritonitis model in mouse. However, no evidence exists in support of a role of PLA_{2b} in airway inflammation.

Of different calcium independent PLA_2 enzymes, most evidence in support of a role in airway inflammation exists for circulating type VII PAF-AH ($LpPLA_2$). Data has been generated in experimental animal models in support of a protective role of rPAF-AH in different experimental setup – PAF induced mortality model [35], allergen induced airway inflammation and airway hyperreactivity model in mouse [36]. Data from human subjects also suggest a role of PAF-AH in asthma pathophysiology. A negative correlation was shown between circulating plasma PAF-AH level and severity of anaphylaxis [37]. Subjects with point mutation in PAF-AH gene (V279F) exhibit an inactive enzyme, greater propensity for asthma prevalence and increase in severity of asthma attack [6]. Inspite of so many positive data, clinical trial of recombinant PAF-AH (1 mg/kg) in fourteen human allergic asthma patients did not offer any protection on early and late allergic response or sputum eosinophil count [38].

Phospholipase A2 Inhibitor:

Indications for which PLA_2 inhibitors are being pursued include sepsis, acute pancreatitis, inflammatory skin and bowel diseases, and rheumatoid arthritis.

Discovery of phospholipase A_2 inhibitor is hampered by complication *in vitro* enzyme assay [5, 39]. Phospholipase A_2 is a soluble enzyme, whereas its substrate lipids are insoluble. For effective enzyme activity, enzyme has to bind to lipid water interface first. This binding is independent of catalytic activity of the enzyme. Bound enzyme will have access to lipid substrate and enzyme activity will again depend on its substrate specificity. Thus enzyme activity is controlled by equilibrium between bound and free enzyme, substrate accessibility, and actual enzyme kinetics. Many compounds that can affect partitioning of the

enzyme between lipid water interface, may come out as false positive. High incidence of false positive in *in vitro* assay leads to poor in vivo efficacy.

Secretory PLA2 Inhibitor:

Many pharmaceutical companies had programs to design and develop secretory phospholipase A_2 inhibitors – Bristol Myers Squib, Yamanouchi, Glaxo Smith Kline, Eli-Lilly [26, 40, 25, 41, 42, 28]. Table 6 lists *in vitro* potency of some of the compounds.

Eli-Lilly had the most advance program for discovery of $sPLA_2$ inhibitor. Two molecules have entered clinical trial from their discovery program. Crystal structure of non pancreatic secretory PLA_2 bound with indole acetamide analog LY31127 was reported [43]. Working with indole acetamide and indole glyoxamide, Dillard *et al* (1996) and Draheim *et al* (1996) established detailed structure activity relationship of this class of compounds for human non pancreatic secretory PLA_2 [44, 41]. Draheim *et al* (1996) reported discovery of LY315920. This molecule has a glyoxamide group at 3 position of indole and an oxyacetic acid group at position 4 as shown in Fig 3 [41]**.** LY315920 inhibited secretory PLA_2 with an IC_{50} of 7 nM and inhibited contraction of guinea pig lung tissue with an IC_{50} of 83 nM and more than 20 fold exhibited selectivity over pancreating PLA_2. Snyder *et al* (1998) have studies LY315920 in greater detail and reported that LY315920 inhibited release of $sPLA_2$ induced release of thromboxane A_2 from guinea pig lung bronchoalveolar lavage cells both *in vitro* and *ex vivo* [28]. When administered by intravenous and oral routes, LY 315920 inhibited serum sPLA2 activity in transgenic mouse expressing human $sPLA_2$. This molecule was administered by intravenous route as an adjunct to disease modifying anti rheumatic drug therapy. Kelly *et al* (2005) reported that LY315920 was safe but did not exhibit any efficacy [45]. An orally active prodrug of LY 315920, LY 333013 was evaluated for efficacy in patients with allergic asthma. However, no protection on allergen induced bronchoconstrictor response was observed [46]. At present, LY 315920 is undergoing phase II clinical trial for atherosclerosis.

