

REVIEW

Interactions of small molecule inhibitors with secreted phospholipase A₂: A review of the structural data

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Abstract

The secreted phospholipase A₂ (sPLA₂) family of enzymes hydrolyzes glycerophospholipid substrates at the *sn*-2 position of the acyl ester bond. Besides their roles in the degradation of phospholipids, these enzymes also play an important role in the immune response by promoting inflammation. The products of sPLA₂-catalysis (lysophospholipids and free fatty acids such as arachidonic acid) as well as secondary metabolites produced by the arachidonic acid cascade are mediators of inflammation and subsequent tissue injury. Based on their physiological functions, the sPLA₂ enzymes are pharmacological targets for the treatment of diseases with an inflammatory component such as atherosclerosis and rheumatoid arthritis. Interestingly, sPLA₂s may also have roles in various types of cancer such as malignant prostate cancer. While a number of sPLA₂ inhibitors have been described, compounds which have been co-crystallized with human sPLA₂ enzymes are of much importance as such three-dimensional structures provide valuable information regarding the structural requirements of good potency inhibitors as well as the molecular interactions between the enzyme and inhibitor. This review selected four structures of human sPLA₂s complexed with inhibitors from the Protein Data Bank to illustrate the important molecular interactions of inhibitor compounds with the enzymes and to highlight the structural features important for interaction with the sPLA₂ active site. In addition, the complex of the sPLA₂ inhibitor, varespladib, with a Lys49-PLA₂-like snake venom toxin is also discussed. Knowledge of the molecular interactions acquired from such structures would greatly aid the discovery of small molecule inhibitors of sPLA₂s for the treatment of snakebite envenoming. The three-dimensional structures presented in this review have been published by various research groups and have been deposited in the Protein Data Bank.

KEYWORDS

inhibitor, interactions, phospholipase A₂, review, sPLA₂, structure-based, varespladib

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1 | CLASSIFICATION OF PHOSPHOLIPASE A₂ (PLA₂) FAMILIES

Phospholipases are enzymes responsible for the hydrolysis of certain ester bonds within glycerophospholipid substrates from cellular membranes and lipoproteins (Figure 1) (Balboa & Balsinde, 2021). The phospholipase A₂ (PLA₂, EC 3.1.1.4) family specifically cleaves the acyl ester bond at the *sn*-2 position to produce lysophospholipids and polyunsaturated free fatty acids (PUFAs) including arachidonic acid, and thus initiates the eicosanoids cascade (Balboa & Balsinde, 2021). The *sn*-2 position of phospholipids is often substituted with PUFAs that could be metabolized to yield a number of eicosanoids and other bioactive lipids, while the lysophospholipid may be a biosynthetic precursor to platelet-activating factor (PAF), a potent mediator of inflammation (Magrioti & Kokotos, 2010; Springer et al., 2000). The PLA₂s are involved in the development and progression of various inflammatory diseases and have been targeted for the development of new medicines (Dennis et al., 2011; Dennis & Norris, 2015). It is estimated that the PLA₂ family consists of more than 50 enzymes in mammals (Murakami et al., 2020). The PLA₂ family have been divided into various types, groups and subgroups based on their various structural and functional characteristics such as the position of disulphide bonds and sequence alignment, as well as catalytic mechanism (His/Asp, Ser/Asp or Ser/His/Asp hydrolase) (Dennis et al., 2011; Schaloske & Dennis, 2006). The six main types of PLA₂s are secreted PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂), calcium-independent PLA₂ (iPLA₂), platelet activating factor acetyl hydrolases (PAF-AH) also known as lipoprotein-associated PLA₂ (Lp-PLA₂), lysosomal PLA₂ (LPLA₂) and adipose-specific PLA₂ (AdPLA) (Dennis et al., 2011). The biochemistry, pharmacology and medicinal chemistry of the PLA₂s and sPLA₂s have been reviewed (Balboa & Balsinde, 2021; Boyanovsky & Webb, 2009; Dennis et al., 2011; Kim et al., 2020; Lambeau & Gelb, 2008; Murakami, 2019; Murakami & Kudo, 2004); however, the focus of this review will be on the sPLA₂s, with particular emphasis

on the interactions of the sPLA₂s with small molecule inhibitors as evidenced by the reported structural data. This review will provide a summary of available X-ray crystal structures of the sPLA₂s complexed with small molecule inhibitors, whereafter the interactions of inhibitors with the enzyme active site will be presented for selected complexes. These may serve as examples for the medicinal chemist that highlight important interactions that are shared by different sPLA₂ isoforms. Finally, based on the structural evidence, strategies for the design of isoform selective inhibitors will be discussed.

A total of 17 different sPLA₂ groups have been identified in animals, plants and microorganisms (Figure 2) (Dennis et al., 2011). Snake venom, in particular, contains a variety of sPLA₂s and were the first sPLA₂s to be isolated and characterized (group IA, elapid and hydrophid snakes and group IIA/IIB, crotalid and viperid snakes) (Dennis et al., 2011; Valentin & Lambeau, 2000). Subsequently, sPLA₂s displaying similar disulphide patterns and sequence homology (Kim et al., 2020; Tonello & Rigoni, 2017; Valentin & Lambeau, 2000) to snake venom sPLA₂s, were identified in mammals (group IB, porcine and human pancreatic sPLA₂; group IIA, human non-pancreatic or synovial fluid sPLA₂) (Dennis et al., 2011; Kim et al., 2020). To date, 10 catalytic active mammalian sPLA₂ isoforms have been identified (group IB, IIA, IIC, IID, IIE, IIF, III, V, X and XIIA) and one inactive isoform (XIIB); however only nine of the active isoforms are expressed in humans, as group IIC exists as a pseudogene in the human genome (Dennis et al., 2011; Nikolaou et al., 2019; Tischfield et al., 1996). The sequence alignments of seven human sPLA₂s have been reported in a recent review, highlighting conserved sequences of the active sites and calcium-binding loops, as well as cysteine residues that are involved in disulphide bonds (Kim et al., 2020). sPLA₂s are typically small proteins of 14–18 kDa, contain 6–8 disulphide bonds and typically function on the extracellular side of cells, primarily using phospholipids as substrates (Batsika et al., 2021; Quach et al., 2014). Compared to other sPLA₂s, groups V and X sPLA₂s are more efficient in hydrolysing phospholipids (Singer et al., 2002). Groups I, II, V and X are considered ‘conventional’ sPLA₂s, while based on their amino acid sequences, groups III and XII

