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Review

Small-molecule inhibitors as potential therapeutics and as tools to understand the role of phospholipases A_2 ☆

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ABSTRACT

Phospholipase A_2 (PLA_2) enzymes are involved in various inflammatory pathological conditions including arthritis, cardiovascular and autoimmune diseases. The regulation of their catalytic activity is of high importance and a great effort has been devoted in developing synthetic inhibitors. We summarize the most important small-molecule synthetic PLA_2 inhibitors developed to target each one of the four major types of human PLA_2 (cytosolic $cPLA_2$, calcium-independent $iPLA_2$, secreted $sPLA_2$, and lipoprotein-associated $LpPLA_2$). We discuss recent applications of inhibitors to understand the role of each PLA_2 type and their therapeutic potential. Potent and selective PLA_2 inhibitors have been developed. Although some of them have been evaluated in clinical trials, none reached the market yet. Apart from their importance as potential medicinal agents, PLA_2 inhibitors are excellent tools to unveil the role that each PLA_2 type plays in cells and *in vivo*. Modern medicinal chemistry approaches are expected to generate improved PLA_2 inhibitors as new agents to treat inflammatory diseases.

1. Introduction

Enzymes are one of the most important classes of drug targets [1]. Small-molecule enzyme inhibitors have transformed human medicine and it is estimated to comprise roughly one-third to half of all marketed drugs [2]. Since the importance of bioactive lipids has been recognized [3], a great attention has been devoted to enzymes involved in lipid metabolism. Phospholipases A_2 (PLA_2s) are a superfamily of enzymes [4] characterized by their ability to hydrolyze the ester bond at the *sn*-2 position of membrane glycerophospholipids releasing free fatty acids, including arachidonic acid (AA), and initiating the eicosanoids cascade [5]. As a matter of fact, PLA_2s are the upstream regulators of the eicosanoid cascade supplying free fatty acids to cyclooxygenases, lipoxygenases, and cytochrome P450 enzymes, which thus produce various inflammatory mediators including prostaglandins, leukotrienes and thromboxanes. Because of their involvement in the development and progression of numerous inflammatory diseases [4,6], PLA_2s have attracted a great interest as medicinal targets for more than twenty years. As a consequence, many small-molecule synthetic inhibitors have been developed in both academia and pharmaceutical companies [4].

In mammals, the phospholipase A_2 superfamily consists of six types of diverse enzymes: GIV PLA_2 [cytosolic calcium-dependent PLA_2 ($cPLA_2$)], GVI PLA_2 [calcium-independent PLA_2 ($iPLA_2$)], several groups of secreted PLA_2 ($sPLA_2$), two groups of platelet-activating factor-acetylhydrolases PLA_2 (PAF-AHs or GVII and GVIII PLA_2), GXV PLA_2 (lysosomal PLA_2), and GXVI PLA_2 (adipose PLA_2) [4]. However, four types of human PLA_2s have been targeted for the development of synthetic inhibitors as new medicinal agents: GIVA $cPLA_2$, $sPLA_2$,

Abbreviations: 3D-QSAR, three-dimensional quantitative structure-activity relationship; AA, arachidonic acid; AD, Alzheimer's disease; AR, acrosome reaction; BEL, bromoenol lactone; BRB, blood-retina barrier; CLP, cecal ligation and puncture; COX, cyclooxygenase; $cPLA_2$, cytosolic PLA_2; DME, diabetic macular edema; DPPC, 1-palmitoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; DXMS, hydrogen/deuterium exchange mass spectrometry; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; ESI, electrospray ionization; FAAH, fatty acid amide hydrolase; HDL, high-density lipoprotein; HUVECs, human umbilical vein endothelial cells; IFD, induced fit docking; IFNγ, interferon gamma; $iPLA_2$, calcium-independent PLA_2; LDL, low-density lipoprotein; LOX, lipoxygenase; LPC, lysophosphatidylcholine; $LpPLA_2$, lipoprotein-associated PLA_2; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; MD, molecular dynamics; MRM, multiple reaction monitoring; mTOR, mammalian target of rapamycin; NOD, non-obese diabetic; NOX2, NADPH oxidase 2; NTD, neglected tropical diseases; PAF-AHs, platelet-activating factor-acetylhydrolases; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PET, positron emission tomography; PGE_2, prostaglandin E_2; PLPC, 1-palmitoyl-2-lauroyl-*sn*-glycero-3-phosphocholine; ROS, reactive oxygen species; SAR, structure-activity relationship; SD rats, Sprague-Dawley rats; SFN, sulforaphane; $sPLA_2$, secreted PLA_2; TXB_2, thromboxane B_2; TNFα, tumor necrosis factor alpha; UCB, unconjugated bilirubin; VEGF, vascular endothelial growth factor

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cytosolic GVIA iPLA$_2$ and lipoprotein-associated PLA$_2$ (LpPLA$_2$ or GVII PLA$_2$). The various classes of small-molecule synthetic PLA$_2$ inhibitors are summarized in two recent review articles [7,8]. Apart from their importance as new potential therapeutics to treat inflammatory diseases, small-molecule PLA$_2$ inhibitors are excellent tools to unveil the role that each PLA$_2$ type plays in cells and *in vivo*.

In the present review article, we discuss the inhibitors targeting each one of GIVA cPLA$_2$, GVIA iPLA$_2$, sPLA$_2$, and LpPLA$_2$. We present the most important small-molecule synthetic PLA$_2$ inhibitors developed and studied, focusing on recent research on PLA$_2$ inhibitors, their applications to understand the role of each PLA$_2$ type in cells and *in vivo* and their therapeutic potential. Computational studies aiming at understanding in depth enzyme-inhibitor interactions as well as recent advances in assaying the activity of PLA$_2$s are discussed. Inhibitors that reached clinical trials are also summarized, although none of them entered the clinical market yet.

2. Inhibitors of cytosolic phospholipase A$_2$

Although multiple subgroups of cytosolic PLA$_2$ have been discovered in different cell types [4], the first purified and sequenced Group IVA cPLA$_2$ (also known as cPLA$_2\alpha$) is the most well studied one. This enzyme is widely distributed in cells throughout most types of human tissue and it exhibits a marked preference for hydrolysis of AA at the *sn*-2 position of phospholipid substrates. GIVA cPLA$_2$ is an 85 kDa protein that is regulated by intracellular calcium. It contains 749 amino acids and consists of an N-terminal C2 domain and a C-terminal catalytic domain. The crystal structure of GIVA cPLA$_2$ was solved in 1999 by Dessen et al. [9] For the hydrolysis of its substrate phospholipid, the catalytic domain of GIVA cPLA$_2$ utilizes an unusual catalytic dyad, Ser-228/Asp-549, located in the α/β hydrolase domain [10,11]. In a recent review article, Leslie has reviewed the role of cytosolic PLA$_2$ in normal physiological processes and disease [12].

Indole derivatives, initially developed by Wyeth, constitute one of the most well studied classes of GIVA cPLA$_2$ inhibitors [4,13]. Ecopladib, presenting IC$_{50}$ values 0.15 μM in a GLU assay and 0.11 μM in a rat whole blood assay [14], displayed oral efficacy in the rat carrageenan air pouch and rat carrageenan-induced paw edema models and advanced to phase I clinical trials. Giripladib (**1**, Fig. 1) was the most promising of this indole series and it was advanced into a Phase II clinical trial for osteoarthritis. However, the trial was terminated due to a failure to differentiate from the standard of care with naproxen because of gastroenterologic effects [15].

The structurally related compound **2** (ZPL-5212372, Fig. 1, formerly known as PF-5212372), is a highly potent and selective inhibitor of GIVA cPLA$_2$ against both isolated enzyme and in whole cell systems (IC$_{50}$ value 7 nM in a GLU assay) [16], that exhibits slow-offset inhibitory kinetics affording long duration of action. It demonstrated excellent efficacy in small and large animal models of airway and skin inflammation and Ziarco Pharma conducted a Phase I single ascending dose study *via* the inhaled route in healthy volunteers. ZPL-5212372 was found to be safe and well tolerated up to high doses. A randomized, adaptive design, double-blind, placebo controlled, sequential group study to determine the safety, tolerability, pharmacokinetics and efficacy of twice daily application of a topical ZPL-5212372 (1.0% w/w) ointment administered for up to 2 weeks in adult healthy volunteers and patients with moderate to severe atopic dermatitis is in progress. Results information has been submitted to ClinicalTrials.gov in February 2018, but is not yet publicly available [17].

Avexxin's ω-3 polyunsaturated fatty acid derivative AVX001 (**3**, Fig. 1), which has been found to inhibit GIVA with an IC$_{50}$ value of 120 nM [18], entered clinical trials. A randomized, double-blind, placebo-controlled, dose-escalation first-in-man study to assess the safety and efficacy of topical AVX001 in patients with mild to moderate plaque psoriasis has been carried out demonstrating that treatment with AVX001 is well tolerated in doses up to 5% [19]. Avexxin has also claimed 2-oxothiazoles and related compounds as anti-inflammatory agents acting through the inhibition of GIVA cPLA$_2$ and AA release [20]. AVX235 (**4**, Fig. 1, known also as GK470) is a potent inhibitor of GIVA cPLA$_2$ (X_I(50) value of 0.011 mol fraction in a mixed micelle assay and IC$_{50}$ of 300 nM in a vesicle assay), which was found to suppress the release of AA with an IC$_{50}$ value of 0.6 μM, in SW982 fibroblast-like synoviocytes [21]. This inhibitor exhibited *in vivo* anti-inflammatory effects comparable to the reference drugs methotrexate and Enbrel in a prophylactic and in a therapeutic collagen-induced arthritis model, respectively. More recently, the anti-angiogenic effects of this inhibitor, in a patient-derived triple-negative basal-like breast cancer model was evaluated and significant tumor growth inhibition was observed after 8 days of treatment [22]. Decreased endothelial cell proliferation and fewer immature vessels in treated tumors were shown by histology.

Asubio Pharma developed another indole-based GIVA cPLA$_2$ inhibitor, ASB14780 (**5**, Fig. 1). This compound was found to be a potent GIVA cPLA$_2$ inhibitor *via* enzyme assay, cell-based assay, and guinea pig and human whole-blood assays (IC$_{50}$ value 0.020 μM in human whole blood assay) [23]. The daily administration of ASB14780 markedly ameliorated liver injury and hepatic fibrosis following 6 weeks of treatment with CCl_4 indicating that a GIVA cPLA$_2$ inhibitor could be useful for the treatment of nonalcoholic fatty liver diseases, including fatty liver and hepatic fibrosis [24].

Another important class of GIVA cPLA$_2$ inhibitors is the pyrrolidine-based compounds, such as pyrrophenone (**6**, Fig. 1) and RSC-3388 (**7**, Fig. 1). This class of inhibitors having 1,2,4-trisubstituted pyrrolidine framework was developed in 2000 by Seno et al. at Shionogi [25]. Both of these inhibitors are commercially available and have been used as tools to understand the role of the enzyme. RSC-3388 exhibited an IC$_{50}$ value of 1.8 nM in a PC/DOG assay [25], while pyrrophenone was found to be a potent and reversible inhibitor of human GIVA cPLA$_2$ (IC$_{50}$ 4.2 nM), that strongly inhibits AA release, prostaglandin E$_2$ (PGE$_2$), thromboxane B$_2$ (TXB$_2$) and leukotriene B$_4$ (LTB$_4$) formation in human whole blood [26]. A combination of extensive molecular dynamics (MD) simulations and deuterium exchange mass spectrometry experimental results was developed as a tool to define more accurate binding sites. Following such a methodology, it has been demonstrated that pyrrophenone is bound to the enzyme through numerous hydrophobic residues located distal from the active site, more precisely bound in the cap region near the interfacial binding surface of the enzyme [27].