$cPLA_2$ Inhibitor:

A lot of effort has gone into designing $cPLA_2$ inhibitors. Several groups have worked with different chemical class and very potent inhibitors have been reported [47-49]. Many of these compounds also block release of arachidonic acid and prostaglandins from cells. Yakash *et al* (2006) have also demonstrated oral efficacy of $cPLA_2$ inhibitors in experimental models of pain [50]. Wyeth had very advanced program to design $cPLA_2$ inhibitor and several molecules have entered preclinical development and two have gone into human trial. Working with an indole chemotype reported discovery of Ecopladib, Giripladib, Efipladib and WAY-19625 as shown in fig.3 [51, 52, 39]. McKew and colleagues have brought in substitutions at positions C2 and C3 of indole ring. Ecopladib has a two carbon ethoxy linker connecting indole ring to benzoic acid [39]. Giripladib, Efipladib and WAY-19625 have a 3 carbon linker at C3 linked to benzoic acid or methyl benzoate. At C2 positions of indole ring, all four compounds have a substituted benzyl sulphonamide group attached with the help of 2 carbon linker. Benzene ring is substituted with 2 trifluoro methyl (Giripladib), 3, 4 dichloro (Ecopladib and Efipladib) and 2, 6 dimethyl groups for WAY-19625. Table, shows *in vitro* and *in vivo* pharmacology data three molecules inhibit $cPLA_2$ enzyme and inhibit release of arachidonic acid breakdown products from intact cells. Ecopladib has much less potency for enzyme compared to Efipladib and WAY-19625. All three molecules exhibit low plasma clearance with poor oral bioavailability in rats – Ecopladib 8%, Efipladib and WAY-19625 4% each. All three molecules have shown oral efficacy in experimental animal models of joint inflammation – carrageenan paw oedema (Table 6).

Whalen *et al* (2008) have shown that WAY-19625 significantly attenuates gene expression induced by allergen when human peripheral blood mononuclear cells from asthma patients are incubated *in vitro* with allergens [53]. Efipladib and WAY-19625 were also tested for efficacy in preclinical models including sheep model of bronchial asthma. At a dose of 10 mg/kg given twice daily and 2 hour before allergen challenge, Efipladib and WAY-19625 had no effect on early phase bronchoconstriction but inhibited late phase bronchoconstriction. A fourth dose was administered 8 hour after allergen challenge and 16 hour later, both compounds offered complete protection of airway from hyperresponsiveness.

Efipladib, Ecopladib and Giripladib have moved into human trial. Giripladib was slotted for phase II osteoarthritis trial. However, it has been terminated. WAY-19625 is undergoing preclinical studies [49, 39].

Calcium Independent PLA_2 Activity Modulation: PAF-AH ($LpPLA_2$):

Inspite of many positive preclinical data, clinical trial of recombinant PAF-AH (1 mg/kg) in fourteen human allergic asthma patients did not offer any protection on early and late allergic response or sputum eosinophil count [38].

Inhibitor of $LpPLA_2$ is being evaluated for efficacy in atherosclerosis. Darapladib, an inhibitor of $LpPLA_2$, is undergoing phase III clinical trial for atherosclerosis.

Expert Summary:

PLA_2s are crucial lipid metabolizing enzymes that play a significant role in etiology and pathology of the inflammatory disorders. Modulation of pro-inflammatory lipid mediator production by inhibition of PLA_2 activity remains a potential target for airways disease. Ample evidence exists now that by inhibiting PLA_2 isoform

activities have resulted in efficacy in animal models and some initial efficacy in humans in clinic. Thus, the current discovery effort should focus on selective inhibition of PLA_2 by small molecules, towards a developing a potential drug for airway diseases.

