Novel therapeutic concepts

Phospholipase A2 enzymes and the risk of atherosclerosis

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Certain members of the phospholipase A2 superfamily of enzymes have established causal involvement in atherosclerosis, thus at least two groups of this family of enzymes have been considered potential candidates for the prevention of cardiovascular events. Recently completed experimental animal studies, human biomarker data, vascular imaging studies, and genome-wide atherosclerosis studies provide the rationale for proceeding with clinical outcome trials directed at inhibition of secretory phospholipase A2 and lipoprotein-associated phospholipase A2. A clinical trial with the sPLA2 inhibitor varespladib methyl was recently terminated, while clinical trials with the Lp-PLA2 inhibitor darapladib are being conducted in coronary heart disease patients. This article reviews the available experimental animal and human trial evidence that serve as the basis for the development of these two classes of phospholipase A2 inhibitors.

Keywords
Secretory phospholipase A2 • Lipoprotein-associated phospholipase A2 • Varespladib • Darapladib • Cardiovascular disease

Introduction

Vascular inflammation is an essential feature in the pathogenesis of atherosclerotic lesion formation, and it contributes to its complications of acute myocardial infarction and stroke. In prospective population-based studies, non-specific systemic inflammatory markers such as the white blood cell count and concentrations of the C-reactive protein, serum amyloid A, and fibrinogen serve to stratify patients into categories of cardiovascular disease (CVD) risk.1–3 However, these non-specific inflammatory risk markers provide limited insight as to the specific inflammatory mediator(s) responsible for the inflammatory response in that particular patient,4 and thus they are less useful targets for therapeutic intervention directed at mitigating risks associated with vascular inflammation.5

Circulating levels and enzymatic activity of two families of phospholipase A2 enzymes, secretory phospholipase A2 (sPLA2) and lipoprotein-associated phospholipase A2 (Lp-PLA2), have been evaluated as biomarkers of cardiovascular risk in population-based studies inclusive of apparently healthy individuals, and patients with established coronary heart disease (CHD).6–11 In the EPIC (European Prospective Investigation of Cancer) Norfolk study, the increased CHD risk associated with the oxidation specific biomarker, oxidized phospholipids on apolipoprotein B (apoB) or lipoprotein(a), was enhanced further among individuals with high sPLA2 concentration and activity than Lp-PLA2 activity.10 This study and other observational studies have shown that both enzyme concentration and activity predict incident cardiovascular events. The evidence that these phospholipase A2 enzymes retain their importance as biomarkers of risk in statin-treated patients is less certain; however, in one trial, LpPLA2 concentration was associated with future CVD events in models that adjusted for baseline covariants and apolipoprotein concentrations.12 The importance of this phospholipase A2 enzyme biomarker data should not be overemphasized, as circulating levels of these two groups of PLA2 enzymes and their various members may not encompass their multifarious downstream pro-atherosclerotic effects in the vessel wall.13,14 Recently, the non-functional (null) V279F allele within the PLA2G7 gene, a common loss of function mutation of Lp-PLA2 in Asians, was associated with reduced CHD risk.15 This Mendelian randomization study provides support for the hypothesis that marked reduction Lp-PLA2 enzymatic activity may be an effective strategy for CVD prevention.

Recently, selective inhibitors of sPLA216 and LpPLA217 have been targeted as potential candidates to reduce incident atherosclerotic cardiovascular events.18,19 The completion of clinical
outcomes trials with selective inhibitors of these phospholipase A₂ enzymes will provide important information on the future role of these crucial enzymes in CVD prevention and treatment. This review examines the biological function of these two groups of phospholipase A₂ enzymes in atherosclerosis through examination of population-based studies, animal studies and human studies. Available evidence that inhibition of sPLA₂ and Lp-PLA₂ is reasonable targets for reducing CVD are presented.

**Phospholipase A₂ superfamily of enzymes**

The phospholipase A₂ superfamily enzymes are characterized by their ability to hydrolyze fatty acids at the sn-2 position of glycerophospholipids and generate multiple classes of bioactive lipids (Figure 1).²⁰ Five main families of phospholipases have defined physiological roles, and they comprise group (G) II or secretory PLA₂ (sPLA₂), GIV or cytosolic PLA₂ (cPLA₂), GXV or lysosomal PLA₂; and two major Ca²⁺-independent groups, GV PLA₂ (iPLA₂) and GVII platelet-activating factor (PAF) acetylhydrolases.

**Secretory phospholipase A₂ structure and biology**

Secretory PLA₂ family consists of 12 isoforms, and with the exception of GII sPLA₂, these proteins have comparable molecular weights of ~16 kDa and three-dimensional structures.²⁰–²² Since the genes encoding GIIA sPLA₂ and GV sPLA₂ are localized in close proximity in homologous regions of human chromosome 1 (and mouse chromosome 4), these group members may represent a gene cluster.²³ Despite common features among human sPLA₂ isoenzymes, they are functionally distinct proteins with specific tissue distributions²¹,²²,²⁴ and enzymatic properties on various phospholipids.²⁴

Each isoform functions both as an enzyme and ligand for receptors (both soluble and membrane-bound) that control and transduce their biological effects or modulate their enzymatic action.²⁵,²⁶ Biological effects include various roles in both physiological and pathophysiological conditions, including inflammatory diseases such as atherosclerosis and rheumatoid arthritis.²⁷

Among the sPLA₂ binding proteins, the M-type receptor (PLA₂R1) is the best-characterized sPLA₂ receptor.²⁸ PLA₂R1 is a type I membrane glycoprotein of 180 kDa that belongs to the superfamily of C-type lectins.²⁹ It is comprised of a single