Recently, it was reported that pyrrophenone blocked calcium release from the endoplasmic reticulum (ER) and concomitant increases in mitochondrial calcium in response to stimulation by ATP, serum and A23187 [28]. This off-target effect, implicating a serine hydrolase in regulation of ER calcium release, highlights the importance of careful dose-response studies with pyrrophenone to study GIVA cPLA$_2$ function. The role of GIVA cPLA$_2$ in local and systemic disease during *S. pneumonia* infection was investigated and it was demonstrated that pharmacological inhibition of GIVA cPLA$_2$ by RSC-3388 blocked *Streptococcus pneumoniae*-induced polymorphonuclear cells transepithelial migration *in vitro* [29]. Those results suggest that the enzyme plays a crucial role in eliciting pulmonary inflammation during pneumococcal infection. GIVA cPLA$_2$ has been shown to increase and mediate various activities in several human cancers including colon, prostate and lung cancers [30–32]. Most recently, the expression level of the enzyme in breast cancer cells in the absence and presence of doxorubicin, and in patients before and after chemotherapy was studied. It was found that blockage of GIVA cPLA$_2$ by either RSC-3388 or pyrrophenone sensitized aggressive breast cancer to doxorubicin through suppressing extracellular signal-regulated kinases (ERKs) and mammalian target of rapamycin (mTOR) kinases [33]. Thus, inhibition of GIVA cPLA$_2$ may be of therapeutic value to overcome chemoresistance in breast cancer.

Recent results have shown that GIVA cPLA$_2$ upregulates CD40

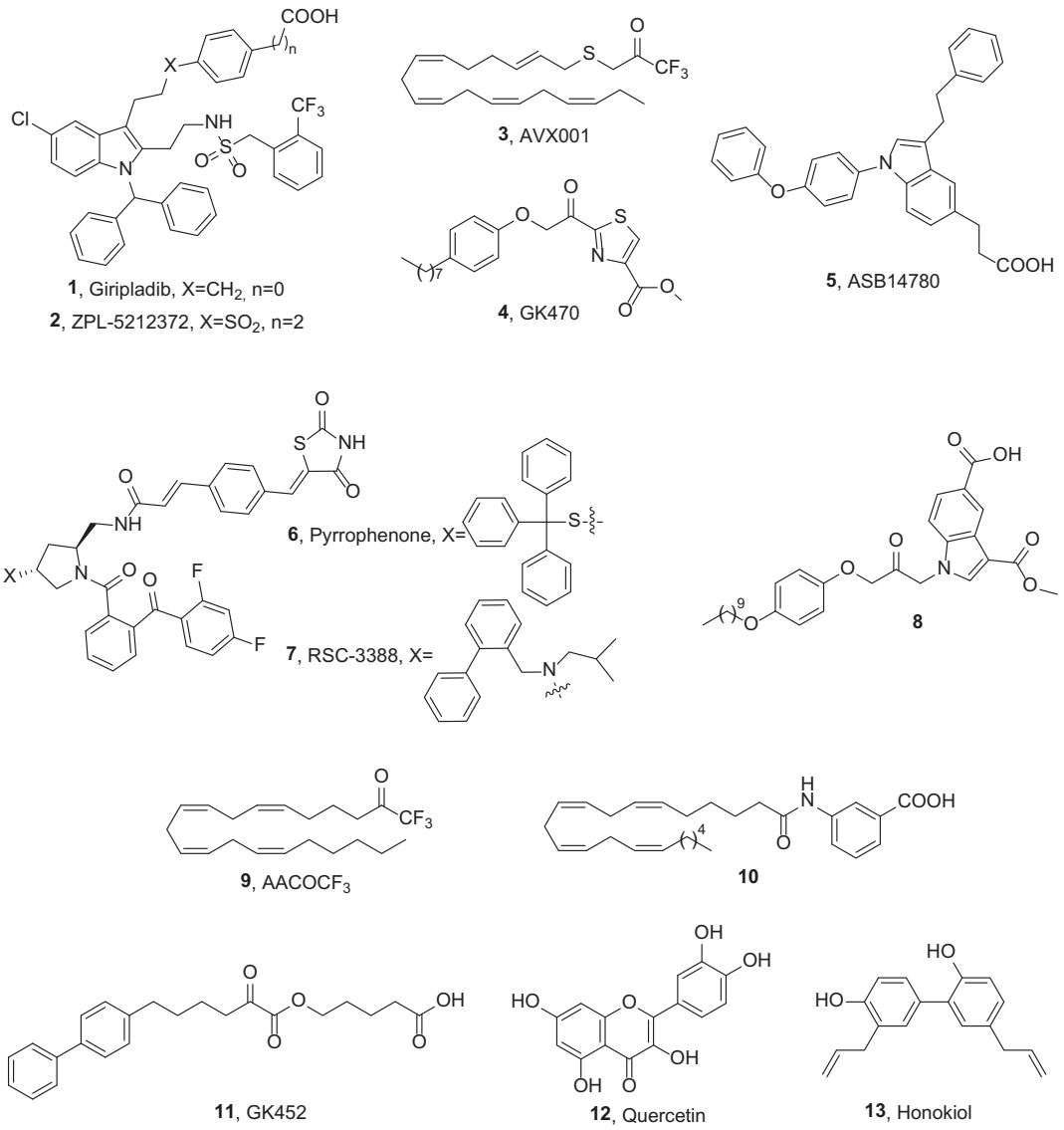

Fig. 1. Structures of GIVA cPLA$_2$ inhibitors.

protein expression induced by either lipopolysaccharide (LPS) or interferon gamma (IFNγ) and this effect is mediated *via* the activation of NOX2-NADPH oxidase and NF-κB [34]. Reduction of the enzyme upregulation by a specific antisense or inhibition by pyrrophenone prevented the induction of CD40 protein expression by either LPS or IFNγ. The results suggest that GIVA cPLA$_2$ has a direct role in CD40 upregulation, a feature of the pro-inflammatory M1-phenotype and indicate that GIVA cPLA$_2$ may serve as a pivotal amplifier of the inflammatory response in the CNS.

Another study has provided evidence that GIVA cPLA$_2$ activity is critically involved in the replication of various +RNA virus families and may thus represent a candidate target for broad-spectrum antiviral drug development [35]. The inhibition of GIVA cPLA$_2$ activity by the low-molecular-weight inhibitor pyrrolidine-2 (RSC-3388) has profound effects on viral RNA and protein accumulation in human coronavirus 229E-infected Huh-7 cells.

Lehr and coworkers have developed 1-heteroarylpropan-2-ones, like compound **8** (Fig. 1), as potent inhibitors of GIVA cPLA$_2$ [36]. In a recent work, they studied in detail structurally related compounds as dual inhibitors of GIVA cPLA$_2$ and fatty acid amide hydrolase (FAAH). Bioisosteric replacement of the carboxylic acid functionality of 1-(5-carboxyindazol-1-yl)propan-2-ones by inverse amides, sulfonylamides, carbamates and ureas showed that the carboxylic acid functionality of the lead compounds is of special importance for a pronounced inhibition of GIVA cPLA$_2$ and FAAH [37]. Since the serine reactive ketone functionality of such compounds is readily metabolized to inactive alcohol derivatives, this moiety was replaced by α-ketoheterocycle, cyanamide and nitrile serine traps, in an effort to obtain metabolically stable inhibitors. However, studies of the inhibitory activity and metabolic stability of these substances revealed that in all cases an increased metabolic stability was accompanied by a loss of inhibitory potency against GIVA cPLA$_2$ and FAAH [38]. Structure-activity relationship studies on 1-(2-oxopropyl)indole-5-carboxylic acids explored the effect of butanoyl and hexanoyl substituents in position 3 of the indole scaffold bearing terminal groups of varying polarity [39]. The inhibitory potency was not considerably affected in most cases, while metabolic phase I and phase II *in vitro* stability and aqueous solubility were influenced and modulated by the structural modifications performed.

Since GIVA cPLA$_2$ plays a critical role in neurodegenerative disorders associated with neuroinflammation, an effective positron emission tomography (PET) radioligand for imaging GIVA cPLA$_2$ in living brain might prove a useful tool for biomedical research. Thus, four high-affinity indole-5-carboxylic acid-based inhibitors of GIVA cPLA$_2$

(IC_{50} 2.1 to 12 nM) were selected for labelling in carboxyl position with carbon-11 ($t_{1/2}$ = 20.4 min) to provide candidate PET radioligands for imaging brain enzyme [40]. However, all the synthesized and tested [^{11}C]arylcarboxylic acids showed low brain penetration and lack of retention in mouse brain *in vivo* showing that these compounds are ineffective brain PET radioligands for GIVA cPLA$_2$.

For better efficiency, an ideal GIVA cPLA$_2$ inhibitor should not only be able to inhibit inflammation, but should also reach the site where inflammatory processes are taking place. Drug delivery systems that target the site of inflammation, or protect PLA$_2$ inhibitors from *in vivo* degradation or detoxification are urgently needed. Arachidonoyl trifluoromethyl ketone (**9**, Fig. 1, AACOCF$_3$, X_I(50) 0.036) is the first GIVA cPLA$_2$ inhibitor reported in 1993 [41], and, although not potent and selective inhibitor, it has been widely used to study the role of this enzyme in cells and in animals. A novel nanoliposomal delivery system of inhibitor AACOCF$_3$ was developed for melanoma treatment [42].This system, called NanoATK, loaded 61.7% of the inhibitor and was stable at 4 °C for 12 weeks. The formulation decreased toxicity-enabling administration of higher doses, which was more effective at killing melanoma cells compared to free AACOCF$_3$.

Recently, a new generation of AA analogues was developed as potential neurological agents targeting GIVA cPLA$_2$ [43]. Among these compounds, inhibitor **10** (Fig. 1) exhibited 5.5-fold stronger GIVA cPLA$_2$ inhibition than AACOCF$_3$. Inhibitor **10** was found to be a GIVA cPLA$_2$ selective inhibitor, non-cytotoxic, cell and brain penetrant and capable of reducing reactive oxygen species (ROS) and nitric oxide (NO) production in stimulated microglial cells.

GIVA cPLA$_2$ inhibitors usually suffer from high lipophilicity. Inhibitors with ClogP values higher than 5 are not expected to present favorable ADME properties, according to Lipinski's rule of five [44]. The ClogP value is a measure of the hydrophobicity, representing the calculated partition coefficient in octanol/water on a logarithmic scale. In addition, the molecular weight of the inhibitor should be lower than 500, again according to Lipinski's rule [44]. The ClogP values, calculated using ChemDraw, together with the molecular weights of inhibitors **1**–**10** are summarized in Table 1. It is obvious that none of them presents a ClogP value lower than 5. Furthermore, a number of them (**1**, **2**, **6**, **7** and **8**) have molecular weights higher than 500. Kokotos and Dennis have developed in the past 2-oxoamides as potent inhibitors of GIVA cPLA$_2$ and demonstrated their *in vivo* activities [45,46]. In a continuation of that research, they have recently reported new 2-oxoamides with reduced lipophilicity [47]. A reduction in the lipophilicity of the *in vivo* active 2-oxoamide inhibitor AX048 was achieved by replacing the long aliphatic chain with a chain containing an aromatic ring along with one or two ether oxygens.