It is well established that arachidonic acid metabolites play important role in the pathophysiology of inflammatory airway diseases like bronchial asthma. Phospholipase A_2 as a target acquires the status of precedented target given the success of downstream mediators in the clinic. Of different PLA_2s, secretory phospholipases are induced. Consequently, these may be an easy target for drug discovery under a background of inflammatory activity. It is not clear why secretory PLA2 inhibitor did not show efficacy in the clinic. It may be possible that more than one PLA2 subtypes may be expressed in human disease, or alternatively, the sPLA2s may have relatively less important role in the propagation of human asthma.

$cPLA_2$ remains central molecule because of $sPLA_2$s need this molecule for their biological effect. $cPLA_2$ is selective for arachidonic acid containing phospholipids. Thus propensity of adverse effect due to nonspecific phospholipase activity may be lower. Very few phospholipase A_2 inhibitors are being pursued for asthma, and other inflammatory airway diseases.

It is not clear why a PLA2 inhibitor did not show efficacy in the clinic. It is possible that inhibiting PLA2 blocks in all likelihood both cyclooxygenase and lipoxygenase pathways. While lipoxygenase derived mediators play important role in airway inflammation, the picture is more complex with cycloxygenase inhibition. Different prostaglandins have mutually antagonistic effects on airway contractility and airway inflammation. Thus inhibition of cyclooxygenase pathway may play a role in masking beneficial effects of lipoxygenase inhibition. It is also emerging that Lipoxin, the natural lipid derived anti-inflammatory molecule, plays important role in inflammation resolution. Inhibition of PLA2 most likely blocks synthesis of lipoxins, this may further complicate anti-inflammatory effect of PLA2 inhibition.

Table 1: Properties of various phospholipase enzymes

	Mol. Wt. KDa	**Ca^{2+}**	**Subtype**	**Localization**	**Substrate**	**Function**
sPLA2	13 - 15	mM range	I, IIA, IID, IIE, III, V, X	Secreted	Non selective	Arachidonic acid release; Cytokine generation; Surfactant breakdown
cPLA2	85	μM range	α; β, γ	Cytosol	Arachidonyl Phospholipid	Arachidonic acid release
iPLA2	85 - 88	None	β, γ;	Cytosol / membrane	Non selective	Lipid remodeling; Arachidonic acid release!
LPLA2	45	None		Lysosome	Phosphatidyl choline and ethanolamine	Surfactant catabolism
aiPLA2	26	None		Cytosol Extracellular fluid;	Dipalmitoyl Phosphatidyl Choline	Surfactant metabolism; Antioxidant
PAF Hydrolase	45	None	LpPLA2	Circulating	PAF	PAF catabolism

Table 2: Distribution and function of sPLA2 subtypes

Secretory Phospholipase A2		
Class	**Distribution**	**Function**
I	Pancreatic juice	Digestion
IIA	Synovial fluid of RA patients; Proinflammatory cells	Antibacterial; Arachidonic acid release
IID	Lung	Unknown
IIE	Lung	Unknown
III		Adaptive Immunity
V	Heart; Lung, epithelialcell, Macrophage, Neutrophil, T lymphocyte	Arachidonic acid metabolism; Surfactant degradation
X	Immune cell; Lung epithelilal cell	Arachidonic acid metabolism;
Ref:		

Table 3: cPLA2 and Experimental Airway Disease

cPLA2 and Experimental Airway Disease			
System	***Experimental Model***	***Response***	**Reference**
KO mouse	Ovalbumin induced allergy	Attenuation of anaphylaxis; Attenuation of airway reactivity	**[19]**
KO mouse	LPS induced lung injury	Gas exchange; Lung oedema; Neutrophil influx	**[22]**
KO mouse	Bleomycin induced fibrosis in mouse	Reduced inflammation; Reduced PG and LT production; Reduced fibrosis	**[23]**
cPLA2 inhibition	LPS induced lung injury	Gas exchange; Lung oedema Neutrophil count and myeloperoxidase activity in BAL; PG and LT in BAL;	**[23]**
cPLA2 inhibition	Guinea pig allergy model	Attenuation of airway eosinophilia; Attenuation of airway reactivity	**[21]**
cPLA2 inhibition	Mouse allergy model	TNF induced eosinophilia and airway reactivity	**[20]**
cPLA2 inhibitor	Allergic mouse model	Protection Ova induced anaphylaxis; Mch induced AHR	**[16]**