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**Figure 1** Phospholipase A₂ enzymatic activity generates lipid products with biological properties and functions. Secretory phospholipase A₂ (sPLA₂) acts to hydrolyze phospholipids from the surface of cell membranes, native lipoproteins and oxidatively-modified lipoproteins to generate multiple bioactive lipids that include arachidonic acid, non-esterified fatty acids (NEFA), lysophospholipids (Lyso-PL), lyso-platelet acting factor (LYSO-PAF), and oxidized non-esterified fatty acids (Ox-NEFA). In contrast, lipoprotein-associated phospholipase A₂ (Lp-PLA₂) or platelet activating factor (PAF) requires oxidized phospholipids as a substrate (oxidized phosphotidylcholine [Ox-Phosphatidylcholine]).
transmembrane domain, a short cytoplasmic tail, and a large extracellular region that includes an N-terminal cysteine-rich domain, a fibronectin-like type II domain, and a tandem repeat of eight distinct C-type lectin-like carbohydrate recognition domains. Currently, the biological functions of PLA2R1, its endogenous ligands other than mammalian sPLA2 isoenzymes, and associated signalling pathways remain unknown.26,28

**Secretory phospholipase A2 and atherosclerosis**

sPLA2 hydrolyzes the sn-2 ester bond in the glycerolylglycerol phospholipids present in lipoproteins and cell membranes, inducing structural and functional changes and forming lysophospholipids and non-esterified fatty acids (NEFAs) with direct proinflammatory effects.31,32 The mammalian sPLA2 isoforms comprise groups named IB, IIA, IID, IIE, IIIF, III, V, X, and XII. Six isoforms are present in human atherosclerotic lesions: IIA, IID, IIE, III, V, and X. The homology between these sPLA2 enzymes varies, but they share a calcium-dependent catalytic mechanism, presence of several disulphide bridges, and a well-conserved overall three-dimensional structure. In addition to their role in atherogenesis, sPLA2 enzymes are implicated in various physiological and pathophysiological functions, including lipid absorption, cell proliferation, myocardial injury, tumour formation, and inflammation. Interestingly, sPLA2 activity is involved in cholesterol homeostasis.30,31 Specifically, GX sPLA2 generates lipolytic products that suppress LXR activation, which negatively regulates ABCA1 and ABCG1 expression and cholesterol efflux from macrophages.33

Secretory PLA2 activity can be proatherogenic in the circulation, and at sites of atherosclerotic plaque development (Figure 2). In plasma, sPLA2 activity hydrolyzes surface phospholipids on lipoproteins, resulting in structural alteration in these particles.34 GV sPLA235 and GX sPLA236 enzymes hydrolyze phosphatidylcholine on the surface of VLDL and LDL at least 20-fold more efficiently than GIIX sPLA2.37 Although group IIIA sPLA2 acts poorly on unmodified LDL, presumably because of its poor binding to phosphatidylcholine-rich membranes, this enzyme shows enhanced ability to hydrolyze oxidized LDL.

As a consequence of phospholipid hydrolysis, conformational changes in apolipoprotein B (apoB) impairs clearance of very low-density lipoprotein remnants and LDL by the apolipoprotein B/E receptor (LDL-R),38 resulting in increased residence time in the circulation and further susceptibility to oxidative modifications (Figure 2).39 Hydrolysis of phospholipids in high-density lipoprotein (HDL) results in impaired cholesterol-efflux capacity. In the arterial vessel wall, the conformational changes in apoB40-44 increase their binding to intimal proteoglycans, promote retention of these atherogenic lipoproteins, and increase intra- and extracellular

![Figure 2](https://academic.oup.com/eurheartj/article-abstract/33/23/2899/539667/figure2) Effects of phospholipase A2 enzymatic activity on circulating lipoproteins. Secretory phospholipase A2 (sPLA2) hydrolyzes phospholipids from the surface of native lipoproteins and oxidatively-modified lipoproteins, whereas lipoprotein-associated phospholipase A2 acts only on oxidatively-modified lipoproteins. Phosphoatidylcholine hydrolysis by sPLA2 results in small VLDL and LDL particles with altered conformation of apolipoprotein B (apoB). The conformational change in apoB reduces binding and internalization of apoB-containing lipoproteins by the apoB/E (LDL) receptor resulting in prolonged residence time in the circulation. This prolonged circulation time of LDL particles increases exposure to reactive oxygen species (ROS) resulting in an oxidized LDL particle (Ox-LDL) that may serve as a substrate for group IIIA sPLA2 (GIIX sPLA2) and Lp-PLA2. Phospholipid hydrolysis of Ox-LDL particles generates oxidized non-esterified fatty acids (Ox-NEFA) and lysophosphatidylcholine (Lyso-PC). sPLA2 acts on cellular membranes resulting in elaboration of arachidonic acid that serves as the substrate for eicosanoids, thromboxanes and leukotrienes.
cholesterol accumulation, a hallmark of atherosclerosis. In addition, reduction in the molar ratio of cholesterol to phospholipid will compromise the solubility of free cholesterol molecules resulting in cholesterol crystal precipitation. Furthermore, cholesterol crystals have been reported to induce inflammation by stimulating caspase-1-activating NLRP3 inflammasome, which results in cleavage and secretion of interleukin-1 family cytokines.47

In addition to indirect effects on fostering cholesterol crystal formation, sPLA2 mediates phospholipid hydrolysis from cell membranes and lipoproteins, and increase oxidative stress through generation of arachidonic acid, lysophospholipids, and non-esterified fatty acids (Figure 1).48,49 These bioactive lipids, including the arachidonic acid-derived metabolites prostaglandins, thromboxanes, and leukotrienes, work collectively with oxidized LDL to recruit inflammatory cells to atherosclerotic lesions and activate inflammatory pathways in multiple cells in the vessel wall (Figure 3).50 Lysophospholipids and NEFAs, the hydrolysis products of sPLA2, also contribute to atherosclerosis plaque progression exerting a broad range of cellular effects, including cytotoxicity.51