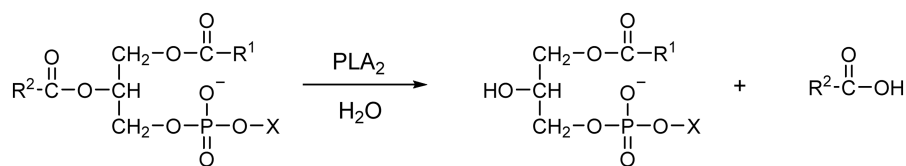


FIGURE 1 The general reaction catalysed by PLA₂. A phospholipid is hydrolyzed at the *sn*-2 position to yield a 2-lysophospholipid and free fatty acid such as arachidonic acid. Arachidonic acid may be converted to several different eicosanoids, including prostaglandins and leukotrienes, with arachidonic acid release being the rate-limiting step in eicosanoid production.

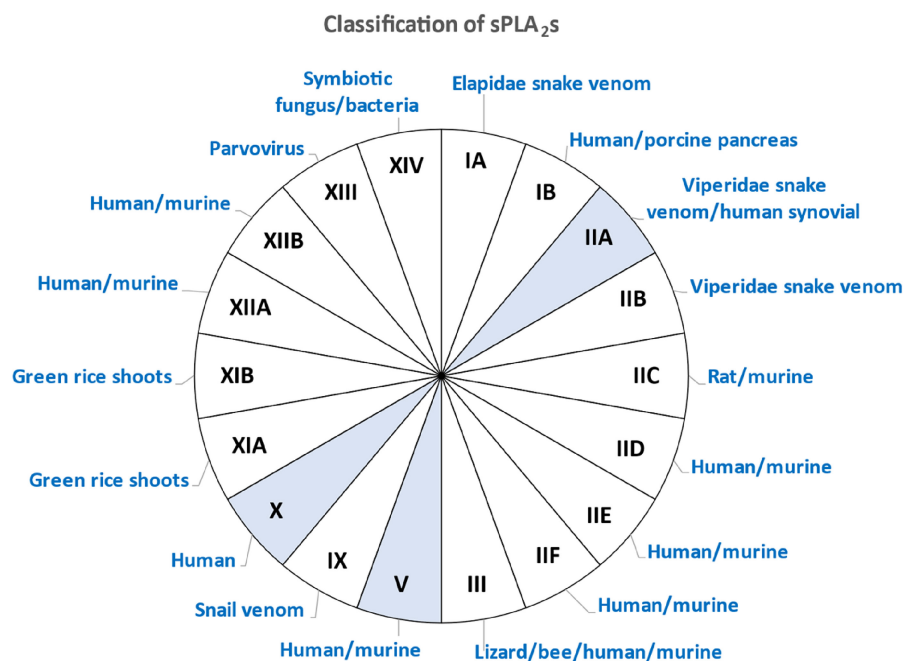


FIGURE 2 The classification of sPLA₂ groups. The human enzymes within the groups that are shaded are currently of most medicinal interest (Dennis et al., 2011; Schaloske & Dennis, 2006).

are structurally distinct from the other groups. All human sPLA₂s, however, have the His/Asp catalytic dyad, a highly conserved Ca²⁺-binding domain and six disulphide bonds which are conserved across the groups (Kim et al., 2020; Quach et al., 2014). sPLA₂s are thus calcium-dependent enzymes with the concentration of calcium required to activate the specific sPLA₂ enzyme often in the micro-to millimolar range, but being dependent on the structure of the phospholipid (Rosenson & Gelb, 2009; Singer et al., 2002). For example, the concentration of calcium required for the activity of sPLA₂ that act on phosphatidylglycerol vesicles is in the low micromolar range (Rosenson & Gelb, 2009; Singer et al., 2002). Based on their roles in inflammation, arteriosclerosis and cancer, human sPLA₂ groups that are of much medicinal importance are groups IIA (non-pancreatic or synovial fluid), V and X (Hou et al., 2017; Knerr et al., 2018; Lee et al., 2013; Rosenson & Hurt-Camejo, 2012).

2 | BIOLOGICAL ROLES OF sPLA₂s IN HUMANS

sPLA₂s are involved in a diverse range of biological processes which varies for each specific group/subgroup based on their substrate specificities (Boyanovsky & Webb, 2009; Dennis et al., 2011; Murakami et al., 2020). Aside from the more well known functions of sPLA₂s such as cell membrane remodeling and degradation of dietary phospholipids, these enzymes also play an important

role in the innate immune response (Boyanovsky & Webb, 2009; Murakami et al., 2020). sPLA₂s contribute to the innate immune response by leading to the production of biologically active molecules that modulate immune responses. The sPLA₂ catalytic reaction generates various precursors (e.g. arachidonic acid) for the biosynthesis of pro-inflammatory lipid mediators such as prostaglandins, leukotrienes, tromboxanes and lipoxins (Boyanovsky & Webb, 2009; Dennis et al., 2011; Triggiani et al., 2006). sPLA₂s may produce arachidonic acid via several mechanisms, including the 'external plasma membrane pathway' where sPLA₂s act directly on the outer leaflet of the plasma membrane to release free fatty acids (Boyanovsky & Webb, 2009). sPLA₂s may, however, also act intracellularly prior to secretion or after internalization (Ni et al., 2006; Rosenson & Gelb, 2009). Bioactive lipid mediators (e.g. lysophospholipids and arachidonic acid) function together to activate multiple inflammatory processes (Rosenson & Gelb, 2009). Experimental evidence has shown that sPLA₂s (groups V, X and to a lesser extent IIA) produce arachidonic acid for the generation of eicosanoids in inflammatory cells such as macrophages, neutrophils, eosinophils and mast cells (Lambeau & Gelb, 2008). sPLA₂s also demonstrate other inflammatory effects that are not mediated by their catalytic activity but rather through interaction with various target cell receptors (Triggiani et al., 2006). These effects include activation of immune cells, immune cell degranulation, activating exocytosis in macrophages and stimulating cytokine/chemokine production (Triggiani et al., 2006).