Most recently, Kokotos and Dennis have developed a novel class of highly potent GIVA cPLA$_2$ inhibitors, namely 2-oxoesters [48]. 2-Oxoester GK452 (**11**, Fig. 1), containing a biphenyl system and a free carboxyl group, led to highly potent and selective GIVA cPLA$_2$ *in vitro* inhibition exhibiting an X_I(50) value of 0.000078. This inhibitor is the first example of a highly potent GIVA cPLA$_2$ inhibitor, which presents a ClogP value lower than 5 (ClogP value 4.70). In RAW264.7 macrophages, treatment with the most potent 2-oxoester inhibitor GK452 resulted in over 50% decrease in KLA-elicited prostaglandin D$_2$ production.

Apart from synthetic small-molecules, some natural products have been found to inhibit GIVA cPLA$_2$. The enzyme has been reported to play an important role in ROS/NO signaling during microglial activation through the lipoxygenase pathway [49]. Botanical polyphenols, such as quercetin (**12**, Fig. 1) and honokiol (**13**, Fig. 1), were effective in inhibiting LPS-induced NO production and phosphorylation of GIVA cPLA$_2$ indicating a potential use of botanical polyphenols to ameliorate the neurotoxic effects [50].

Table 1
ClogP values and molecular weights of some GIVA cPLA$_2$ inhibitors.

Inhibitor	ClogP[a]	MW
Giripladib (**1**)	10.50	745.25
ZPL-5212372 (**2**)	7.85	823.34
AVX001 (**3**)	7.58	386.52
GK470 (**4**)	5.96	389.51
ASB14780 (**5**)	8.98	461.56
Pyrrophenone (**6**)	9.19	876.01
RSC-3388 (**7**)	8.92	838.97
Inhibitor **8**	8.50	523.63
AACOCF$_3$ (**9**)	7.94	356.47
Inhibitor **10**	9.31	437.62
GK452 (**11**)	4.70	382.46

[a] Calculated using ChemDraw.

3. Inhibitors of calcium-independent phospholipase A$_2$

The distinctive features of the many isoforms belonging to group VI iPLA$_2$ are the fact that they are calcium-independent and that they do not show specificity for AA [51]. They are the most widespread PLA$_2$s throughout human tissues. The first and most widely described member of this family, GVIA iPLA$_2$ (also known as iPLA$_2$β), purified from macrophages in 1994 [52], also possesses lysophospholipase, transacylase and acyl-CoA thioesterase activity [53,54]. According to homology models and sequence alignments, the lipid hydrolysis is executed by a Ser/Asp catalytic dyad and the sequence consists of a linker region, seven ankyrin repeats and a catalytic domain [55]. Very recently, the crystal structure of GVIA iPLA$_2$ revealed that the protein forms a stable dimer mediated by catalytic domains with both active sites in close proximity, positioned for cooperative activation and internal transacylation [56]. Both active sites of the dimer are wide open and provide sufficient space for phospholipids to access the catalytic centers. The ankyrin repeat domains are oriented toward the membrane-binding interface, so that they can interact with membrane proteins. The structure of GVIA iPLA$_2$ monomer (PDB ID: 6AUN) is presented in Fig. 2. The missing loops were filled with Modeller [57] and the residues of the active site are represented with balls (yellow: C atoms, red: O atoms, blue: N atoms, white: H atoms).

In a recent review article, Ramanadham et al. discuss in detail how increased or decreased expression of iPLA$_2$s may affect the metabolic state, CNS function, cardiovascular performance and cell survival [58]. Since dysregulation of iPLA$_2$s may play a critical role in the development of many diseases, such as diabetes, Barth syndrome, ovarian cancer, ischemia, multiple sclerosis, research for the discovery of potent small-molecule inhibitors is therefore considered crucial [4,7,8,13].

Polyfluoroketones constitute the most important class of potent and selective small-molecule GVIA iPLA$_2$ inhibitors [59–61] and they have been used to study the role of the enzyme *ex vivo* and *in vivo* and in particular in autoimmune diseases [62–64]. FKGK11 (**14**, Fig. 3), presenting an X_I(50) value of 0.0014 [60], caused a strong reduction in the clinical severity and progression of experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis, showcasing that GVIA iPLA$_2$ plays a key-role in both the onset and the progression of the disease [62]. FKGK18 (**15**, Fig. 3) was found to be seven times more potent inhibitor of GVIA iPLA$_2$ (X_I(50) value of 0.0002) than FKGK11 and 195 and 455 times more potent for GVIA iPLA$_2$ than for GIVA cPLA$_2$ and GV sPLA$_2$, respectively [60]. GK187 (**16**, Fig. 3) is even more potent than FKGK18 (X_I(50) value of 0.0001) [61].

FKGK18, a reversible inhibitor which is 100-fold more selective for iPLA$_2$β (GVIA iPLA$_2$) than iPLA$_2$γ (GVIB iPLA$_2$) and does not inhibit other serine proteases, is not cytotoxic and inhibits β-cell apoptosis [65], has been investigated as for its impact on autoimmune diabetes development [66]. When administrated to spontaneous diabetes-prone non-obese diabetic (NOD) mice, FKGK18 was found to significantly decrease incidence of diabetes in association with reduced insulitis, improved glucose homeostasis, higher circulating insulin, and preservation of β-cell area. Moreover, FKGK18 reduced tumor necrosis

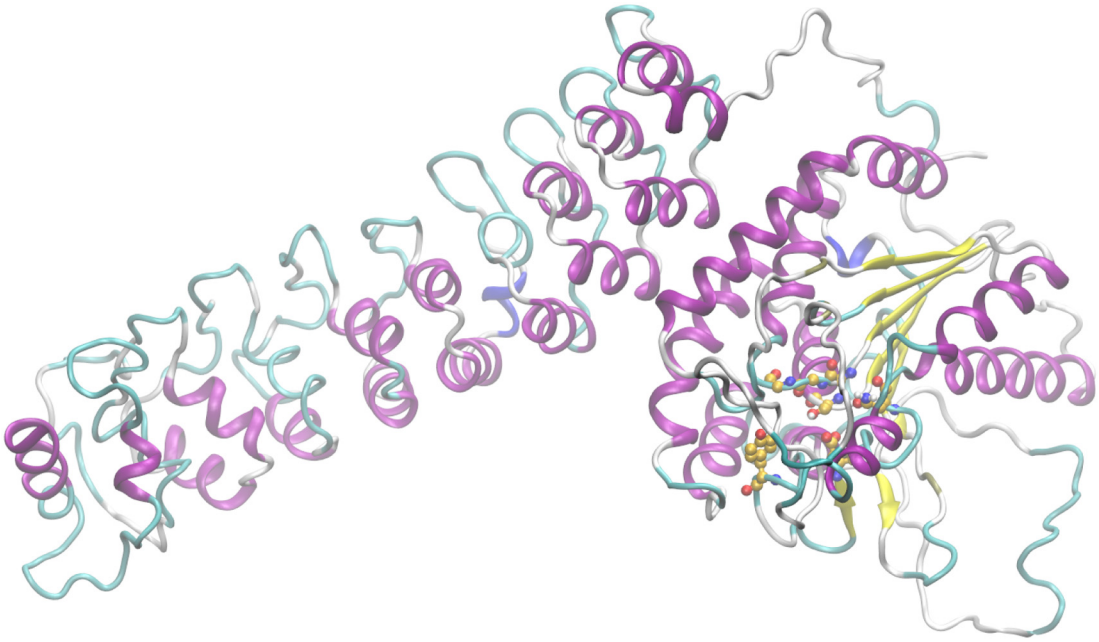

Fig. 2. Structure of GVIA iPLA$_2$ monomer.

factor alpha (TNFα) production from CD4$^+$ T-cells and antibodies from B-cells, suggesting modulation of immune cell responses by iPLA$_2$β or GVIA iPLA$_2$-derived products. Further, adoptive transfer of diabetes by CD4$^+$ T-cells to immunodeficient and nondiabetogenic NOD.*scid* mice was moderated by FKGK18 pretreatment and TNFα production from CD4$^+$ T-cells was reduced by inhibitors of cyclooxygenase (COX) and 12-lipoxygenase (12-LOX). Taken together, the observations suggest that GVIA iPLA$_2$ activation promotes immune responses, while GVIA iPLA$_2$ inhibition may be beneficial in ameliorating diabetes.

FKGK18 was also used, along with other inhibitors, so that the role of three different PLA$_2$ types in spontaneous and progesterone (P4)-induced acrosome reaction (AR) could be studied. More specifically, pyrrolidine-1 was used as a GIVA cPLA$_2$ inhibitor, LY329722 as a sPLA$_2$ (group X) inhibitor, while BEL and FKGK18 as GVIA iPLA$_2$ inhibitors. According to the results, GVIA iPLA$_2$ is crucial for spontaneous AR, both GVIA iPLA$_2$ and GX sPLA$_2$ are involved in P4-induced AR, while GIVA cPLA$_2$ is dispensable in both types [67]. Moreover, progesterone-induced AR was found to be a long lasting process, spreading over 30 min in the mouse and kinetic analyses suggest the presence of different sperm subpopulations, using distinct PLA$_2$ pathways to achieve AR. At low physiological P4 concentration (2 μM), sperm undergoing early AR (0–5 min post-P4) rely on GVIA iPLA$_2$, whereas sperm undergoing late AR (20–30 min post-P4) rely on GX sPLA$_2$. The role of PLA$_2$s in AR seemed to depend on P4 concentration, with the PLA$_2$s being key actors

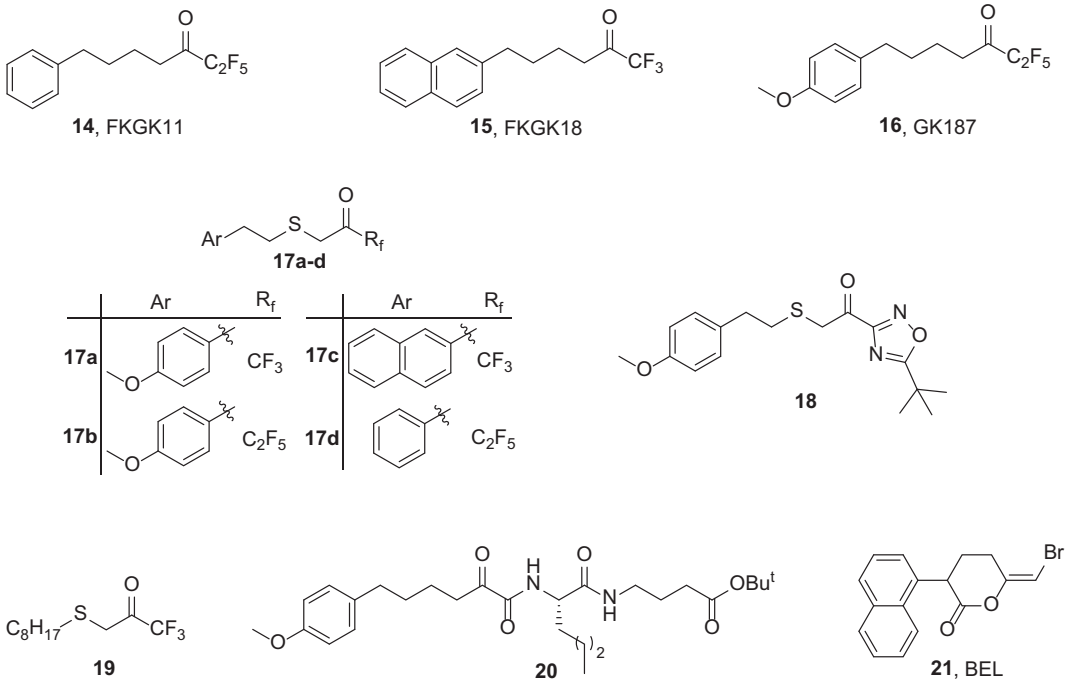

Fig. 3. Structures of GVIA iPLA$_2$ inhibitors.