It should be stressed that the total hydrolysis of phospholipids from one apoB particle will generate more than 500 molecules of lysophospholipids and free fatty acids. Thus, in atherosclerotic plaques, these products can reach high local concentrations. These bioactive lipid products can induce different proatherogenic and proinflammatory cellular processes, and cell membrane perturbation (Figure 3).31,32,51

Several sPLA2 isoenzymes members have been detected in murine and human atherosclerotic lesions (Figure 4).22,51,52 Additionally, recent data suggest that sPLA2 isoenzymes-IIId also contribute to atherosclerosis.53 Experimental animal studies using gain-of-function and loss-of-function approaches have established GIIA, III, V, and X as causal factors in atherogenesis.54–58

Transgenic

Figure 3  Cellular mechanisms of action of sPLA2 enzymes in eicosanoid production. In this model, hydrolysis of phospholipids (PL) by sPLA2 enzymes may occur on the cell membrane and on extracellular lipoproteins. Lipoproteins can bind to cell surface proteoglycans (PG) and PG in the extracellular space facilitating their interaction with sPLA2s such as group sPLA2 II (sPLA2-II) and group V sPLA2 (GV-sPLA2) that also bind to PG. This binding enhances the hydrolytic activity of GV-sPLA2 on lipoproteins. Binding to cell surface PG, glypican, results also in internalization of sPLA2. Group X sPLA2 (GX-sPLA2) does not bind to PG; however, it binds tightly to zwitteronic phospholipid membranes relative to all other mammalian sPLA2s and can act on the outer plasma membrane leaflet. Expression and secretion of sPLA2s can be modulated by different stimulus, i.e. GV-sPLA2 expression is enhanced by high fat diet, and sPLA2-II and X by inflammatory stimuli. On the other hand, chemical inhibition of extracellular GIIA-sPLA2 by varespladib decreases its own secretion, by a yet unknown mechanism. Several sPLA2s, including groups IIA, V and X, are likely to be involved in the release of arachidonic acid (AA) and other fatty acids as well as lysophospholipids (Lyso-PC) that can be further metabolized into various eicosanoids and related bioactive lipid mediators by different intracellular pathways and in different cell types, i.e. in cells such a s neutrophiles and monocytes that express 5-LO, AA is converted in leukotriene-B4 (LTB4). Lyso-PC may mobilize intracellular calcium and further activate intracellular eicosanoid pathways by upregulating cytoplasmic PLA2 (cPLA2) and other enzymes such as 5-lipoxygenase (5-LO) and cyclooxygenases (COX).
mice that constitutively overexpress human GIIA sPLA2 develop atherosclerotic lesions on standard chow and atherogenic diets. In addition, human GIIA sPLA2 transgenic mice with macrophage-specific overexpression showed increased lesion area and enhanced collagen deposition. Transplanting bone marrow cells from these transgenic mice into LDL-R-deficient mice (LDL-R$^{-/-}$) increased atherosclerotic lesion formation. Macrophage-specific overexpression of human GIIA sPLA2 increased foam cell formation in LDL-R$^{-/-}$ mice transplanted with sPLA2 bone marrow of transgenic mice fed a western-type diet.

GV sPLA2 has been identified as a potential factor in promoting atherosclerosis in human and murine atherosclerosis. This sPLA2 isoenzyme is expressed in human atherosclerotic lesions and human vascular cells. In human vascular cells, GV sPLA2 is found extracellularly around foam cells in lipid core areas, and it is associated with smooth muscle cells in the neointima and media of intermediate and advanced lesions. Experiments in mouse models showed that diet- or genetic-induced hyperlipidaemia in mouse models increase the expression of GV sPLA2 2 but not of GII sPLA2 in the aorta. On the other hand, lipopolysaccharide-induced acute inflammation augmented the expression of GII sPLA2 but not of GV sPLA2. These differences in stimulus-mediated expression indicate that GII and GV sPLA2 enzymes can have different roles in the atherosclerosis.

Overexpression of mouse GV sPLA2 by retrovirus-mediated gene transfer increased the lesion area in LDL-R$^{-/-}$ mice, whereas mice deficient in bone marrow-derived GV sPLA2 had reduced the lesion area. Transgenic GV sPLA2 overexpression increased lipid deposition and collagen deposition in LDL-R$^{-/-}$ mice. In apoE$^{-/-}$ mice fed a high-fat diet GV sPLA2 promotes lipid deposition; however, unlike the experiments in LDL-R$^{-/-}$ mice, GV sPLA2 deficiency did not reduce atherosclerosis even though there was a reduction in the collagen formation. A potential explanation for these differences may relate to the increased sphingomyelin content of LDL particles isolated from apoE$^{-/-}$ mice, which decreases GV-mediated phosphatidylcholine hydrolysis.

Cholesterol accumulation in the vessel wall is one mechanism for GV sPLA2-mediated atherosclerosis. In murine macrophages, group V sPLA2-modified LDL induced cholesterol ester accumulation that was independent of LDL-R, SR-A, and CD36 scavenger receptors or the pathway that clears LDL aggregates (Figure 4). The mechanism for the increased uptake of GV-modified LDL involves initial binding to heparan sulfate proteoglycans of the extracellular matrix, and perhaps directly binding to macrophages.