Various sPLA₂ groups have demonstrated antibacterial (Degousee et al., 2002; Gronroos et al., 2001; Koduri et al., 2002) and antiviral activity (Kim et al., 2007; Mitsuishi et al., 2006). The bactericidal effects of these enzymes are due to the disruption of the bacterial cell membrane. Group IIA sPLA₂s have high affinity for phosphatidylglycerol, one of the main components of bacterial cell membranes and via the degradation of bacterial membranes provides the first-line of defence as an antibacterial sPLA₂, particularly for Gram-positive bacteria (Boyanovsky & Webb, 2009; Laine et al., 1999; Weinrauch et al., 1998). Although sPLA₂s mainly target Gram-positive bacteria, these enzymes have demonstrated activity against some Gram-negative bacteria in the presence of bactericidal/permeability-increasing protein (BPI) (Elsbach et al., 1994; Koduri et al., 2002). BPI disrupts the outer lipopolysaccharide membrane of Gram-negative bacteria, allowing sPLA₂s access to the inner phospholipid membrane (Koduri et al., 2002). The antiviral mechanism of sPLA₂s involves either degradation of the viral membrane (Kim et al., 2007) or modification of the host cell membrane to prevent viral fusion with host cells (Mitsuishi et al., 2006). The high affinity of group V and X sPLA₂s for phosphatidylcholine, allows these enzymes to specifically target phosphatidylcholine-rich viral and mammalian cell membranes (Kim et al., 2007; Mitsuishi et al., 2006). Interestingly, group IIA sPLA₂ might protect against the malaria parasite, *Plasmodium falciparum*, by hydrolysing 'oxidized' lipoproteins to inhibit parasite growth (Dacheux et al., 2019).

3 | BIOLOGICAL ROLE OF sPLA₂S IN SNAKE VENOM

Snake venom consists of various enzymatic and non-enzymatic proteins and peptides, that aids in the capture and digestion of prey, as well as protection against predators (Munawar et al., 2018). sPLA₂ enzymes are one of the major components of snake venom and are responsible for inducing a variety of toxic effects (Tonello & Rigoni, 2017). These toxic effects can be classified as neurotoxic (targeting the peripheral nervous system), myotoxic (targeting the skeletal muscles) and hemostasis-impairing effects (hemolytic, pro- or anticoagulant activity) (Tonello & Rigoni, 2017). Similar to human sPLA₂s, the snake venom enzymes induce their toxic effects by means of catalytic-dependent and/or -independent mechanisms. Catalytically active venom sPLA₂s may cause damage to nerve or muscle cells through hydrolysis of membrane phospholipids or by releasing lysophospholipids and free fatty acids. However, the localization and potency of their catalytic action are determined by the binding affinity of

each sPLA₂ for specific target sites (Kini, 2003; Kini & Evans, 1989; Montecucco et al., 2008).

4 | POTENTIAL THERAPEUTIC APPLICATIONS OF sPLA₂ INHIBITORS

Abnormal sPLA₂ levels have been implicated in the pathophysiology of various conditions, particularly those associated with increased inflammation such rheumatoid arthritis, atherosclerosis and sepsis (Dennis et al., 2011; Kim et al., 2020; Quach et al., 2014). sPLA₂s have been associated with the pathogenesis of numerous disorders with an inflammatory component and group IIA, V and X sPLA₂s have been found to be overexpressed in these conditions (Table 1). Inflammatory conditions may be mediated by PLA₂-generated products (free fatty acids and lysophospholipids) as well as by secondary metabolites from the arachidonic acid cascade which are mediators of inflammation and subsequent tissue injury (Kim et al., 2020; Kramer et al., 1989). For example, group IIA sPLA₂ are significantly increased in patients with severe infections (peritonitis and sepsis) (Nyman et al., 1996). Group IIA concentrations is increased in synovial fluids of patients with rheumatoid arthritis (Seilhamer et al., 1989), and the sPLA₂ inhibitor varespladib methyl (or LY333013) has been evaluated in clinical trials for the treatment of this disorder (Bradley et al., 2005). In fact, other sPLA₂ enzymes are also expressed in rheumatoid arthritis (Masuda et al., 2005). Evidence indicated that at least three sPLA₂s, group IIA, V and X may possess proatherogenic properties (Hanasaki et al., 2002; Kimura-Matsumoto et al., 2008; Rosengren et al., 2006). Inflammatory reactions in atherosclerosis, in particular, have been linked to high concentrations of bioactive lipid mediators (e.g. fatty acids and lysophospholipids) in atherosclerotic plaques (Kim et al., 2020), which has led to the development of sPLA₂ inhibitors (e.g. varespladib methyl, varespladib and AZD2716) for the treatment of cardiovascular disease (Draheim et al., 1996; Giordanetto et al., 2016; Karakas & Koenig, 2009; Snyder et al., 1999). However, in a clinical trial varespladib methyl failed to demonstrate efficacy in the treatment of rheumatoid arthritis (Bradley et al., 2005). Similarly, varespladib (or LY315920), the non-prodrug precursor of varespladib methyl, was evaluated for the treatment of sepsis. Although Phase I trials found that varespladib has an acceptable safety profile, its development was terminated due to poor efficacy in patients with severe sepsis (Abraham et al., 2003; Kearney & Naum, 1999). Similarly, a trial with varespladib for the treatment of acute coronary syndrome was terminated as the drug did not display efficacy and

TABLE 1 Examples of inflammatory and other conditions that have been associated with increased levels or activity of sPLA₂s.