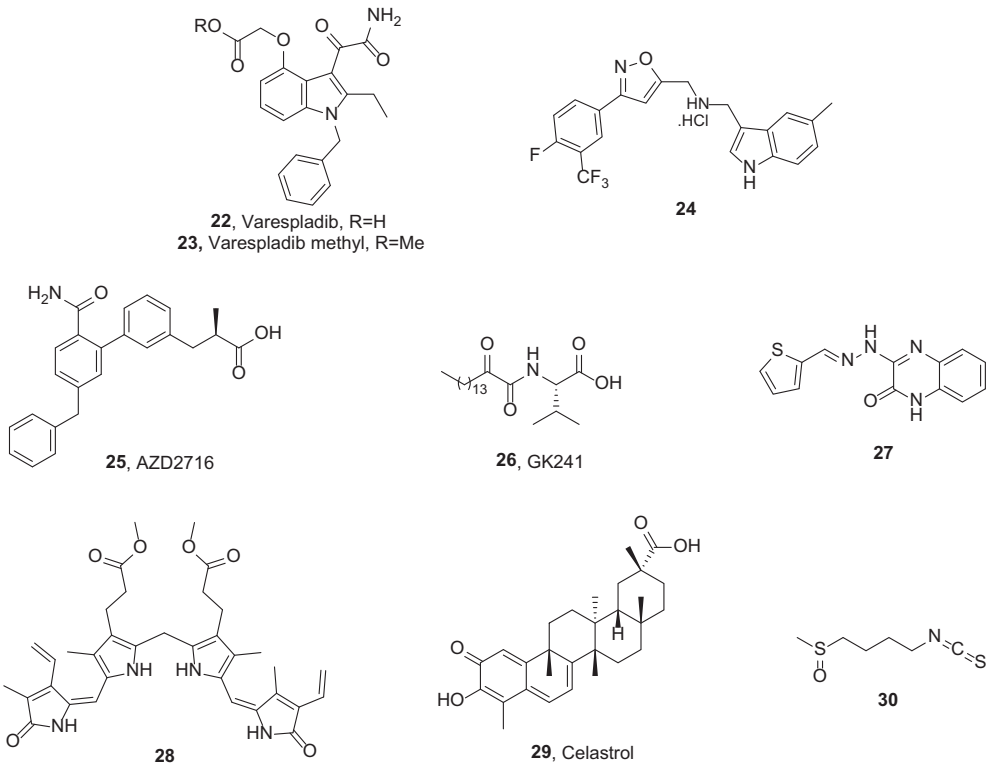

Fig. 4. Structures of sPLA$_2$ inhibitors.

at low physiological P4 concentrations (≤ 2 μM), but not at higher ones (~10 μM).

In 2014, GIVA cPLA$_2$ and GVIA iPLA$_2$ were studied for their substrate specificities during inflammatory activation of macrophages with zymosan, using selective inhibitors, more specifically, GIVA cPLA$_2$ inhibitor pyrrophenone (**6**, Fig. 1) and GVIA iPLA$_2$ inhibitor FKGK18 (**15**, Fig. 3) [68]. The data showed that the two enzymes act on different phospholipid pools. Unlike GIVA cPLA$_2$, GVIA iPLA$_2$ does not participate in AA release, but shows specificity for choline glycerophospholipid (PC) species containing palmitic acid at the *sn*-1 position to generate lysoPC (LPC) (16:0), which is a major acceptor for AA incorporation back into phospholipids.

Molecular docking calculations and MD simulations were employed so that a structure-activity relationship (SAR) between GVIA iPLA$_2$ and previously synthesized inhibitors could be established. Based on the SAR model, new compounds were synthesized and tested for the *in vitro* inhibitory activity against GIVA cPLA$_2$, GV sPLA$_2$ and GVIA iPLA$_2$. Among them, the thioether fluoroketone compounds **17a-d** (Fig. 3) and thioether keto-1,2,4-oxadiazole compound **18** (Fig. 3) potently inhibited GVIA iPLA$_2$ and were quite selective relative to GIVA cPLA$_2$ and GV sPLA$_2$ [69].

Linear aliphatic beta-substituted trifluoromethyl ketones were tested against human GVIA iPLA$_2$, GIVA cPLA$_2$ and GV sPLA$_2$. The beta-thiotrifluoromethyl ketone **19** (Fig. 3) was found to be highly potent inhibitor of GVIA iPLA$_2$ with an X_I(50) value of 0.0002 mol fraction (IC_{50}110 nM), while it did not show significant inhibition toward GIVA cPLA$_2$ and GV sPLA$_2$ [70]. Additionally, combination of hydrogen/deuterium exchange mass spectrometry (DXMS) with computer-aided design techniques, led to an enzyme-inhibitor complex that disclosed the binding mode of **19** (Fig. 3) in the binding site of the enzyme.

New 2-oxoamides based on dipeptides and pseudodipeptides were synthesized and studied for their *in vitro* inhibitory activity against human GVIA iPLA$_2$, as well as for their selectivity over the other PLA$_2$ types. Compound **20** (Fig. 3), a 2-oxoamide based on Nle-GABA-OBu^t, presented significant inhibition of GVIA iPLA$_2$ (X_I(50) 0.007), as well as selectivity over GIVA cPLA$_2$ and GV sPLA$_2$ [71].

Bromoenol lactone (**21**, Fig. 3, BEL) is an irreversible, covalent inhibitor of GVIA iPLA$_2$, exhibiting 1000-fold selectivity for iPLA$_2$ over cPLA$_2$ and sPLA$_2$ [72,73]. Over the years, BEL has been used to discern the involvement of GVIA iPLA$_2$ in biological processes at cellular level as well as *in vivo*. However, several features of BEL decrease its feasibility for in cells and *in vivo* use: (a) irreversible inhibition of iPLA$_2$, (b) inactivation of other serine proteases, and (c) high toxicity due to its interaction with cysteines. In 2016, it was reported that iPLA$_2$β and iPLA$_2$γ are expressed and are active in murine cells of the osteoblastic phenotype and that their inhibition with BEL results in an initial increase in PGE$_2$ formation, due to iPLA$_2$-independent AA accumulation, followed by a decrease at higher BEL concentrations [74]. Moreover, BEL seems to react with intracellular cysteine and glutathione leading to glutathione depletion, which is responsible for the decrease in PGE$_2$ production. These findings suggest that BEL must be used with caution in a cellular environment, since all data presented resemble conditions of extreme oxidative stress.

4. Inhibitors of secreted phospholipase A$_2$

Secreted PLA$_2$s were the first type of PLA$_2$ enzymes identified and in mammals constitute the largest family, containing 10 catalytically active isoforms (IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XIIA) and one inactive isoform (XIIB) [4,75–77], that differ in source, structure and function. They are typically small proteins (14–18 kDa), which require Ca^{2+} in mM concentration for their catalytic activity. They utilize an Asp/His dyad in the active site and they are stabilized by six common disulfide bonds and one or two variable additional ones. Individual members of sPLA$_2$s exhibit unique tissue and cellular distributions and enzymatic properties, suggesting their distinct biological roles. They can act either by catalyzing reactions as enzymes or by binding to receptors and hence, they are involved in the activation of several biological pathways. Maintaining sPLA$_2$ homeostasis appears critical for several physiological functions, as up-regulation or down-regulation of

the expression of some $sPLA_2$ isoforms is related to pathological conditions, such as atherosclerosis, immune disorders and cancer, as summarized in several review articles [78–80]. As a result, a variety of synthetic inhibitors targeting $sPLA_2$ have been developed [4,7,8,13].

The most known $sPLA_2$ inhibitors are the indole-based varespladib (**22**, Fig. 4) [81] and its orally bioavailable prodrug, varespladib methyl (**23**, Fig. 4), which entered clinical trials for inflammatory diseases (*e.g.* sepsis, rheumatoid arthritis, acute coronary syndrome), but failed demonstrating poor efficacy [82–84].

Recently, the World Health Organization (WHO) has added snakebite envenoming to the priority list of Neglected Tropical Diseases (NTD) [85]. It is estimated that > 75% of the fatal snakebites occur before victims can reach the hospital for antivenom treatment. Thus, small molecule therapeutics seem attractive tools for initiating the treatment of snakebite in the pre-hospital environment and as adjuncts to antivenom therapy [86]. Varespladib and varespladib methyl have shown high-level $sPLA_2$ inhibition at nano-and picomolar concentrations against 28 medically important snake venoms using chromogenic assays [87]. *In vivo* proof-of-concept studies with varespladib had striking survival benefit against lethal doses of *Micrurus fulvius* and *Vipera berus* venom and suppressed venom-induced $sPLA_2$ activity in rats challenged with 100% lethal doses of *M. fulvius* venom.

Inhibitory potential of varespladib for snake envenomation was also evaluated by another research group. Treatment with varespladib showed a significant inhibitory effect to snake venom PLA_2, estimated by IC_{50} *in vitro* and ED_{50} *in vivo* (*Deinagkistrodon acutus*: IC_{50} 0.0037 μg/μL, ED_{50} 1.14 μg/g, *Agkistrodon halys*: IC_{50} 0.0016 μg/μL, ED_{50} 0.45 μg/g) [88]. In animal models, the severely hemorrhagic toxicity of *D. acutus* and *A. halys* venom was almost fully inhibited after administration. Moreover, signs of edema in gastrocnemius muscle were remarkably attenuated by administration of varespladib, with a reduced loss of myonecrosis and desmin. Serum levels of creatine kinase, lactate dehydrogenase isoenzyme 1, aspartate transaminase and alanine transaminase were down-regulated after treatment with varespladib, which indicated the protection to viscera injury.

In an effort to combine the PLA_2 inhibitory activities of indoles and isoxazoles, a series of indole containing isoxazole derivatives were studied as $sPLA_2$ inhibitors. Among them, compound **24** (Fig. 4) showed $sPLA_2$ inhibitory activity with an IC_{50} value of 10.23 μM *in vitro*. Tested on a group of rats using carrageenan model and paw volumes, **24** (Fig. 4) showed 75.67% and 76.54% edema inhibition at 3 h and 4 h, respectively. Further studies demonstrated that **24** (Fig. 4) showed *in vitro* antiproliferative activity, when tested against MCF-7 breast and DU145 prostate cancer cells in MTT assay [89].