Human GIID sPLA2 is expressed in the intima of human atherosclerotic lesions where it colocalizes with foam cells and smooth muscle cells that resemble myofibroblasts. GIID and GIID sPLA2 appear as an anti-atherogenic factor that inhibits macrophage activation and inflammatory responses by reducing cell adhesion, NO, and TNF-alpha levels, while augmenting the production of the anti-inflammatory IL-10 cytokine.

**Figure 4** Phospholipase A2 enzymatic activity and foam cell formation. Secretory phospholipase A2 (sPLA2) and lipoprotein-associated phospholipase A2 (Lp-PLA2) increase oxidation of LDL particles allowing for enhanced internalization into the macrophage via the conventional scavenger receptor resulting in foam cell formation. In addition, group V (GV sPLA2) and group X sPLA2 (GX sPLA2)-modified LDL particles are incorporated into macrophages via a putative M-type receptor, which contributes to cholesterol content of tissue macrophages via this distinct pathway.

**Secretory phospholipase A2 and ischaemic injury**

Groups II, V, and X sPLA2 have been implicated in ischaemic myocardial tissue damage. Elevated plasma levels of GII sPLA2 are associated with the increased CHD risk. During an acute coronary syndrome, the plasma concentration of GII sPLA2 rises, and this biomarker has been shown to useful to discriminate between
ACS patients who develop a myocardial infarction vs. those patients who do not. The localization pattern of GII sPLA2 in infarcted myocardium and its temporal course in plasma, in relation to those of C-reactive protein, are in line with a postulated pro-inflammatory role during acute myocardial infarction for GII sPLA2 which serves as a generator of lysophospholipids that serve as ligands for C-reactive protein.

GV sPLA2 is expressed in high concentrations in various tissues including cardiomyocytes where its expression is further increased in human-infarcted myocardium. GV sPLA2 activates extracellular signal-related kinase 1/2 (ERK1/2) that, in turn, activates cPLA2 (Figure 3). Cytoplasmic PLA2 is a major regulator of eicosanoid generation. When compared with controls, GV sPLA2−/− mice had lower content of leukotriene B4 and thromboxane B2 in ischemic myocardium, and smaller myocardial infarctions and preserved left ventricular function.

In experimental myocardial infarction, the cardiomyocyte plasma membrane loses its asymmetry resulting in exposure of the anionic phospholipids phosphatidylserine and phosphatidylethanolamine to the outer membrane leaflet. Structural re-organization in membrane phospholipids enhances sPLA2 binding, and consequently increases complement-mediated myocardial necrosis. The use of a specific sPLA2 inhibitor PX-18 (2-N,N-Bis(oleoyloxyethyl)amino-1-ethanesulfonic acid) reduced cardiomyocyte apoptosis and limited experimental infarct size. In addition, myocardial ischemia/reperfusion injury is attenuated in GX-deficient GX-sPLA2 mice partly through the suppression of neutrophil cytotoxic activities. Together, this human and experimental in vivo evidence suggest a pathogenic role of these three sPLA2 isoenzymes in infarcted myocardium.

**Lipoprotein-associated phospholipase A2 structure and biology**

Group VIIA or PAF is a potent phospholipid activator that is secreted by multiple inflammatory cells including monocytes/macrophages, T lymphocytes, and mast cells. It mediates many leukocyte functions, in particular inflammation. Two groups of PLA2 enzymes, designated GVII and GVIII, catalyse 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 PAF acetylhydrolases (PAF-AH) (Figure 1). GVIIA PLA2 is a secreted enzyme with a molecular weight of 45 kDa that associates with LDL and HDL in human plasma. Therefore, the enzyme is also known as plasma PAF-AH (pPAF-AH) or Lp-PLA2. GVIIIB PLA2, also referred to as PAF-AH II, is an intracellular enzyme with a molecular weight of 45 kDa that has an N-terminal myristoylation site and which shares 41% amino acid sequence identity with GVIIA PLA2. GVIII PLA2 is a brain intracellular heterotrimeric protein complex that is also referred to as PAF-AH Ib.
although this enzyme also displays significant phospholipase A\(_2\) activity.\(^{70}\)

Lp-PLA\(_2\) binds to two distinct domains on apoB, and after secretion it is primarily transported by LDL and lipoprotein (a) [Lp(a)].\(^{13,14}\) About 30% of Lp-PLA\(_2\) binds to apolipoprotein A-I, and transported by HDL particles.\(^{58}\)

Lp-PLA\(_2\) cleaves oxidized lipids from the sn-2 position of the apoB100-containing lipoproteins LDL and lipoprotein (a) [Lp(a)].\(^{14}\) The bioactive lipids include oxidized non-esterified fatty acids and lysophosphatidylcholine (Figures 1 and 4). Saturable PAF receptor-independent transport rather than intravascular hydrolysis modulates the pro-inflammatory and pro-apoptotic oxidized lipid mediators.\(^{71}\) In a diabetic, hypercholesterolaemic porcine model of atherosclerosis, upregulation of Lp-PLA\(_2\) increased peripheral blood mononuclear cell expression of ICAM-1 and IL-6, and coronary artery expression of ICAM-1 and VCAM-1.\(^{72}\)