Disease state	sPLA ₂ implicated	Clinical trials	Supporting reference
Rheumatoid arthritis (autoimmune disease)	IIA	Phase I and II	Bradley et al. (2005), Murakami et al. (2011)
Asthma	IIA, X		Hallstrand et al. (2011)
Chronic obstructive pulmonary disease (COPD)	IID		Igarashi et al. (2009)
Acute respiratory distress syndrome (ARDS)	IIA		Touqui and Arbibe (1999)
Inflammatory bowel disease (Crohn's disease and ulcerative colitis)	IIA		Haapamäki et al. (1997), Haapamäki, Gronroos, Nurmi, Irjala, et al. (1999), Minami et al. (1994)
Other acute inflammatory conditions – pancreatitis, peritonitis and sepsis	IIA, IB	Phase I and II for sepsis: NCT00034476	Abraham et al. (2003), Nevalainen et al. (2005), Tong et al. (1998), Zeiher et al. (2005)
Atopic dermatitis	IIF, III	Phase I: NCT05729074	Murakami et al. (2002), Sato et al. (2009)
Atherosclerosis/coronary heart disease	IIA, III, V, X	Phase II: NCT00455546, NCT00525954, NCT00743925 Phase III: NCT01130246	Kimura-Matsumoto et al. (2008), Nicholls et al. (2014), Rosenson et al. (2009, 2010, 2011), Rosenson & Hurt-Camejo (2012), Sato et al. (2008)
Psoriatic lesions	IIA, IIF		Andersen et al. (1994), Miki et al. (2022), Yamamoto et al. (2015)
Prostate cancer	IIA		Graff et al. (2001), Jiang et al. (2002), Kallajoki et al. (1998), Sved et al. (2004)
COVID-19 Severe acute respiratory syndrome-coronavirus (SARS-CoV)	IIA, IID	Phase II for COVID-19: NCT04969991	Snider et al. (2021), Vijay et al. (2015)

may in fact increase the risk of myocardial infarction in this group of patients (Nicholls et al., 2014). In spite of this, it is noteworthy that preclinical evidence supports an antiatherogenic effect for varespladib methyl (Fraser et al., 2009; Shaposhnik et al., 2009). Group IIA, V and X sPLA₂s are present in human and mouse atherosclerotic lesions (Rosenson & Gelb, 2009), while overexpression or presence of groups IIA, V and X sPLA₂s increases atherosclerotic lesions and may promote lipid accumulation due to the hydrolysis of lipoprotein phospholipids (Ivandic et al., 1999; Kimura-Matsumoto et al., 2008; Rosenson & Gelb, 2009). Intriguingly, mice that express the human group IIA sPLA₂ enzyme, developed atherosclerosis even when maintained on a low-fat diet, which demonstrates that this enzyme promotes atherogenesis and is not simply a marker for the disease (Ivandic et al., 1999).

Interestingly, sPLA₂s may also have roles in various types of cancer where they may have either a pro- or anti-tumorigenic role (Laye & Gill, 2003). For example, group IIA is elevated in malignant prostate cancer and may promote tumour growth and metastasis through the

increased production of eicosanoids (Graff et al., 2001; Jiang et al., 2002; Sved et al., 2004). sPLA₂s have also been implicated in the pathology of adult respiratory distress syndrome (ARDS) (Boyanovsky & Webb, 2009; Furue et al., 2001), as well as in inflammation in brain tissue and neurodegenerative disorders (Farooqui & Horrocks, 2006; Sun et al., 2004), inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Haapamäki, Gronroos, Nurmi, Alanen, et al., 1999; Haapamäki, Gronroos, Nurmi, Irjala, et al., 1999; Minami et al., 1994), and endotoxin-induced septic shock (Hanasaki et al., 1997).

Small molecule sPLA₂ inhibitors also have potential therapeutic use for snakebite envenomation. Due to their abundance in snake venoms (Tasoulis & Isbister, 2017), sPLA₂s are attractive broad-spectrum therapeutic targets for snakebite envenomation. Two drugs originally developed for treatment of coronary artery disease (Draheim et al., 1996; Karakas & Koenig, 2009), varespladib and varespladib methyl, were repurposed to determine their activity against snake venom sPLA₂s (Lewin et al., 2016).

Both drugs demonstrated potent *in vitro* sPLA₂ activity (in the pico- to nanomolar range) against 28 different snake venoms (Lewin et al., 2016). In animal models, varespladib and varespladib methyl treatment increased survival and significantly reduced the toxic effects of several different snake venoms (Bryan-Quiros et al., 2019; Gutiérrez et al., 2020; Lewin et al., 2016; Lewin, Gilliam, et al., 2018; Lewin, Gutiérrez, et al., 2018; Wang et al., 2018; Xiao et al., 2017, 2018). These results suggest that sPLA₂ inhibitors have potential as initial adjuvant treatment for snakebite envenomation.

5 | THE STRUCTURE OF sPLA₂

While X-ray crystal structures have been recorded for various groups of sPLA₂s, for the human enzymes three-dimensional structures have been reported for only groups IB, IIA, IIE and X. The three-dimensional structures of groups IIA and X are similar and contain three α -helices and a β -wing, which consist of two-stranded β -sheets (Dennis et al., 2011). The sPLA₂ enzymes have at least six highly conserved disulphide bonds that stabilize the folded three-dimensional structure (Kim et al., 2020). The enzymes are dependent on calcium for activity and have a calcium-binding loop (X-Cys-Gly-X-Gly-Gly) where the calcium ion is coordinated by interactions with the main chain carbonyl oxygens of amino acid residues (His27, Gly29 and Gly31) as well as with the β -carboxylate oxygen of the catalytic aspartate (Asp48) in human sPLA₂-IIA (Figure 3) (Dennis et al., 2011; Kim et al., 2020). A second calcium ion is present in most crystal structures of sPLA₂s (Scott et al., 1990). The sPLA₂s also have a conserved active site sequence (Asp-X-Cys-Cys-X-X-His-Asp) where the histidine and a remote aspartate (Asp99) form the catalytic dyad (Dennis et al., 2011; Kim et al., 2020). The role in hydrolysis of a substrate is similar to that of the classical catalytic triad of serine hydrolase enzymes which consists of serine, an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine) (Kim et al., 2020). Both residues are charged (histidine – basic, aspartate – acidic) thus an electrostatic interaction exists between the two residues while the calcium ion which is bound to the calcium-binding loop is in close proximity to the catalytic dyad. As will be discussed in the section below, this arrangement is of catalytic significance.