Astra Zeneca developed a series of biphenyl derivatives as $sPLA_2$ inhibitors. AZD2716 (**25**, Fig. 4) inhibited GIIA, GV and GX $sPLA_2$ with IC_{50} values of 10, 40, and 400 nM, respectively, and demonstrated high plasma $sPLA_2$ inhibition ($IC_{u,50}$ 0.1 nM) [90]. *In vivo*, a dose of 30 mg orally administered to cynomolgus monkeys, generated a concentration-dependent inhibition of $sPLA_2$ activity in plasma ($IC_{u,80}$ 13 nM). When incubated with HepG2 cells, **25** (Fig. 4) inhibited $sPLA_2$ activity (IC_{50} < 14 nM) and suppressed production of GIIA $sPLA_2$ (IC_{50} 176 nM). In addition, **25** (Fig. 4) showed $sPLA_2$ inhibition (IC_{50} 56 nM) in atherosclerotic plaque homogenates, as obtained from carotid endarterectomy of coronary artery disease patients. Exhibiting excellent preclinical pharmacokinetic properties across different animal species and minimized safety risk, inhibitor **25** (Fig. 4) was selected as a clinical candidate for the treatment of coronary artery disease.

Since 2002, 2-oxoamide inhibitors have been synthesized and evaluated for their inhibitory activity against various PLA_2 types [45,46,91–93]. A continuation of this research, guided by molecular docking simulations, led to derivative **26** (Fig. 4, GK241), a long chain 2-oxoamide based on (*S*)-valine. Compound **26** (Fig. 4) exhibited high potency for inhibition of GIIA $sPLA_2$ (IC_{50} 143 nM and 68 nM against human and mouse GIIA $sPLA_2$, respectively). The inhibitor was proven ten times more selective for GIIA than GV $sPLA_2$ and did not exhibit any appreciable inhibition against other human and mouse $sPLA_2$ enzymes [94]. Additionally, inhibitor **26** (Fig. 4), as well as other $sPLA_2$ inhibitors, presented a significant suppression of IL-1β-stimulated PGE_2 release in rat renal mesangial cells, indicating that $sPLA_2$ plays a predominant role in the production of PGE_2 [95].

Quinoxalinone derivatives with dual activities on biochemically unrelated enzymes, but mutually involved in diabetes and its complications, were presented [96]. All of the studied compounds showed low micromolar IC_{50} values against the different $sPLA_2$ isozymes (IC_{50}s < 20 μM) and satisfactory IC_{50} values (IC_{50}s < 50 μM) against pancreatic α-glucosidase. For instance, compound **27** (Fig. 4) inhibited human GIIA, human GV, human GX and human GXIIA $sPLA_2$ with IC_{50} values of 2.81, 6.28, 4.43 and 3.81 μM, respectively, as well as α-glucosidase (IC_{50} 9.99 μM).

Unconjugated bilirubin (UCB), an endogenous antioxidant, has been previously reported as a GIIA $sPLA_2$ inhibitor [97]. Chemical modifications led to more hydrophobic derivatives, which were then evaluated for their inhibitory activity against AA cascade enzymes. Dimethyl ester of bilirubin (**28**) inhibited GIIA $sPLA_2$ activity in a concentration dependent manner with an IC_{50} value of 4.0 μM (~3 fold lower compared to UCB) [98]. *In vivo*, **28** (Fig. 4) reduced GIIA $sPLA_2$ induced edema in a dose dependent manner. Compound **28** (Fig. 4) also inhibited 5-LOX and COX-2 peroxidase activity in a concentration dependent manner with IC_{50} values of 2.0 μM and 1.0 μM, respectively. Furthermore, **28** (Fig. 4) inhibited AA induced platelet aggregation, as well as dose dependently decreased carrageenan induced mouse paw edema.

Celastrol (**29**, Fig. 4), a quinine methide triterpene, was found to modulate inflammation through inhibition of the catalytic activity of mediators of AA pathway. Briefly, celastrol inhibited GIIA $sPLA_2$ (IC_{50} 6 μM) and 5-LOX activity (IC_{50} 5 μM), in a concentration-dependent manner, and COX-2 peroxidase activity (IC_{50} 20 μM) *in vitro*. It, additionally, inhibited carrageenan-induced edema and GIIA $sPLA_2$-induced edema in mice. Co-injection of different concentrations of celastrol with 4 μg of human GIIA $sPLA_2$ into hind paws of mice decreased the edema ratio in a dose-dependent manner, while at 20 μg concentration, the edema was completely inhibited. Celastrol also inhibited LPS-stimulated production of PGE_2 in human neutrophils and exhibited strong antioxidant activities [99].

Various natural products have been claimed as $sPLA_2$ inhibitors. Maslinic acid, a natural pentacyclic triterpenoid, was proved to interact directly with human GIIA $sPLA_2$ and inhibit the enzyme activity in a concentration-dependent manner, by binding to the calcium binding and phospholipid interfacial site. Maslinic acid also inhibited human GIIA $sPLA_2$-induced THP-1 cell differentiation and migration [100].

Sulforaphane (**30**, Fig. 4, SFN), a natural isothiocyanate present in cruciferous vegetables, was examined for its effects on the expression and activity of GIIA $sPLA_2$ *in vitro* and *in vivo*. First, the effects of SFN on the expression and activity of GIIA $sPLA_2$ induced by LPS in human umbilical vein endothelial cells (HUVECs) were determined. Post-treatment of SFN (at 10–30 mM) potently inhibited the expression and activity of GIIA $sPLA_2$. *In vivo*, in a cecal ligation and puncture (CLP) model of sepsis, post-treatment with SFN markedly reduced GIIA $sPLA_2$ expression in both LPS-injected and CLP-induced sepsis mice. SFN also suppressed the activation of GIVA $cPLA_2$ and ERK1/2 by LPS [101].

5. Inhibitors of lipoprotein-associated phospholipase A_2

The phospholipases belonging to Group VII and Group VIII catalyze the hydrolysis of the acetyl group from the *sn*-2 position of PAF, a potent phospholipid mediator that plays a major role in inflammation [102], therefore were named PAF acetylhydrolases (PAF-AH). GVIIA PLA_2 associates with both low- and high-density lipoproteins (LDL and HDL) in human plasma, which led to the name lipoprotein-associated PLA_2 ($LpPLA_2$) [103]. $LpPLA_2$ is a calcium independent, secreted extracellularly enzyme of 45 kDa, which uses the catalytic triad Ser/His/

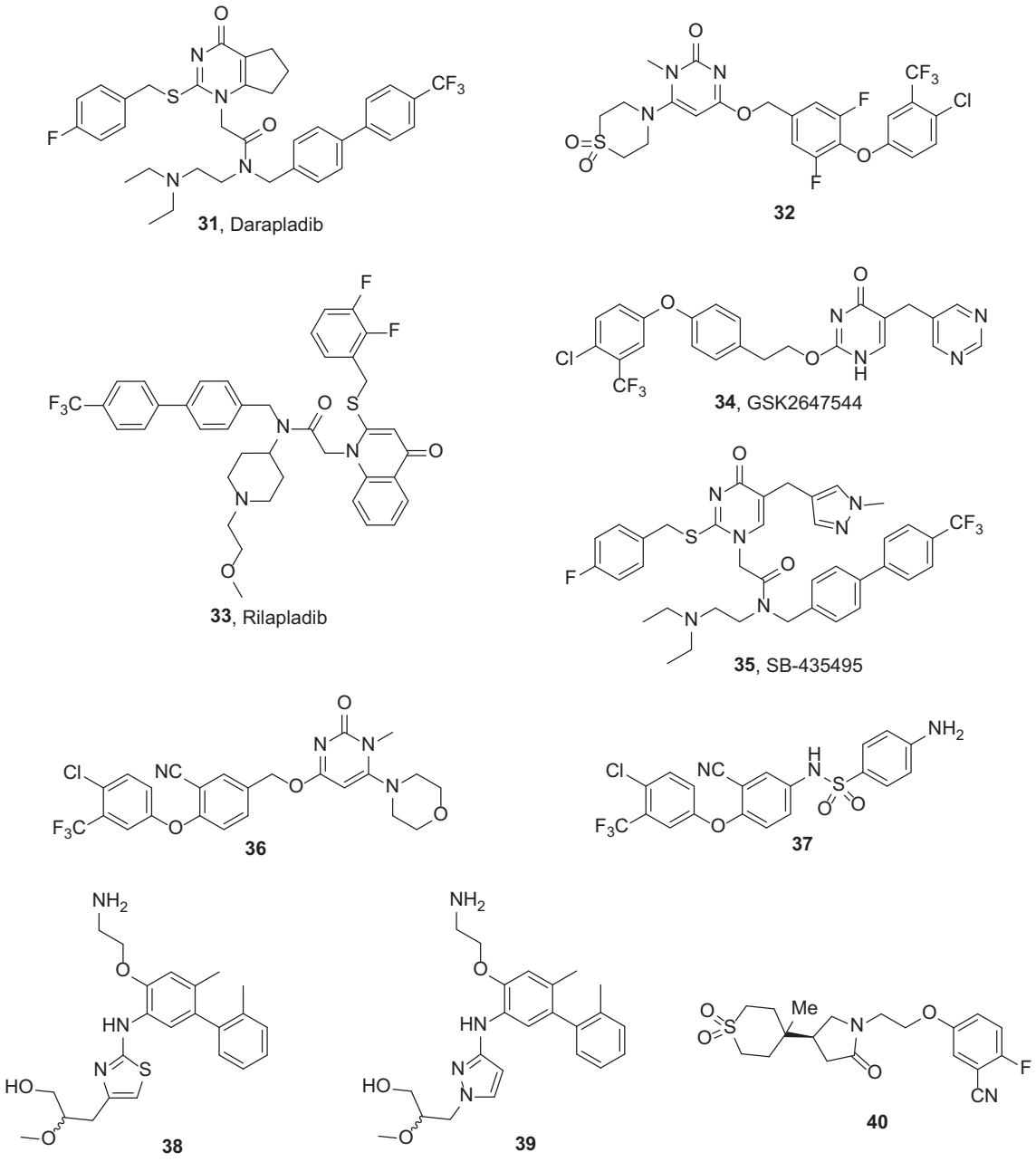

Fig. 5. Structures of $LpPLA_2$ inhibitors.

Asp [104]. $LpPLA_2$ shows broad substrate specificity; apart from PAF, it can also hydrolyze phosphatidylcholines with short-chain *sn*-2 residues, as well as oxidized phospholipids [105]. When cloned from human plasma in 1995, it was shown to have anti-inflammatory activity *in vivo* [106]; however subsequent clinical studies of $LpPLA_2$ levels in patients established this enzyme as a definitive marker of coronary heart disease [107]. Recently, a reagent for measuring $LpPLA_2$ activity received FDA approval [108]. Elevated levels of $LpPLA_2$ are associated with coronary heart disease and stroke [109,110]. Thus, further research is crucial for the development of new inhibitors of $LpPLA_2$.