### Lipoprotein phospholipase A\(_2\) concentration and activity, and risk of cardiovascular disease events

The Lp-PLA\(_2\) Studies Collaboration examined the associations between Lp-PLA\(_2\) concentration and activity and CVD events from 79,036 participants who participated in 32 prospective studies.\(^8\) In the 32 prospective studies, Lp-PLA\(_2\) activity was measured in 57,931 participants from 18 studies, and Lp-PLA\(_2\) mass was measured in 58,224 participants from 21 studies. Overall, risk ratios for CHD and ischaemic strokes increased progressively for every 1-standard deviation higher Lp-PLA\(_2\) activity or concentration in models that adjusted for age, sex, baseline history of vascular disease, and other non-lipid and lipid risk factors. Risk ratios for CHD events were 1.10 [95% confidence interval (CI) 1.05–1.16] for Lp-PLA\(_2\) activity and 1.11 (1.07–1.16) for Lp-PLA\(_2\) concentration; 1.08 (0.97–1.20) and 1.14 (1.02–1.27) for ischaemic stroke; 1.16 (1.09–1.24) and 1.13 (1.05–1.22) for vascular mortality; and 1.10 (1.04–1.17) and 1.10 (1.03–1.18) for non-vascular mortality. However, the associations between Lp-PLA\(_2\) activity and CHD events were significant only for patients with stable CHD [1.17 (0.78–1.76)], whereas the association was not significant in subjects with no history of CHD [1.03 (0.95–1.12)]. In contrast, Lp-PLA\(_2\) mass was predictive of CHD events in both groups of participants. Subsequent to this meta-analysis, an analysis from the Nurses’ Health Study reported that Lp-PLA\(_2\) activity was associated with incident myocardial infarction (RR 1.75, 95% CI 1.09–2.84) after multivariable adjustment for clinical, lipid, and inflammatory risk factors.\(^7\)

PLASG7 is the gene for LpPLA\(_2\) or group VII phospholipase A\(_2\). A missense mutation (Val279Phe, exon 9, position 994;G\(\rightarrow\)T) of the LpPLA\(_2\) gene results in complete loss of enzymatic activity,\(^{70}\) whereas the VV genotype is associated with increased LpPLA\(_2\) activity.\(^{81}\) The associations between the LpPLA\(_2\) gene polymorphism V279F and CHD was recently investigated in two meta-analyses. In 26,000 individuals, PLAG2 polymorphisms 379V was associated with changes in enzymatic activity, but not with coronary atherosclerosis or CHD events.\(^{82}\) In an analysis of seven studies involving predominantly Asian individuals (7705 from a total of 7948), there was no overall association with CHD risk [OR = 1.14; (95% CI 0.86–1.52)];\(^{83}\) However, the association with CHD events was significant within the Japanese subgroup [OR = 1.38; (95% CI 1.21–1.56)]. In a larger study from two data sets of Korean men, one copy of 279F null allele was associated with a 20% reduction in the CHD risk.\(^{15}\)

These gene association studies provide evidence that reduced LpPLA\(_2\) activity is associated CVD in populations with high prevalence of this loss-of-function mutation.

As discussed earlier, Lp-PLA\(_2\) circulates in a complex with apoB, thus apoB levels may confound its association with the CVD risk.\(^{13,14}\) The Heart Protection Collaboration Group investigated associations between baseline Lp-PLA\(_2\) concentration and activity with incident CVD events in a randomized, placebo-controlled clinical trial of simvastatin.\(^{12}\) Baseline Lp-PLA\(_2\) concentration and...
activity were associated with CHD events in analyses that adjusted for non-lipid risk factors (hazard ratio (HR) per 1-SD Lp-PLA2 concentration, 1.08 (1.03–1.12); and HR per 1-SD Lp-PLA2 activity, 1.11 (1.06–1.15)). After adjustment for apoB, the associations between Lp-PLA2 activity and CHD events became non-significant (HR 1.02, 0.97–1.06), whereas associations between Lp-PLA2 concentrations remained significant (HR 1.05, 1.01–1.09). This report did not investigate on-trial associations between Lp-PLA2 activity and a cardiovascular event, which is relevant because statins reduce LpPLA2 concentrations by 25% and essentially all high-risk individuals will be treated with statin therapy.

**Pharmacology of phospholipase A2 inhibitors**

Inhibitors of sPLA2 and Lp-PLA2 have distinct chemical structures and pharmacological properties that target the specific enzymatic site for the two enzymes. Thus, the mechanisms of action for the two classes of inhibitors will be discussed separately in this section. An overview of the current status of the phospholipase A2 inhibition programs is summarized in Table 1.

Secretory PLA2 enzymes share a common catalytic dyad that is distinct from other phospholipases that have a classic serine-based catalytic triad (Asp-His-Ser). This unique catalytic dyad served as the target for design of sPLA2 inhibitors. Varespladib sodium ([3-(aminooxoacetyl)-2-ethyl-1-(phenylmethyl)-1H-indol-4-yl]oxyacetate; A-001; Anthera Pharmaceuticals Inc., Hayward, CA, USA) is an intravenous formulation and varespladib methyl ([3-(aminooxoacetyl)-2-ethyl-1-(phenylmethyl)-1H-indol-4-yl]oxyacetate; A-001; Anthera Pharmaceuticals Inc., Hayward, CA, USA) is an oral formulation of indole-based sPLA2 inhibitors. Varespladib is a pan inhibitor of sPLA2 enzymes with half maximal inhibitory concentrations (IC50) in the low nM range (group IIA IC50: 9–14 nM; group V IC50: 77 nM; and group X IC50: 15 nM). The methylated prodrug, varespladib methyl, is used for oral administration and is rapidly metabolized by esterases to varespladib (A-001). Recent findings showed that sPLA2 enzymes, GII, V ad X, can participate in intracellular arachidonic acid release and as a consequence in the intracellular inflammatory signalling and production of bioactive lipid mediators by different pathways. Thus, to inhibit the intracellular inflammatory component of sPLA2 activity contributing to atherosclerosis and plaque rupture, sPLA2 inhibitors should penetrate cells. Indole-based sPLA2, such as varespladib acid, is cell impermeable, and therefore incapable of blocking intracellular effects. Furthermore, one may extrapolate that poor cell permeability may also compromise the transport of varespladib across arterial endothelial cells at places of atherogenesis. The clinical failure of this compound to produce beneficial results in rheumatoid arthritis and sepsis patients may in part be due to issues of poor cell permeability.