6 | MECHANISM OF ACTION OF sPLA₂

Two different catalytic mechanisms have been proposed for sPLA₂s, namely the triad model and the

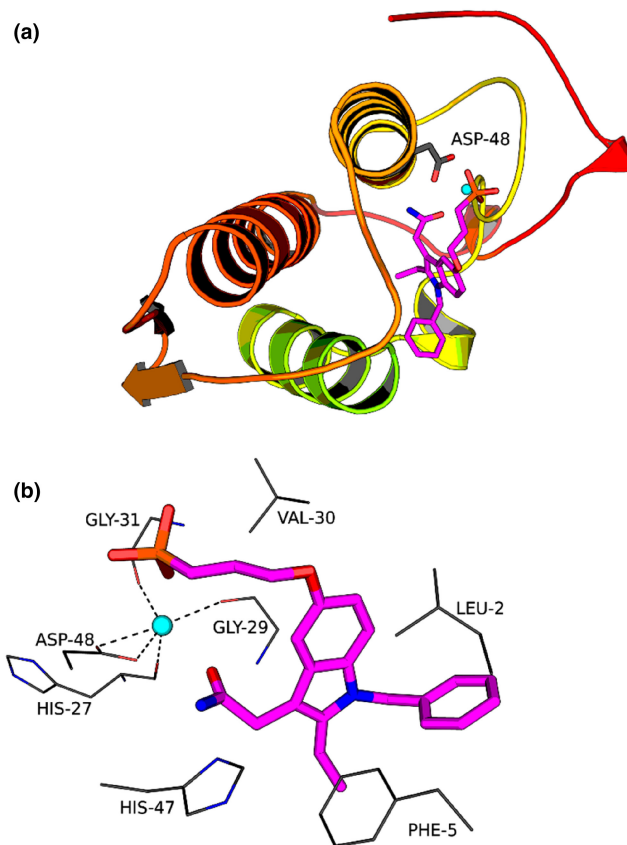


FIGURE 3 The three-dimensional structure of the human sPLA₂-IIA (PDB code: 3U8D) with the inhibitor LY311727 (magenta) bound. (a) The inhibitor located in the hydrophobic channel of the enzyme; (b) A model of the active site showing the coordination of the calcium ion (cyan sphere) to surrounding residues. In inhibitor-bound complexes, two additional coordination bonds to the calcium are provided by the ligand, in this instance the amide carbonyl oxygen and phosphonic acid of the ligand are involved (see Figure 6) (Lee et al., 2013).

calcium-coordinated oxyanion model (Figure 4) (Berg et al., 2001; Kim et al., 2020; Rogers et al., 1996; Scott et al., 1990; Yu et al., 1998; Zambelli et al., 2017). For the triad model, the N^{δ1} atom of the catalytic histidine (His48) polarizes a structurally conserved water molecule. This water molecule then acts as nucleophile during the hydrolysis reaction and attacks the *sn*-2 positioned carbonyl group of the phospholipid substrate. His48 thus acts as a proton acceptor to facilitate the attack of water on the carbonyl carbon for the ester function of the substrate. The proton is subsequently transferred to the carboxylate of Asp99. The calcium ion bound to the calcium-binding loop which is located near the active site, also participates in the reaction by stabilizing the transition state intermediate by coordinating the negatively charged phosphate oxygen and the *sn*-2 ester carbonyl group (Montecucco et al., 2008). The calcium-coordinated oxyanion model proposes that two water molecules are involved in the

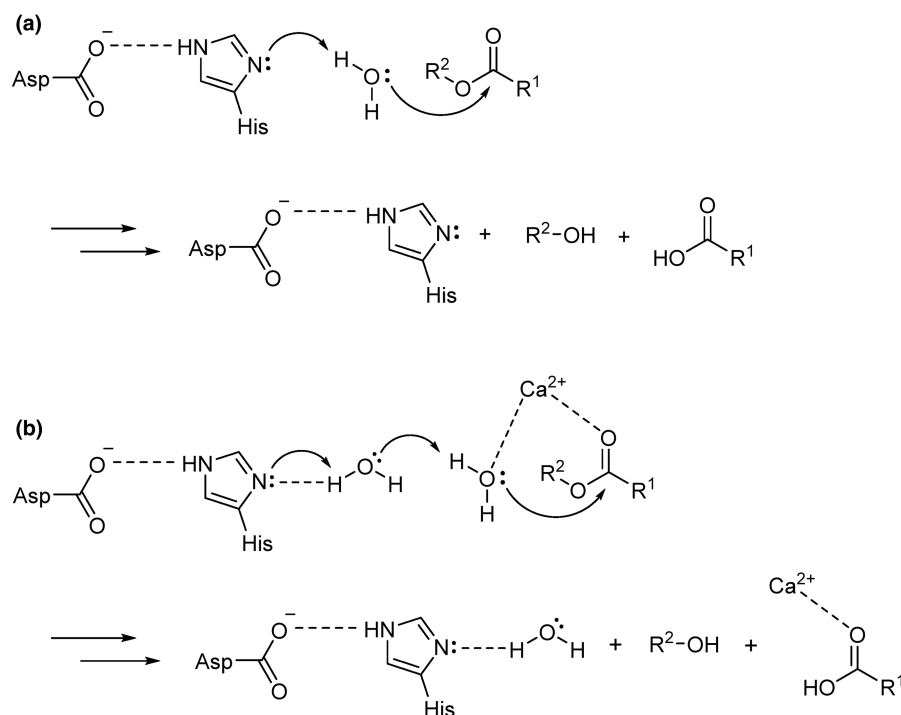


FIGURE 4 The first step of the proposed catalytic mechanisms for sPLA₂s illustrating the nucleophilic attack of a structural water on the ester of the phospholipid substrate: (a) the triad model and the (b) calcium-coordinated oxyanion model (Kim et al., 2020).