Table 4: PLA2 and Inflammatory Mediator Release

PLA2 and Inflammatory Mediator Release		
System	**Response**	**Reference**
cPLA2 ko mouse	Loss of stimulus induced arachidonic acid and PAF release from macrophage;	**[19]**
cPLA2 inhibitor	Arachidonic acid release from PLA2a expressing cell CHO cell;	**[16]**
sPLA2 antisense	PGE2 release from macrophage cell line	**[55]**
sPLA2 inhibitor	Release of LTB4 and superoxide from neutrophils Neutrophil degranulation	**[27]**
sPLA2 inhibitor	Arachidonic acid, LTB4 and PAF release from PMN	**[26]**

Table 5: sPLA2 and Experimental Airway Disease

sPLA2 and Experimental Airway Disease			
System	***Experimental Model***	***Response***	**Reference**
sPLA2 X KO mouse	Ovalbumin induced allergy	• Attenuation of LT and PG production; • Reduced cytokine production; • Decreased airway inflammatory cell number • Decrease airway remodeling	**[31]**
sPLA2 III KO mouse		Reduced arachidonic acid release from cell	**[4]**
sPLA2 V KO mouse	Allergic mouse model	Reduced cell migration; Reduced AHR Reduced eicosanoid production from macrophage ex vivo	**[29]** **[54]**
sPLA2 Inhibitor		Reduced sPLA2 induced TxA2 production in BAL fluid ex vivo and in vitro	**[28]**
gIIsPLA2 inhibitor	LPS induced surfactant reakdown	LPS induced PLA2 expression; PLA2 inhibitor block LPS induced surfactant breakdown	**[33]**
Human asthma patient	Allergen challenge	Elevated sPLA2	**[46]**

Table 6: Pharmacological characterization of cPLA2 inhibitors

	Cell Free Assay IC_{50} (nM)	**Cell Based Assay** IC_{50} (nM)		***In Vivo* Assay** ED_{50} **(mg/kg)**			PK	
	Enzyme Assay	LTB4	PG	CPE	Air Pouch	AIA	Cl (ml/min/kg)	% F
Effipladib	40	20	20	35	-	25		4
Way 196025	10	12	6	8	-	> 25		4
Ecopladib	150	<300	<300	40	8		14	8
Giripladib								

Table 7: In-Vitro & In-vivo characterization of sPLA2 inhibitors

	Cell Free Assay IC_{50} **(μM)**	**sPLA2 Mediated Response** IC_{50} **(μM)**	***In vivo* Studies** (μg/ear)	**Reference**
	Enzyme Assay	**Thromboxane Release**	**Mouse Ear Oedema**	
LY 315920 (Varespladib)	0.009	0.790		[28]
LY 311727	0.023	1.8		[42]
BMS 181162	8	10 (*)	0.180 / ear	[26]
YM 26567	6.7	-	0.028 / ear	[40]
Variabilin	6.9	-	0.133 / ear	[27]
SB 203347	0.5	1 (*)	~100 mg/kg($)	[25]

(*) A23187 induced arachidonic acid release
($) Mouse endotoxemia model by intraperitoneal route