A novel class of GIVA cPLA2 inhibitors, the 2-oxamides, has been shown to inhibit sPLA2 activity. The long-chain 2-oxamide GK126 inhibited human group IIA sPLA2 activity (IC50 300 nM) and GV sPLA2 activity (IC50 440 nM), while it had no effect on GX sPLA2 activity.

Several selective and highly potent azetidinone inhibitors have been developed as pharmacological tools, and one agent, darapladib (SB480848), has entered into two large Phase III clinical outcomes trials. Azetidinones target the active-site serine residue of the enzyme. The Lp-PLA2 inhibitor darapladib has an IC50 of 270 pM. In phase II trials, the reduction in circulating Lp-PLA2 activity has achieved 80%. Rilapladib is a second oral inhibitor of the azetidinones class that has entered phase II studies.

**Table 1** Comparison between LpPLA2 and sPLA2 inhibition as potential anti-atherosclerosis therapies

<table>
<thead>
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<th>Phospholipase A2 inhibition programs</th>
<th>Darapladib</th>
<th>Varespladib</th>
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<td><strong>Target: enzymatic activity inhibition</strong></td>
<td>Lp-PLA2 (PAF-Ach)</td>
<td>sPLA2 (II &gt; X &gt; V)</td>
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<td><strong>Target biology validation</strong></td>
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<td>Pig</td>
<td>Yes (Diabetic)</td>
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<td><strong>Target is a biomarker/clinical association with CVD events/risk</strong></td>
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<td>Yes (8 studies)</td>
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<td><strong>Clinical data with compound</strong></td>
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Experimental animal studies of secretory phospholipase A\textsubscript{2} inhibition

The anti-atherosclerotic effects of varespladib methyl have been investigated in several non-clinical models that include an ApoE\textsuperscript{-/-} mouse model,\textsuperscript{96} an accelerated ApoE\textsuperscript{-/-} mouse model,\textsuperscript{97} and a guinea pig atherosclerosis model.\textsuperscript{98} In ApoE\textsuperscript{-/-} mice, the combination of varespladib methyl and pravastatin effectuated a synergistic effect on reducing atherosclerosis.\textsuperscript{99} Varespladib methyl treatment also increased fibrous cap thickness resulting in more stable plaque architecture. However, the CS7Bl6 murine model used for generation of apoE\textsuperscript{-/-} mouse is limited by its lack of GIIA sPLA\textsubscript{2} expression, thus the anti-atherosclerotic effect of varespladib is due to inhibition of other sPLA\textsubscript{2} enzymes and/or a specific unknown sPLA3 enzyme that is expressed in the mouse as a compensatory mechanism for lacking GIIA sPLA\textsubscript{2} expression. In a guinea pig model, animals were fed a high-fat, high-cholesterol diet to induce atherosclerosis.\textsuperscript{99} Varespladib methyl (150 mg/kg/day) reduced cholesterol accumulation in aortic tissue without changing serum cholesterol levels; however, atherosclerosis was non-significantly reduced. Together, these rodent studies show that varespladib has a beneficial effect on atherosclerosis.

Clinical trials of phospholipase A\textsubscript{2} inhibitors

Pharmacological approaches with a lipoprotein-modulating and anti-inflammatory agent are potential novel approach to treating patients with atherosclerosis. Inhibition of sPLA\textsubscript{2} by means of administering a pan sPLA\textsubscript{2} inhibitor is one such approach. Clinical trials with the sPLA\textsubscript{2} inhibitor varespladib methyl have included phase II trials in patients with stable CHD and post-ACS. The recently terminated phase III trial with varespladib methyl was designed to investigate the short-term effects of varespladib methyl in ACS patients (Press release Anthera Pharmaceuticals, Inc., 9 March 2012).

Phospholipase levels and serological markers of atherosclerosis (PLASMA) was a phase II, randomized, double-blind, placebo controlled parallel arm dose-ranging study of four doses of varespladib methyl (50, 100, 250, and 500 mg twice daily) in 396 patients with stable CHD.\textsuperscript{99} After 8 weeks, varespladib methyl treatment reduced GIIA sPLA\textsubscript{2} levels from 69 to 96% in a dose-dependent manner (\(P < 0.0001\)). Varespladib methyl treatment also lowered concentrations of LDL cholesterol, oxidized LDL, and hs-C-reactive protein (Figure 5). The most common adverse reactions were non-specific and transient with no-dose response relationship.\textsuperscript{99–101} In PLASMA II, varespladib methyl 250 mg and 500 mg given once daily was evaluated in 135 stable CHD patients.\textsuperscript{100} When compared with placebo, 8 weeks treatment with varespladib methyl lowered GIIA sPLA\textsubscript{2} protein concentrations by 73 to 84%. Varespladib methyl 500 mg once daily reduced concentrations of LDL cholesterol by 15% (\(P < 0.001\)), non-HDL cholesterol by 15% (\(P < 0.0001\)), and ApoB by 15% (\(P < 0.01\)); however, the lipid effects were non-significantly changed with the lower dosage. In the Fewer Recurrent Acute coronary events with Near-term Cardiovascular Inflammatory Suppression (FRANCIS) trial, the effects of varespladib methyl were evaluated on plasma biomarkers in 625 ACS patients who were treated with atorvastatin 80 mg daily.\textsuperscript{103} Compared with placebo, varespladib methyl significantly lowered GIIA sPLA\textsubscript{2}, LDL cholesterol, and hs-C-reactive protein levels at each time point except at week 8 for hs-C-reactive protein. More varespladib methyl treated subjects (51%) had combined LDL cholesterol levels <1.81 mmol/L and hs-C-reactive protein <3 mg/L at 24 weeks than those treated with placebo (34%, \(P = 0.0013\)). At the same point, 27% subjects treated with varespladib methyl had combined LDL cholesterol levels <1.81 mmol/L (70 mg/dL) and hs-C-reactive protein <1 mg/L compared with 16% on the placebo (\(P = 0.01\)). In a subgroup analysis of 624 diabetics patients, the anti-inflammatory effect of varespladib methyl was larger in patients with diabetes.\textsuperscript{102} Further corroboration of the differences in the anti-inflammatory effects of varespladib methyl in diabetic and non-diabetic patients requires further investigation in the larger Vascular Inflammation to Suppression to Treat Acute coronary syndromes at 16 weeks (VISTA-16) trial.\textsuperscript{103,104}