catalytic reaction. The first water molecule is coordinated by the calcium ion and forms a hydrogen bond with the second water molecule that is bound to the N^{δ1} atom of the catalytic histidine. Polarization of the first water molecule initiates the nucleophilic attack on the carbonyl group of the substrate, resulting in the formation of an oxyanion that coordinates the calcium ion. Due to the involvement of two water molecules, the required activation energy is lower for this model than for the triad model (Kim et al., 2020).

7 | EXAMPLES OF INHIBITORS AND INHIBITOR CLASSES OF THE sPLA₂s

To date, several different classes of small molecule sPLA₂ inhibitors (Table 2) have been studied for their potential therapeutic use, as well as to investigate the role of specific sPLA₂s in various diseases. Many of these inhibitors have also entered clinical trials, however, efficacy has been limited and none have reached the market. One of the most extensively studied inhibitor classes is the indole derivatives. The pharmaceutical companies, Eli Lilly and Shionogi, developed a range of indole derivatives (**1**, **2**, **3**, LY311727, **4**, **5** and varespladib) for the inhibition of human sPLA₂-IIA (Dillard et al., 1996a, 1996b; Draheim et al., 1996; Schevitz et al., 1995). Varespladib

and its prodrug, varespladib methyl, are potent sPLA₂-IIA inhibitors and demonstrated acceptable safety profiles in Phase I clinical trials for the treatment of sepsis (Abraham et al., 2003). Both drugs have entered various Phase II and III clinical trials for treatment of several inflammatory diseases; however, efficacy of these drugs have been limited thus far (Abraham et al., 2003; Bradley et al., 2005; Kearney & Naum, 1999; Nicholls et al., 2014). Most recently, these drugs have been repurposed and entered Phase II trials for treatment of snakebite envenomation (NCT04996264) (Carter et al., 2022) as well as COVID-19-associated acute respiratory distress syndrome (NCT04969991 – study was terminated). Shionogi also studied the inhibitory activity of various indolizine and indene derivatives (Hagishita et al., 1996). The indolizine derivative, indoxam, demonstrated nanomolar sPLA₂-IIA activity (Hagishita et al., 1996; Yokota et al., 1999). In addition, indoxam also blocked pro-inflammatory cytokine production in a murine endotoxic shock model via PLA₂-independent mechanisms (Yokota et al., 1999). A structurally similar indole analog, methyl indoxam or Me-indoxam, demonstrated potent (low nanomolar) activity against human sPLA₂-IIA, -IIE and -V; intermediate activity against human sPLA₂-IB; and minimal activity against human sPLA₂-IID, -IIF, -X and -XII (Singer et al., 2002). A series of Me-indoxam analogs was developed, including compounds **6**, **7** and **8**, which demonstrated a similar pattern of activity towards human sPLA₂-IIA, -IIE and -V

(Smart et al., 2004). As these analogs were slightly more selective towards sPLA₂-IIE than -IIA, Hou et al. (2017) used the crystal structure of human sPLA₂-IIE in complex with these inhibitors to identify key interactions necessary for activity. Oslund et al. (2008) also designed a series of indole- and indolizine-based compounds in an attempt to identify inhibitors that are highly specific for human sPLA₂-X. During the study they identified compound **9** which demonstrated potent activity towards all human and mouse sPLA₂s.

Compounds BMS-181162 and BMS-188184, belonging to the dicarboxylic acid class of PLA₂ inhibitors, were developed by Bristol-Myers Squibb as anti-inflammatory agents (Burke et al., 1995; Springer et al., 1997; Tramposch et al., 1992). These compounds demonstrated modest in vitro activity against sPLA₂-IIA and reduced inflammation markers in vivo in a mouse model (Springer et al., 1997; Tramposch et al., 1992). BMS-181162 progressed to Phase II clinical trials for the topical treatment of psoriasis. However, this compound failed to

TABLE 2 Different classes and activities of sPLA₂ small molecule inhibitors.