Darapladib (**31**, Fig. 5) is a potent and selective inhibitor of $LpPLA_2$ (IC_{50} 0.25 nM against recombinant human $LpPLA_2$ in a DNPG assay), developed by GlaxoSmithKline [111], that has undergone two phase 3 trials for cardiovascular disease, but has been discontinued because of failure to reduce the risk of major coronary events [112,113]. Recently, darapladib was found to reduce elevated $LpPLA_2$, Rho kinase activity, and cardiomyocyte apoptosis in atherosclerosis, which can lead to cardiovascular protection [114]. In an *in vivo* rat type 2 diabetes mellitus model, darapladib was proven to reduce inflammation [115], to decrease vascular cell adhesion molecule-1 and intercellular adhesion molecules-1 aorta expression in early stages of atherosclerosis [116] and to improve insulin resistance occurring in the metabolic disorder [117]. Moreover, investigation of the effects of chronic diabetes mellitus and hypercholesterolaemia on pig retina revealed increased permeability of the blood-retina barrier (BRB) coupled with alterations in retinal architecture, including a leak of plasma components into the retina, selective immunoglobulin G binding to neurons in the ganglion cell layer, thinning of retinal layers due to cell loss and increased glial fibrillary acidic protein expression in Müller cells, which were all curtailed by treatment with darapladib [118].

Recently, the crystal structures of human $LpPLA_2$ bound with darapladib and inhibitor **32** (Fig. 5, IC_{50} 1.7 nM against recombinant human $LpPLA_2$) [119] were determined. Briefly, structural investigation into the $LpPLA_2$/darapladib and $LpPLA_2$/**32** complexes identified a

fairly open, large, relatively hydrophobic and rigid binding pocket. Key interactions between inhibitors and $LpPLA_2$ were revealed. The structure of $LpPLA_2$ proved very rigid and the binding of darapladib and **32** (Fig. 5) into $LpPLA_2$ did not change the overall conformation of the protein. Furthermore, isothermal titration calorimetry experiments revealed that the binding of these two inhibitors to $LpPLA_2$ is driven by enthalpic effects while the influence of entropy on the binding is negative [120].

GlaxoSmithKline has also introduced rilapladib (**33**, Fig. 5, SB-659032) and GSK2647544 (**34**, Fig. 5), which have both entered into clinical trials as potential treatment of Alzheimer's disease (AD). Rilapladib has completed a phase IIa study [121] to evaluate its effect in AD, where it demonstrated improved cognitive outcomes and changes to a number of mechanism- and disease-related biomarkers, suggesting that rilapladib and inhibition of $LpPLA_2$ may have the potential to slow the progression of AD and alter the underlying pathology in a subpopulation of AD patients with neuroimaging evidence of cerebrovascular disease [122]. GSK2647544 has been evaluated as for the safety, tolerability, pharmacokinetics and pharmacodynamics in healthy volunteers in phase 1 clinical trials [123]. The compound was generally well tolerated and had a reasonable PK-PD profile [124]. In order to refine therapeutic dose predictions and confirm brain penetration, a radiolabelled form of the inhibitor [^{18}F]GSK2647544 was manufactured for use in a PET biodistribution phase I study [125]. According to the results, GSK2647544 is able to cross the blood brain barrier and enter the human brain. An exploratory analysis of the data indicated that a dose of 102 mg, twice daily would be sufficient to inhibit ~80% brain $LpPLA_2$ [126].

Another study has investigated whether $LpPLA_2$ and lysophosphatidylcholine (LPC), are involved in BRB damage during diabetic retinopathy, using an analog of darapladib. Systemic $LpPLA_2$ inhibition using SB-435495 (**35**, Fig. 5) [127] at 10 mg/kg (i.p.), effectively suppressed BRB breakdown in streptozotocin-diabetic Brown Norway rats. This inhibitory effect was comparable to intravitreal vascular endothelial growth factor (VEGF) neutralization and the protection against BRB dysfunction was additive when both targets were inhibited simultaneously. Mechanistic studies in primary brain and retinal microvascular endothelial cells, as well as occluded rat pial microvessels, showed that luminal but not abluminal LPC potently induced permeability and that this required signaling by the VEGF receptor 2. The results suggested that $LpPLA_2$ could be an efficacious therapeutic target for diabetic macular edema (DME), either alone or in combination with anti-VEGF therapeutics [128].

Another series of pyrimidone derivatives was tested for the $LpPLA_2$ inhibitory activity and the effect on DME. Compound **36** (Fig. 5) demonstrated decent pharmacokinetic profile and robust inhibitory potency against $LpPLA_2$ in male Sprague-Dawley (SD) rats. The derivative presented IC_{50} values of 1.0 and 2.2 nM against rat plasma and recombinant human $LpPLA_2$, respectively. Moreover, **36** (Fig. 5) significantly inhibited retinal thickening in streptozotocin-induced diabetic SD rats, as a model of DME, after oral dosing for 4 weeks [119].

Combination of fragment screening, crystal structure determination, virtual screening and medicinal chemistry led to the identification of novel sulfonamide $LpPLA_2$ inhibitors. Compound **37** exhibited high inhibitory activity (IC_{50} 14 nM), good stability, as well as good permeability *in vitro* [129]. Additionally, inhibitor **37** (Fig. 5) showed favorable oral bioavailability in male SD rats and maintained the inhibitory activity for 24 h after oral administration, which is superior to that of darapladib.

An X-ray fragment screening was conducted in order to identify $LpPLA_2$ inhibitors, which do not make a direct interaction with the catalytic residues of the enzyme. This screening led to the identification of numerous fragment hits that effectively mapped the active site of the enzyme and occupied a binding surface similar to that defined by darapladib. However, a subset of fragment hits were revealed to bind in a novel pocket, approximately 13 Å from the oxyanion hole, formed by rotation of the Phe357 side chain. For instance, compounds **38** (Fig. 5) and **39** (Fig. 5) exhibited low IC_{50} values, molecular weights < 500 Da, and ClogP values of 3.6 and 3.4, respectively. In addition, they have comparable human plasma protein binding and artificial membrane permeability to darapladib. Finally, the work was halted because neither thiazole **38** (Fig. 5) nor pyrazole **39** (Fig. 5) possessed PK properties consistent with once-daily dosing in humans. Nonetheless, the success of fragment based drug discovery in identifying ligand efficient compounds with vastly improved physicochemical properties was demonstrated [130].

Based on the above fragment-based approach, lactam inhibitors of $LpPLA_2$ were developed [131]. Compound **40** (Fig. 5), a γ-lactam that bears a sulfone moiety, presented an IC_{50} value of 32 nM in whole human plasma assay, an inhibitory potency similar to that of darapladib. However, **40** (Fig. 5) has significantly lower MW and is less lipophilic than darapladib. Moreover, inhibitor **40** (Fig. 5) has promising pharmacokinetic properties and was found to bind in the oxyanion hole of the enzyme [131].

6. Computational studies on phospholipase A_2 inhibitors

Computational studies not only contribute to better understanding of the enzyme-substrate and enzyme-inhibitor interactions, but also constitute a powerful tool to discover novel inhibitors. The applications of rational drug design on the development of PLA_2 inhibitors have been reviewed in the past [132]. The combination of computational and experimental studies have been proven useful in defining inhibitor binding sites, facilitating the generation of improved enzyme inhibitors. Such a combination of MD simulations with DXMS helped to understand how pyrrophenone and 2-oxoamide inhibitors interact with GIVA $cPLA_2$ [27] and how fluoroketones interact with GVIA $iPLA_2$ [133].

Computational studies have been also employed to simulate the interactions of PLA_2s with substrates and cell membrane for the membrane-associated members [134]. The binding mode of a phospholipid into the active site of GIVA $cPLA_2$ and GVIA $iPLA_2$ has been explored using MD simulations driven by DXMS data [135]. This study highlighted the importance of the interactions between protein-membrane for the substrate binding and the catalytic mechanism. Since an X-ray structure was not available at that time, a homology model of GVIA $iPLA_2$ was created. The results showed that the polar head of the substrate 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) occupies the hydrophilic area close to the catalytic center. The phosphate group creates a hydrogen bond with Arg200 in GIVA $cPLA_2$ and Lys489 in GVIA $iPLA_2$ and the carbonyl group at the *sn*-2 position is located near the catalytic Ser288 in GIVA $cPLA_2$ and Ser519 in GVIA $iPLA_2$. The aliphatic chains of the fatty acids are accommodated in the hydrophobic areas of the channel of both proteins (Fig. 6).

In a continuation of that work, Mouchlis et al. further explored the substrate specificity by lipidomics and MD simulations [136]. They discovered that a unique hydrophobic area in the binding site accommodates the cleaved fatty acid and it thus drives the substrate specificity. Based on structural analysis and MD simulations, they identified an optimal phospholipid binding mode in the case of GIVA $cPLA_2$ and they could explain the substrate specificity differences between GVIA $iPLA_2$ and GV $sPLA_2$.

Computational studies, organic synthesis and *in vitro* assays led to the development of potent and selective GVIA $iPLA_2$ inhibitors. A homology model of GVIA $iPLA_2$ was used and the binding pocket was defined by the Gly486, Gly487, Lys489, Ser519, Val548, Phe549, Leu560, Tyr643, Phe644, Asp652, Lys729, and Leu770 residues [69]. The binding modes of trifluoromethyl ketone GK187 and pentafluoroethyl ketone FKGK18 were generated with IFD (Induced Fit Docking) calculations and the poses were subjected to MD simulations with NAMD [137] for 300 ns. According to the results (Fig. 7), although the carbonyl group of each compound interacts with the “oxyanion hole” Gly486/Gly487, it does not create any interaction with the