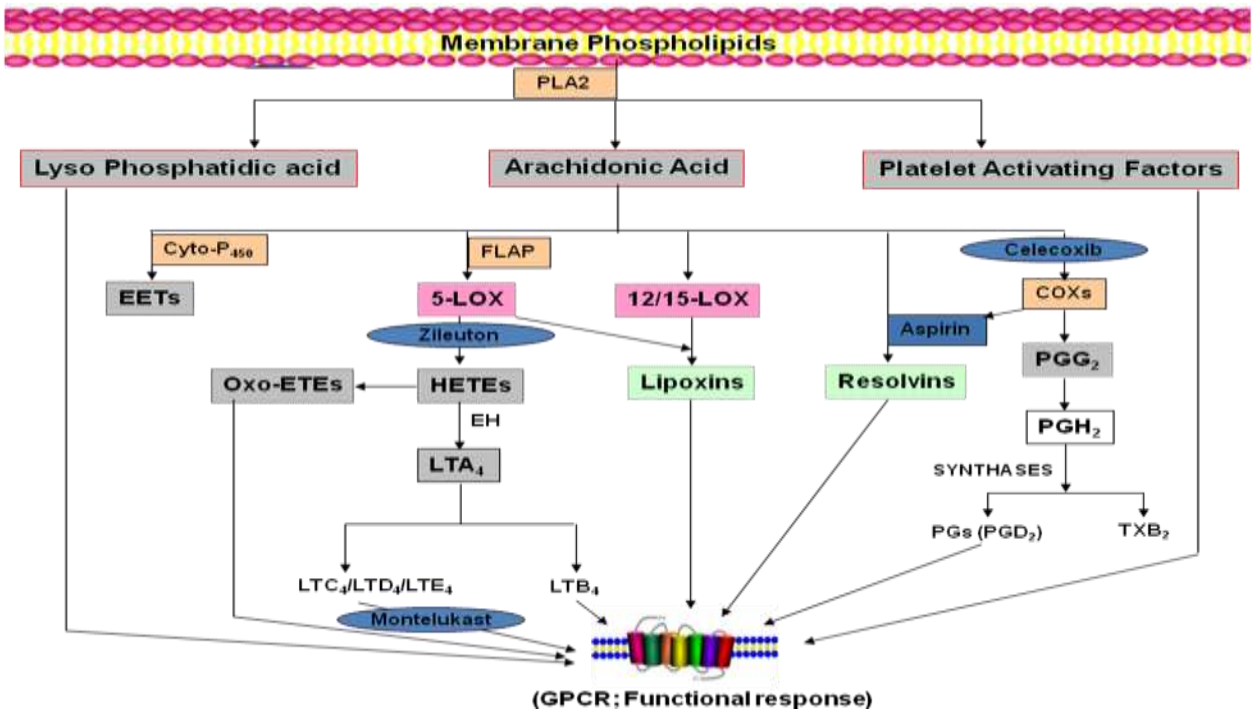

Figure 1: Lipid metabolism pathway and current marketed drugs interfering lipid metabolism pathway. Membrane phospholipid is metabolized by PLA2 inhibitors leading to three major metabolites, namely Lysophosphatidic acid (LPA), Archidonic acid (AA) and Platelet activating factors (PFA). LPA and PFA bind to their respective GPCRs and exert biological functions. AA is metabolized further through various distinct pathways and lead to both pro-inflammatory metabolistes (like LTB4, LTC4, EETs, PGs etc) and anti-inflammatory mediators (Lipoxins and Resolvins). Through intervening of these pro-inflammatory pathways various drugs have been produced in clinic, e.g., *Zileuton* by inhibiting 5-Lipoxygenase enzyme, *Montelukast* by antagonizing CysLT receptor functions, *Clelcoxib* and *Aspirin* by inhibiting COX enzymes.