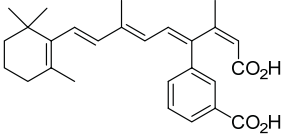
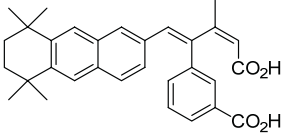
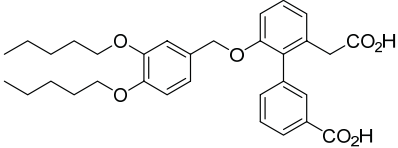
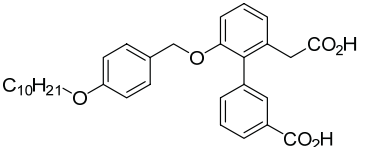
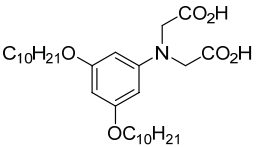
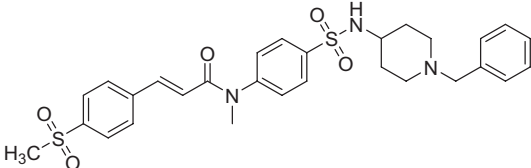
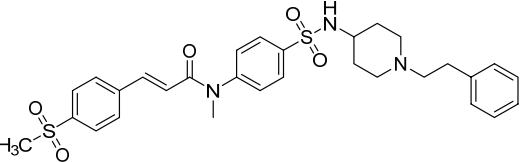
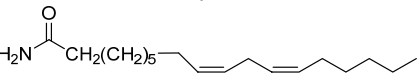
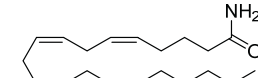
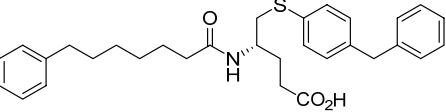
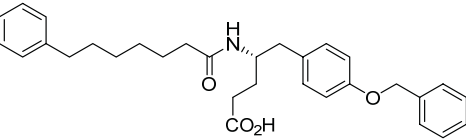
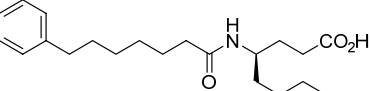
Inhibitor class and structure	sPLA ₂ isoform	In vitro activity	Reference
Dicarboxylic acids  BMS-181162	IIA	IC ₅₀ : 40 μM ^a	Tramposch et al. (1992)
 BMS-188184	IIA	IC ₅₀ : 17 μM ^a	Springer et al. (1997)
 10	IIA	IC ₅₀ : 8 μM ^a	Springer et al. (2000)
 11	IIA	IC ₅₀ : 4 μM ^a	Springer et al. (2000)
 Ro 23-9358	IIA	IC ₅₀ : 0.23 μM ^a	LeMahieu et al. (1993)

TABLE 2 (Continued)

Inhibitor class and structure	sPLA ₂ isoform	In vitro activity	Reference
Benzenesulfonamides  12 (ER-3826)	Membrane-bound PLA ₂	IC ₃₀ : 0.028 μM ^a	Oinuma et al. (1991)
 13	Membrane-bound PLA ₂	IC ₃₀ : 0.009 μM ^a	
Primary amides  Synthetic γ-linolenoyl amide	IIA	X _I (50): 0.002 ^b	Jain et al. (1992)
 Synthetic arachidonyl amide	IIA	X _I (50): 0.004 ^b	
Non-phospholipid amides  FPL67047XX	IIA	IC ₅₀ : 0.021 μM ^a	Beaton et al. (1994)
 KH064	IIA	IC ₅₀ : 0.029 μM ^c	Hansford et al. (2003)
 14	V	X _I (50): 0.003 ^a	Antonopoulou et al. (2008)

(Continues)

TABLE 2 (Continued)

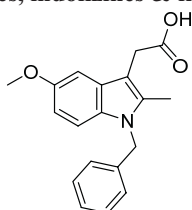
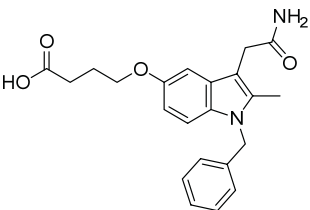
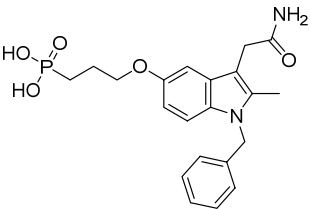
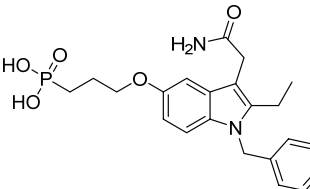
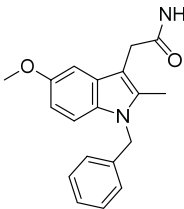
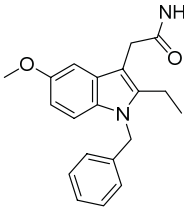
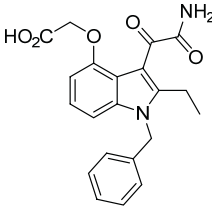
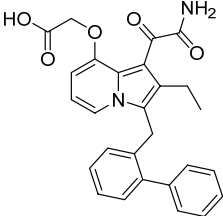
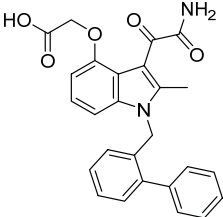
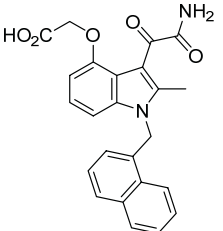
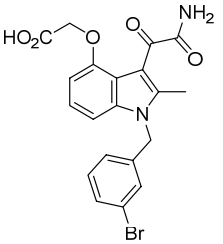
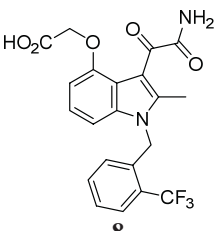
Inhibitor class and structure	sPLA ₂ isoform	In vitro activity	Reference
Indoles, indolizines & indenenes			
Indoles, indolizines & indenenes	IIA	X _I (50): 0.00068 ^d	Schevitz et al. (1995)
 1			
 2	IIA	X _I (50): 0.00012 ^d	
 3	IIA	X _I (50): 0.000045 ^d	
 LY311727	IIA	X _I (50): 0.000019 ^d IC ₅₀ : 0.0538 μM ^c	Lee et al. (2013)
 4	IIA	IC ₅₀ : 0.84 μM ^d	Dillard et al. (1996a)
 5	IIA	IC ₅₀ : 0.26 μM ^d	

TABLE 2 (Continued)

Inhibitor class and structure	sPLA ₂ isoform	In vitro activity	Reference
 Varespladib (LY315920)	IIA	IC ₅₀ : 0.009 ^d /0.007 ^a μM	Draheim et al. (1996)
 Indoxam	IIA	IC ₅₀ : 0.006 ^d /0.003 ^a μM	Hagishita et al. (1996)
 Me-Indoxam	IIA	IC ₅₀ : 0.03 ^e /0.006 ^a μM	Singer et al. (2002)
	IIE	IC ₅₀ : 0.008 ^e /0.01 ^a μM	
	V	IC ₅₀ : 0.025 ^e /0.006 ^a μM	
 6	IIA	IC ₅₀ : 0.0115 μM ^c	Lee et al. (2013)
	IIE	IC ₅₀ : 0.28 μM ^c	
 7	IIE	IC ₅₀ : 0.22 μM ^c	Hou et al. (2017)
 8	IIE	IC ₅₀ : 0.10 μM ^c	