In the SPIDER-PCI (Secretory PLA\textsubscript{2} Inhibition to Decrease Enzyme Release after Percutaneous Coronary Intervention) trial, stable CHD patients were given high-dose varespladib methyl (500 mg twice daily) 3 to 5 days before elective PCI.\textsuperscript{105} After 18–24 h, sPLA\textsubscript{2} activity was significantly reduced. Five days after PCI, sPLA\textsubscript{2} activity was significantly reduced, but this reduction was not associated with reduced myocardial necrosis.

Several phase II trials with varespladib methyl demonstrate significant reductions in concentrations of GIIA sPLA\textsubscript{2}, LDL cholesterol, non-HDL cholesterol, and apoB. Limitations of these studies include lack of sPLA\textsubscript{2} activity that may have resulted from the use of an assay with insufficient sensitivity at the reduced enzyme mass.\textsuperscript{99} Although the mechanism for the reduction in GIIA sPLA\textsubscript{2} concentration requires further investigation, it has been proposed that varespladib interferes with the sPLA\textsubscript{2} synthesis and secretion.\textsuperscript{106} The involvement of ligand-receptor-mediated signalling in this autocrine pathway remains unknown.

Secretory phospholipase A\textsubscript{2} inhibition and cardiovascular disease events

The effects of sPLA\textsubscript{2} at the interface of lipoprotein modification and vascular inflammation while extending myocardial ischaemic injury served as the basis for the concept that sPLA\textsubscript{2} inhibition may be particularly useful for the prevention of cardiovascular morbidity and mortality in high-risk CHD patients and particularly in those patients presenting with ACS.\textsuperscript{18,19,103} VISTA-16 (at 16 weeks, NCT01130246) was an international multicentre double blind, placebo-controlled Phase III trial in 6500 high-risk ACS patients.\textsuperscript{103,104} This trial investigated the short-term efficacy of varespladib methyl therapy (500 mg daily) on cardiovascular morbidity and mortality when added to standard of care and atorvastatin. The ACS study population had been enriched by inclusion of other prior cardiovascular event or...
intervention and other risk factors that may include either diabetes mellitus, metabolic syndrome, HDL cholesterol <1.1 mmol/L, estimated GFR <60 mL/min. The primary endpoint was the composite of cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, or documented unstable angina with objective evidence of ischaemia requiring hospitalization. Study subjects were randomized within 96 h of the index event, and followed for a minimum 16 weeks. Survival status was ascertained at 6 months. Limitations of the VISTA-16 trial design included inclusion of only 6500 subjects in a short-term trial that was time-limited and not event driven. On 9 March 2012, this trial was halted by the Data and Safety Monitoring Board due to a lack of efficacy (Press release Anthera Pharmaceuticals Inc., 9 March 2012).

Another population for the future use of a sPLA2 inhibitor are patients with type 2 diabetes who often manifest the atherogenic dyslipidemia and low-grade inflammation. The inflammation in type 2 diabetes is associated with central obesity and accumulation of inflammatory cells within the visceral adipose tissue. Plasma sPLA2 activity is reported to be elevated in obesity. Inhibition of sPLA2 would be expected to decrease inflammatory cytokines such as IL-6, which is elevated in type 2 diabetes. Moreover, sPLA2 inhibition reduces NEFA levels, which would be expected to reduce glucose levels in the condition. Elevated concentration of small LDL particles in type 2 diabetes is associated with an increased risk for CHD. sPLA2 activity can generate small-dense LDL particles, and its inhibition reverses the effect. Therefore, pharmacological approaches with a dual effect that target inflammation and lipoprotein modulating effect reducing small-dense LDL particle formation could therefore provide a novel approach to treat patients with type 2 diabetes and its cardiovascular complications.

Experimental animal studies of lipoprotein-associated phospholipase A2 inhibition

Lipoprotein-associated phospholipase A2 inhibition with darapladib reduced formation of atherosclerotic lesions in experimental animal studies, and improves stability of atherosclerotic plaques through reduced vascular inflammation. In hypercholesterolaemic diabetic pigs, darapladib treatment (10 mg/kg/day) reduced vascular Lp–PLA2 activity (by 84%), plaque area and incidence of necrotic cores compared with controls. In addition, treatment with darapladib reduced expression of multiple genes related to leucocyte activity (CD68, chemokine receptor/ligand mRNAs). These data suggest that treatment with darapladib has important effects in mitigating key proinflammatory pathways (Figure 5).