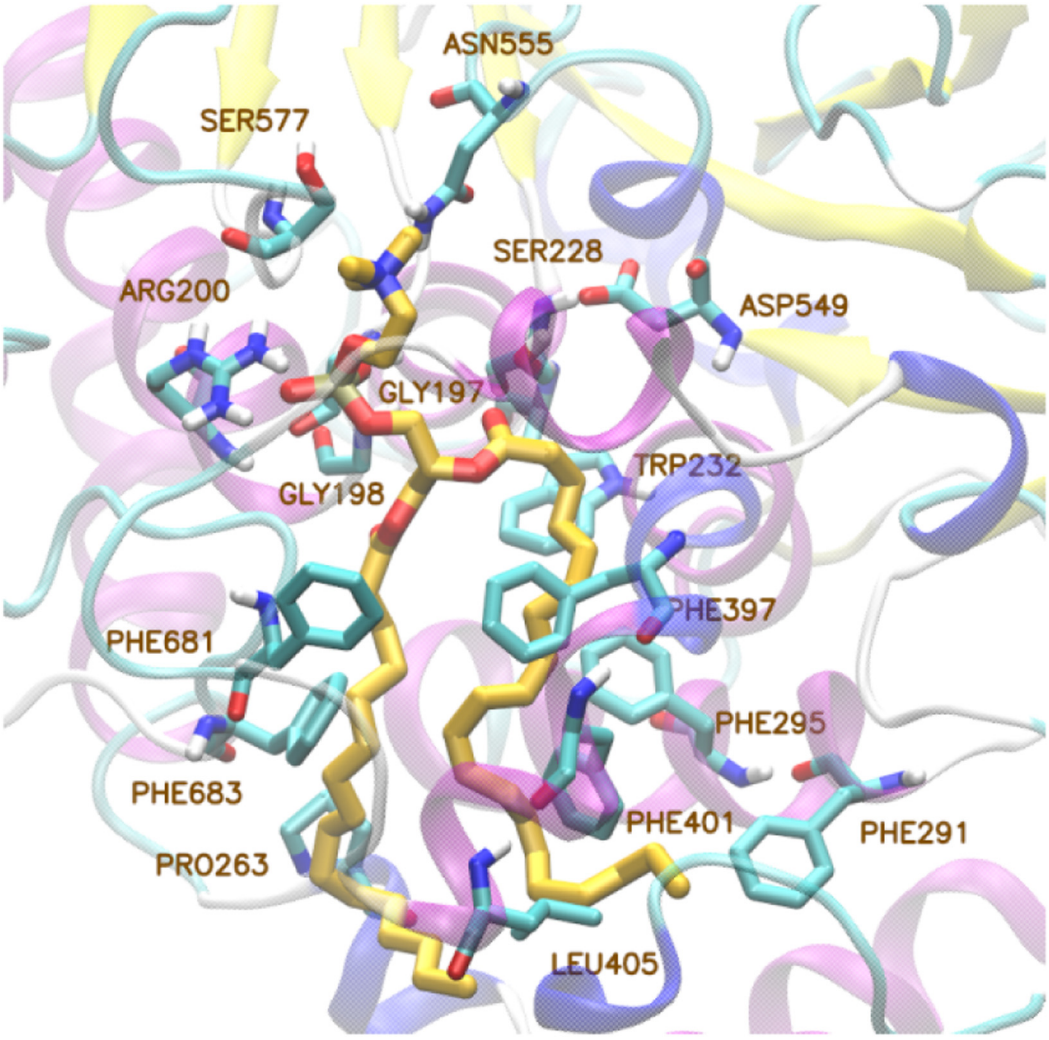

Fig. 6. Binding mode of PAPC substrate in the active site of GIVA cPLA$_2$.

catalytic Ser519. Also, the interactions the fluorine atoms create with Asn658 do not seem to be critical for the binding. Interestingly, the methoxyphenyl group of inhibitor GK187 and the naphthalene group of inhibitor FKGK18, which were located in the entrance of the binding pocket in the initial pose, moved to the hydrophobic region during the simulation.

The binding mode of a fluoroketone inhibitor to GIVA cPLA$_2$ was also studied [69]. The results showed that the same fluoroketone functional group can be used to construct inhibitors exhibiting selectivity for either GVIA iPLA$_2$ or GIVA cPLA$_2$. The size of the hydrophobic chain is very critical for both activity and selectivity. Short-chain compounds tend to selectively inhibit GVIA iPLA$_2$, while long-chain compounds also inhibit GIVA cPLA$_2$. That study, together with studies reported by Mouchlis et al. [70], indicated the importance of the presence of a sulfur atom at the β-position to the activated carbonyl group of fluoroketones.

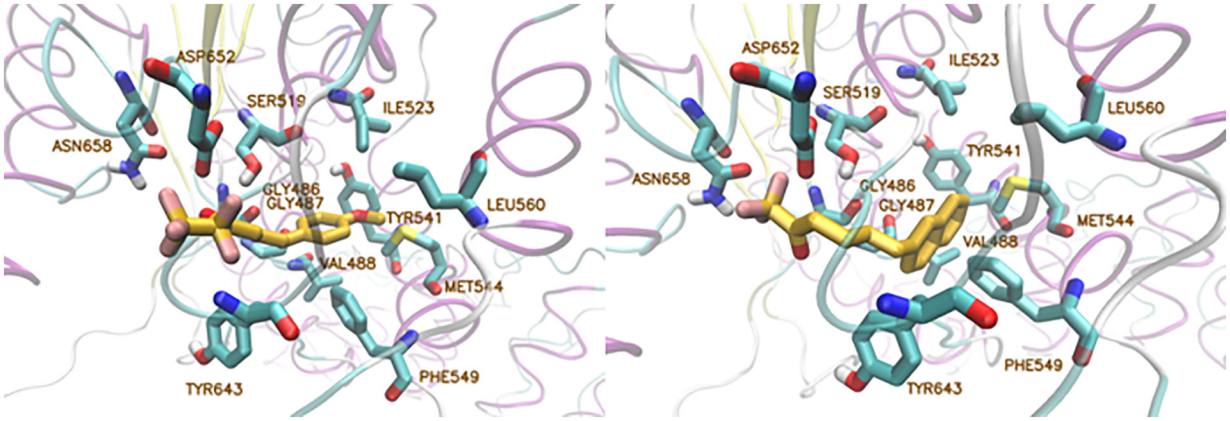

Fig. 7. Left: Binding mode of inhibitor GK187 in GVIA iPLA$_2$. Right: Binding mode of inhibitor FKGK18 in GVIA iPLA$_2$.

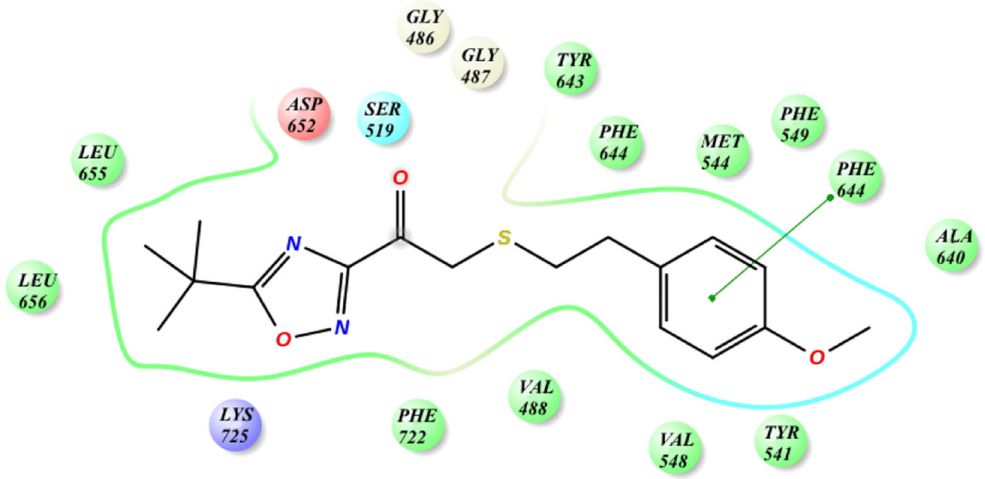

Fig. 8. Interactions of thioether **18** with the residues of GVIA iPLA$_2$.

The binding mode of the novel thioether derivative **18** to GVIA iPLA$_2$ was also studied [69]. Again the carbonyl group interacts with the "oxyanion hole" Gly486/Gly487, while Asn658 interacts *via* a hydrogen bond with the oxadiazole ring. The beneficial insertion of a sulfur atom is depicted by the higher affinity and, according to MD simulations, this is due to the interactions with the residues Tyr643, Phe722 and Leu770 (Fig. 8).

Recently, a library of small molecules, which contain the 2-oxoester functionality, was generated [48]. Inhibitor GK452, a 2-oxoester derivative with a biphenyl group and a free carboxyl group, presented high inhibitory activity against GIVA cPLA$_2$. According to its binding mode described in that paper, the carboxyl group of the molecule interacts with Arg200 and the 2-oxoester moiety interacts with the oxyanion hole (Gly197/Gly198) *via* a hydrogen bond (Fig. 9).

Structural modifications on known sPLA$_2$ inhibitors using computational tools have been reported for the design of new derivatives with improved potency. Docking calculations for the evaluation of derivatives of co-crystallized inhibitor FPL67047XX suggested the synthesis of new derivatives, which reproduced the key interactions with the residues of the binding pocket presenting lower binding energy [138]. In addition, Mouchlis et al. [139] reported the use of a three-dimensional quantitative structure-activity relationship (3D-QSAR) model for the design and evaluation of new indole derivatives against sPLA$_2$. The construction of the 3D-QSAR model was based on a set of 34 indole inhibitors and the generated new indole derivatives reproduced the key interactions.

The binding mode of 2-oxoamide inhibitor GK126 to GIIA sPLA$_2$ has been studied using GOLD [93]. Molecular docking calculations and MD simulations with AMBER led to the development of the more potent GIIA sPLA$_2$ inhibitor GK241 (Fig. 10) [94,140]. Docking calculations have been also employed to simulate the interaction in GIVA cPLA$_2$ – 2-oxoamide inhibitor complexes [141]. Inhibitor GK241 interacts with the calcium ion of GIIA sPLA$_2$ *via* its carboxyl group and the 2-carbonyl group of the amide functionality. During the MD simulation of GK241-enzyme complex, two hydrogen bonds are formed between the amide group and the residues His47 and Gly29, resulting in the stable binding of GK241 to the enzyme.

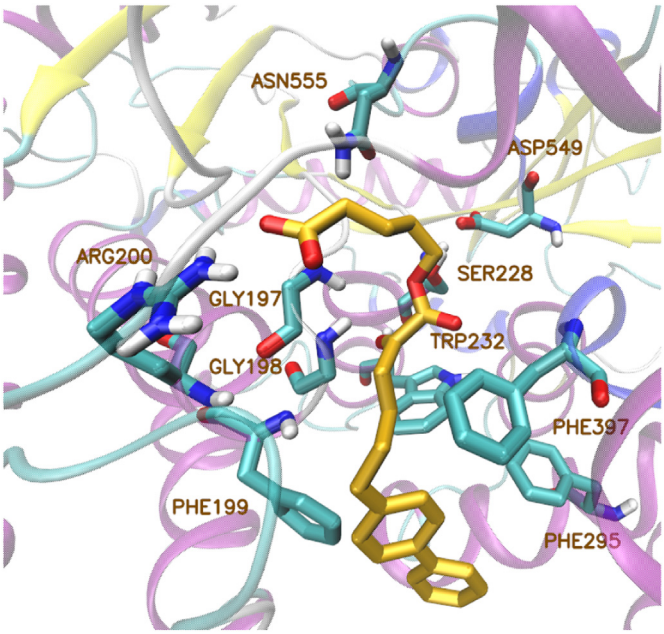

Fig. 9. Binding mode of inhibitor GK452 in the active site of GIVA cPLA$_2$.

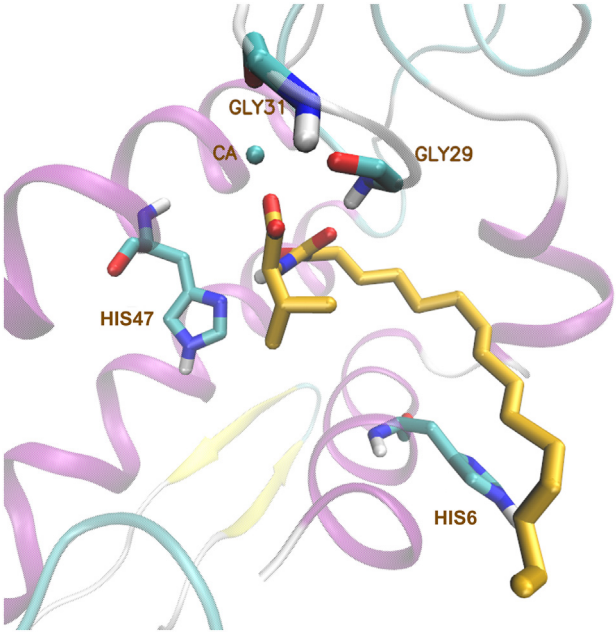

Fig. 10. Conformational arrangement of GK241 in the binding pocket of GIIA sPLA$_2$.