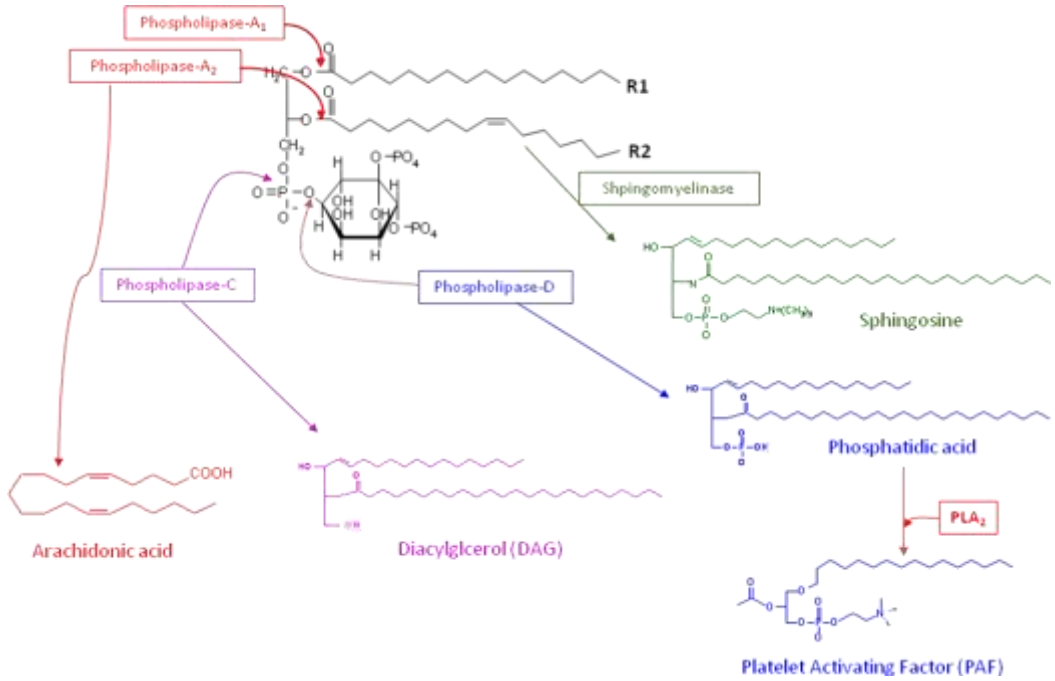

Figure 2: Mechanism of action of PLA2s. The glycerol moiety of phospholipid consists of three major parts, two of long chain fatty acids and one polar head group. The various phospholipases cleave phospholipid at distinct places to produce distinct metabolites. PhospholipaseA1 and A2 cleave the ester bond of first and second fatty acid attached, respectively leading to release fattya cid. Phospholipase-C cleaves the phosphate bond of the polar group to produced DAG. Phospholipase-D cleaves the polar heard group to produced PAF.

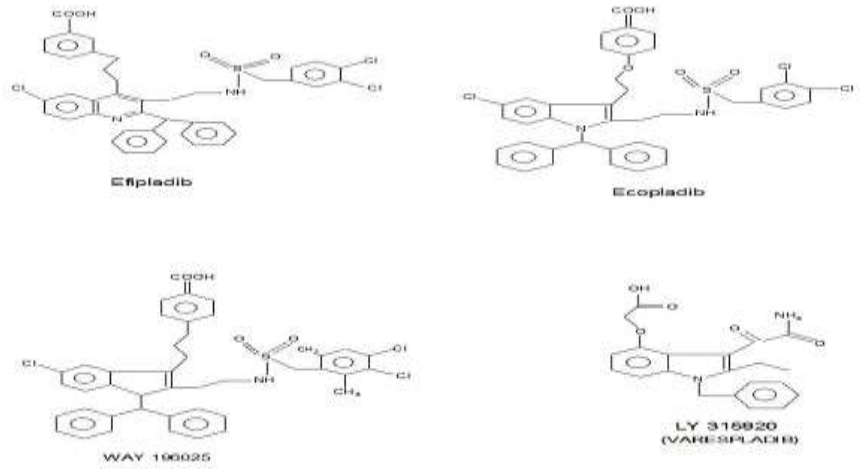

Figure 3: Various PLA2 inhibitors tested in clinic. List of various drugs tested in clinic as PLA2 inhibitors till date.

Declaration of interest:

The authors state no conflict of interest and have received no payment in preparation of his manuscript.

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