Human biomarker studies of lipoprotein-associated phospholipase A2 inhibition

The clinical trial data with the Lp–PLA2 inhibitor darapladib includes a biomarker study and an atherosclerosis imaging trial. In stable CHD patients with achieved LDL cholesterol concentrations <115 mg/dL on atorvastatin, treatment with darapladib 40 mg, 80 mg, and 160 mg reduced Lp–PLA2 activity by 43, 55, and 66%, respectively. Lp–PLA2 concentration was reduced by 6, 12.9, and 9.3% at darapladib dosages of 40, 80, and 160 mg daily. Dissimilar to varespladib (sPLA2 inhibitor), treatment with darapladib was not accompanied by changes in lipid concentrations or hs-C-reactive protein. However, interleukin-6 levels (IL-6) were reduced by 12.3% (95% CI 22–1%; P = 0.028).

The Integrated Biomarker and Imaging Study (IBIS-2) investigated the effects of selective Lp–PLA2 inhibition with darapladib on plaque characteristics using intravascular ultrasound-based pahography, and C-reactive protein levels. Secondary outcome measures include endothelial function and plaque volume by quantitative coronary arteriography and virtual histology by intravascular ultrasonography. IBIS-2 enrolled 300 patients who had successful PCI or uncomplicated diagnostic catheterization and a suitable non-intervened coronary artery with IVUS. Treatment with darapladib had no effect on the co-primary endpoints that included coronary atheroma deformability assessed by intravascular palography and plasma hs-C-reactive protein concentration. A secondary endpoint, lipid necrotic core assessed by virtual histology, increased in the placebo group (4.5 ± 7.9 mm3; P = 0.009), while darapladib halted this increase (0.5 ± 13.9 mm3; P = 0.71), resulting in a significant treatment difference of −5.2 mm3 (P = 0.012). These findings from these phase II trials are consistent with the results from the porcine model, and suggest that selective Lp–PLA2 inhibition may improve stability of rupture-prone plaques.

In phase II clinical studies, darapladib is well tolerated at dosages of 40, 80, and 160 mg daily. The most common adverse reactions, reported in 16–36% of study subjects, include diarrhoea and malodour of the urine and faeces.

Lipoprotein-associated phospholipase A2 inhibitors and cardiovascular events

The clinical efficacy of darapladib will be investigated in two large-scale cardiovascular event trials that investigate the effects of selective Lp–PLA2 on non-fatal myocardial infarction, non-fatal stroke, and cardiovascular death in CHD patients. Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy Trial (STABILITY) is a multicentre, randomized, double blind, placebo-controlled trial that evaluates the effects of darapladib or placebo on cardiovascular events in 15 500 patients with stable CHD. Stabilization Of Plaques using Darapladib Thrombolysis in Myocardial Infarction (SOLID-TIMI52) is designed to investigate the effects of darapladib 160 mg daily vs. placebo on major cardiovascular events in 11 500 patients with a cardiovascular event (myocardial infarction or stroke) within the preceding 30 days. These two trials will continue until a requisite number of major CVD events have occurred.

In the two ongoing secondary prevention trials with darapladib, it will be important to investigate the relationships between baseline and on-trial Lp–PLA2 concentration and activity, and
cardiovascular events. Similar data are relevant for future clinical trials with the sPLA₂ inhibitor varespladib methyl.

Conclusions

The two major phospholipase A₂ enzymes that have been studied as biomarkers of cardiovascular risk and targets of pharmacological intervention differ in several important aspects. Secretory phospholipase A₂ groups IIa, V, and X hydrolyze native lipoproteins resulting in smaller, denser, and more electronegative lipoprotein particles that are less avidly internalized by the hepatic apoB/E receptor resulting in increased residence time in the circulation that allows for further physicochemical alterations, increased intimal proteoglycan binding, and incorporation by macrophage scavenger receptors and specific PLA₂R. Lp-PLA₂ requires oxidized LDL as a substrate, and it has no effect on native lipoproteins. The hydrolysis of phospholipids on lipoproteins and cell membranes results in bioactive lipids (NEFAs, lysophospholipids, and eicosanoids) that activate pro-inflammatory redox-sensitive transcription factors and enhance proapoptotic effects.

Varespladib is a selective inhibitor of sPLA₂ that has efficacy against multiple group IIa isoenzymes (pan-inhibitor). For this reason, some investigators refer to varespladib as non-specific inhibitor even though it is highly specific for sPLA₂. In stable CHD patients, varespladib methyl 500 mg daily reduced concentrations of LDL cholesterol, non-HDL cholesterol, and apoB. In addition, there were reductions in LDL and VLDL particle concentrations. In these trials, oxidized LDL levels were reduced, but concentrations of systemic inflammatory biomarkers were nonsignificantly reduced. However, in ACS patients, varespladib methyl reduced hs-C-reactive protein concentrations. Despite the favourable short-term biomarker data in varespladib methyl-treated subjects, therapy had no effect on cardiovascular events in the VISTA-16 trial. One reason may be the short duration of treatment to test an antiatherosclerotic mechanism.

Lp-PLA₂ inhibition with darapladib has undergone evaluation in a biomarker study, and a human atherosclerosis trial. In the biomarker trial, darapladib had no effects of plasma lipids, lipoproteins, or hs-C-reactive protein. In the coronary atherosclerosis imaging trial, there were no reductions in atheroma volume but the lipid necrotic core was reduced. In aggregate, the studies with darapladib indicate that this agent may reduce vulnerability of rupture-prone atherosclerotic lesions. The ongoing phase III clinical trials with the Lp-PLA₂ inhibitor will provide important information about the potential effects of targeting vascular inflammation through inhibition of Lp-PLA₂ in high-risk CHD patients. Future trials with sPLA₂ inhibitors may require use of a lipophilic agent and design of a trial with sufficient duration to investigate atherosclerosis mediated cardiovascular events.

Conflict of interest: E.H.-C. is employed by AstraZeneca, R&D, CVGI iMed, Sweden. R.S.R. has no conflicts of interest.

References


Phospholipase A2 enzymes