Table 2
Clinical trials of PLA_2 inhibitors.

Inhibitor	Clinical trial	Condition
Varespladib ($sPLA_2$)	Phase III clinical trial VISTA-16 clinicaltrials.gov Identifier: NCT01130246	Acute coronary syndrome
Giripladib ($cPLA_2$)	Phase II clinical trial clinicaltrials.gov Identifier: NCT00396955	Osteoarthritis
	Phase I clinical trial clinicaltrials.gov Identifier: NCT00440492	Rheumatoid arthritis
PF-5212372 (ZPL-5212372) ($cPLA_2$)	Phase I/II study clinicaltrials.gov Identifier: NCT02795832	Atopic dermatitis
Darabladib ($LpPLA_2$)	Phase III clinical trial STABILITY clinicaltrials.gov Identifier: NCT00799903	Atherosclerosis
	Phase III clinical trial SOLID-TIMI 52 clinicaltrials.gov Identifier: NCT01000727	Acute coronary syndrome
	Phase III clinical trial clinicaltrials.gov Identifier:NCT01067339	Endothelial dysfunction/ Coronary atherosclerosis
	Phase II clinical trial clinicaltrials.gov Identifier:NCT01506895	Diabetic retinopathy
Rilapladib ($LpPLA_2$)	Phase II clinical trial clinicaltrials.gov Identifier: NCT01428453	Alzheimer's disease
	Phase II clinical trial clinicaltrials.gov Identifier: NCT00695305	Atherosclerosis
GSK2647544 ($LpPLA_2$)	Phase I clinical trial clinicaltrials.gov Identifier: NCT01702467, NCT01978327, NCT01924858	Alzheimer's disease

7. Assaying the activity of phospholipases A_2

Assaying the activity of PLA_2s is challenging, because although these enzymes are water-soluble themselves, they act on phospholipid substrates, which aggregate in aqueous solution to form micelles, vesicles, or liposomes [142]. When the inhibition of each PLA_2 type is compared, one must note the particular assay conditions and aggregated form of substrate used. Almost twenty years ago, group-specific radiolabel-based assays able to distinguish between the four major types of mammalian PLA_2s have been developed [143]. The purpose of this subchapter is to highlight some of the recently reported assays.

Most recently, a novel mass spectrometric-based high-throughput assay (96 well-plate assay) toward both natural and synthetic membrane phospholipids in mixed micelles with a nonionic surfactant has been reported [136]. In this lipidomics-based HPLC/MS assay, a HILIC column and multiple reaction monitoring (MRM) were used for targeted quantification of the assay components including the surfactant (octaethylene glycol monododecyl ether, C12E8), a free fatty acid (AA), a phospholipid (1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, PAPC), a lysophospholipid (1-palmitoyl-*sn*-glycero-3-phosphocholine, 16:0 LPC), and an internal standard (1-heptadecanoyl-*sn*-glycero-3-phosphocholine, 17:0 LPC). AA was detected in negative electrospray ionization (ESI) mode, while lysophospholipids, phospholipids and surfactant (C12E8) in positive ESI mode. Unexpected head group and acyl chain specificity for three major PLA_2 types was discovered. These studies allowed a detailed understanding of the preference of GIVA $cPLA_2$ for cleavage of pro-inflammatory AA, and indicated the preference of GIVA $iPLA_2$ for cleavage of linoleic acid and the preference of GV $sPLA_2$ for linoleic acid, saturated fatty acids and phosphatidylglycerol.

Barbour and Ramanadham reported a detailed procedure to measure GVIA $iPLA_2$-specific activity in cell lines or tissue preparations using a simple radiolabel-based assay and to evaluate the impact of small-molecule inhibitors on resting- and disease-state GVIA $iPLA_2$ activity [144]. The assay provides rapid, selective and quantifiable measurement of GVIA $iPLA_2$-specific activity, without requiring purification of the enzyme. Although PAPC is a common choice for the radiolabeled substrate, 1-palmitoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-lauroyl-*sn*-glycero-3-phosphocholine (PLPC), and 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-$^3H_2$]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC] are also suitable. The plasmalogen phospholipid (16:0p/18:1)-PC, containing a vinyl ether linkage in the *sn*-1 position, is an extremely favored substrate by GVIA $iPLA_2$.

A fluorometric high-throughput screening assay for $sPLA_2$s using phospholipid vesicles was developed and used to identify human GIII $sPLA_2$ inhibitors on a library of 370,276 small molecules [145]. The substrate is present in phospholipid vesicles, because this matrix more closely resembles the natural substrate of human GIII $sPLA_2$, as opposed to phospholipid/detergent mixed micelles. A phospholipid analogue containing BODIPY fluorophores dispersed as a minor component in vesicles of nonfluorescent phospholipids was used as substrate. An increase in fluorescence was observed, when the enzyme acted and liberated a free fatty acid from the phospholipid, because a reduction in quenching of the fluorophore occurred. The assay uses optical detection in a 1536-well plate format.

A colorimetric assay using PAF analog 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine as substrate (chemical $LpPLA_2$ activity assay) is the most commonly used $LpPLA_2$ activity assay in current clinical settings [108,146]. The colorimetric assay proved to be accurate and robust with acceptable linearity through a wide range of activity levels, allowing for adoption of $LpPLA_2$ activity in clinical practice [108]. Most recently, a novel enzymatic method for assaying $LpPLA_2$ in serum has been developed [147]. This $LpPLA_2$ activity assay used 1-O-hexadecyl-2-acetyl-*rac*-glycero-3 phosphocholine (*rac* C16 PAF) as a substrate, which was hydrolyzed by $LpPLA_2$ to produce 1-O-hexadecyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LysoPAF). LysoPAF was then hydrolyzed by lysoplasmalogen-specific phospholipase D (lysophospholipase D) to generate choline, which was detected by choline oxidase. Regression analysis of $LpPLA_2$ activity measured by the enzymatic $LpPLA_2$ activity assay *vs.* two chemical $LpPLA_2$ activity assays, *i.e.* $LpPLA_2$ FS and PLAC® test, and ELISA, gave the following correlation coefficients: 0.990, 0.893 and 0.785, respectively (n = 30).

8. Conclusion

Up to now, several small-molecule inhibitors of $sPLA_2$, $cPLA_2$ and $LpPLA_2$ have been studied in clinical trials for their safety and efficacy in humans. The most advanced clinical trials of PLA_2 inhibitors, together with the disease indications, are summarized in Table 2.

Varespladib ($sPLA_2$ inhibitor) was the first PLA_2 inhibitor, which was advanced into clinical trials as an intravenously-administered therapy for sepsis-induced systemic inflammatory response syndrome. Although at the end of the Phase I study it was found to have an acceptable safety profile in patients with severe sepsis, the trial was

terminated because the Phase II study showed poor efficacy [82]. A double-blinded placebo-controlled clinical trial of varespladib methyl failed to show efficacy in the treatment of rheumatoid arthritis [83]. Later on, varespladib methyl was evaluated for cardiovascular disease. FRANCIS (Fewer Recurrent Acute Coronary Events with Near-Term Cardiovascular Inflammation Suppression) study demonstrated that treatment with varespladib methyl reduced concentrations of LDL-C, hs-CRP and $sPLA_2$ in ACS patients treated with evidence-based therapies inclusive of high-dose atorvastatin [148]. VISTA-16 (Vascular Inflammation Suppression to Treat Acute Coronary Syndrome for 16 Weeks) clinical trial evaluated the safety and efficacy of 16 weeks of treatment with varespladib methyl on morbidity and mortality when added to atorvastatin and standard of care in subjects with an ACS [149]. However, in 2012 the study was terminated due to lack of efficacy.

Giripladib, an indole-based GIVA $cPLA_2$, was advanced into a Phase II clinical trial for osteoarthritis, but in 2007 the trial was terminated due to gastrointestinal events [15]. A randomized, double-blind, placebo controlled study to determine the safety, tolerability, pharmacokinetics and efficacy of a topical ZPL-5212372 (another indole-based GIVA $cPLA_2$ inhibitor) ointment in adult healthy volunteers and patients with moderate to severe atopic dermatitis is in progress and the results information is not yet publicly available [17].

Darapladib, a potent and selective $LpPLA_2$ inhibitor, has undergone two phase III trials for cardiovascular disease, but it failed to reduce the risk of major coronary events [112,113]. More recently, a clinical trial of rilapladib, another $LpPLA_2$ inhibitor, has completed and demonstrated improved cognitive outcomes and changes to a number of mechanism- and disease-related biomarkers, suggesting that inhibition of $LpPLA_2$ may have the potential to slow the progression of AD [124]. In addition, GSK2647544 ($LpPLA_2$ inhibitor) has been evaluated as for the safety, tolerability, pharmacokinetics and pharmacodynamics in healthy volunteers in phase I clinical trials [123].

Overall, although several small-molecule PLA_2 inhibitors have been evaluated in clinical trials, none of them reached the market yet. The trials for $sPLA_2$ inhibitors varespladib and varespladib methyl failed to exhibit the expected *in vivo* efficacy, while the $cPLA_2$ inhibitor giripladib caused undesired side effects. Recent results showing high-level of $sPLA_2$ inhibition by varespladib and varespladib methyl against 28 medically important snake venoms [87] suggest that these $sPLA_2$ inhibitors may find use in initiating the treatment of snakebite in the prehospital environment. In the case of $cPLA_2$ inhibitors, as we previously noticed, the high lipophilicity of the known $cPLA_2$ inhibitors is a serious drawback. Thus, it seems that such inhibitors may be more suitable for topical use, for example for the treatment of dermatitis, rather than for systemic use. Fortunately, potent $cPLA_2$ inhibitors having ClogP values lower than 5, for example 2-oxoesters, have been recently discovered [48]. As for $LpPLA_2$ enzyme, the failure of the potent $LpPLA_2$ inhibitor darapladib to reduce the risk of major coronary events, as demonstrated in both STABILITY and SOLID-TIMI 52 phase III studies, indicates that the enzyme may be a biomarker of vascular inflammation rather than a target for pharmaceutical treatment with beneficial effects.

In conclusion, although both pharmaceutical companies and academic institutions have devoted huge efforts to develop PLA_2 inhibitors, it seems that still fundamental issues about the connection of structure with cellular function and the involvement of each PLA_2 type in particular diseases have not been clearly understood. For example, most recently a combination of LC/MS lipidomics and MD simulations revealed surprising and previously unrecognized substrate specificity for $sPLA_2$ and $iPLA_2$. It is expected that advanced analytical lipidomic approaches and computational methods may contribute to increase our knowledge on the PLA_2 superfamily of enzymes. In addition, the recently solved crystal structure of GVIA $iPLA_2$ may help in designing novel inhibitors for this enzyme. Classical medicinal chemistry approaches, including organic synthesis and structure-activity relationship studies, as well as modern approaches employing functional lipidomics and computer-aided drug design may provide new chemical entities as potential novel pharmaceutical agents.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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