(Continues)

TABLE 2 (Continued)

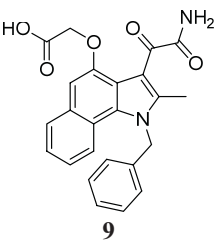
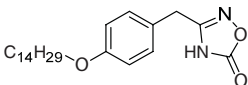
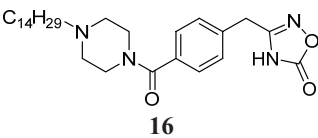
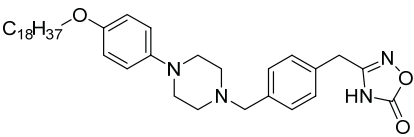
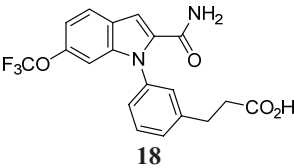
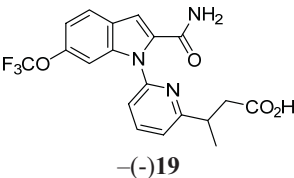
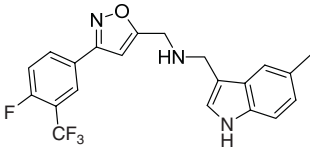
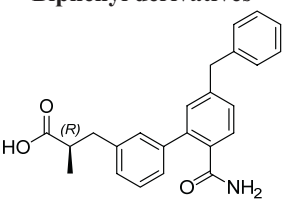
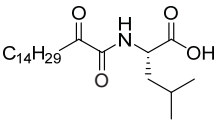
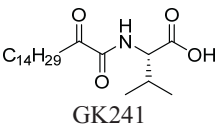
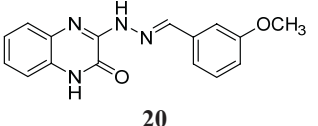
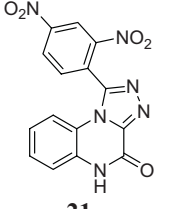
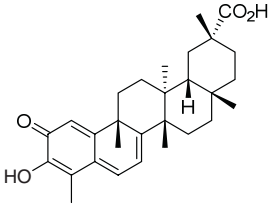
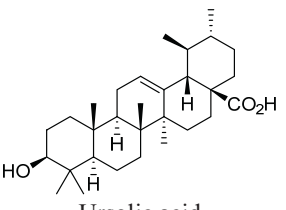
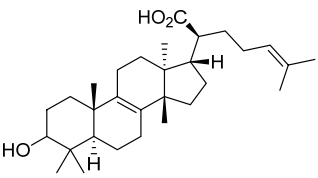
Inhibitor class and structure	sPLA ₂ isoform	In vitro activity	Reference
 9	IB	IC ₅₀ : 0.084 μM ^e	Oslund et al. (2008)
	IIA	IC ₅₀ : 0.04 μM ^e	
	IID	IC ₅₀ : 0.007 μM ^e	
	IIE	IC ₅₀ : 0.007 μM ^e	
	IIF	IC ₅₀ : 0.05 μM ^e	
	V	IC ₅₀ : 0.035 μM ^e	
	X	IC ₅₀ : 0.02 μM ^e	
Oxadiazolones  15 (PMS1062)	IIA	IC ₅₀ : 4 μM ^e	Assogba et al. (2005)
 16	IIA	IC ₅₀ : 9 μM ^e	
 17	IIA	IC ₅₀ : 0.03 μM ^e	
Indole-2 carboxamides  18	X	IC ₅₀ : 0.026 μM ^f	Knerr et al. (2018)
 (-)19	X	IC ₅₀ : 0.014 μM ^f	
Isoxazole  22	IIA	IC ₅₀ : 10.23 μM ^c	Pedada et al. (2016)

TABLE 2 (Continued)

Inhibitor class and structure	sPLA ₂ isoform	In vitro activity	Reference
Biphenyl derivatives  AZD2716	IIA V X	IC ₅₀ : 0.01 μM ^f IC ₅₀ : 0.04 μM ^f IC ₅₀ : 0.4 μM ^f	Giordanetto et al. (2016)
2-Oxamides  GK126	IIA V	IC ₅₀ : 0.30 μM ^e IC ₅₀ : 0.44 μM ^e	Mouchlis et al. (2011)
 GK241	IIA	IC ₅₀ : 0.143 μM ^e	Vasilakaki et al. (2016)
Quinoxalinones  20	IIA V X XIIA	IC ₅₀ : 1.90 μM ^g IC ₅₀ : 1.22 μM ^g IC ₅₀ : 0.33 μM ^g IC ₅₀ : 0.84 μM ^g	Alasmmary et al. (2017)
 21	IIA V X XIIA	IC ₅₀ : 0.76 μM ^g IC ₅₀ : 1.53 μM ^g IC ₅₀ : 1.05 μM ^g IC ₅₀ : 2.58 μM ^g	
Triterpenoids  Celastrol	IIA	IC ₅₀ : 6 μM ^a	Joshi et al. (2016)
 Ursolic acid		IC ₅₀ : 12 μM ^a	Nataraju et al. (2007)

(Continues)

TABLE 2 (Continued)

Inhibitor class and structure	sPLA ₂ isoform	In vitro activity	Reference
 Elemolic acid		IC ₅₀ : 5.7 μM ^a	Giresha et al. (2022)

Note: Enzymatic activity measured with the following assays.

^aRadiometric assay.

^bInterfacial catalysis in scooting mode.

^cColorimetric assay kit (Cayman Chemical).

^dChromogenic assay.

^eFluorometric assay.

^fColorimetric assay – NEFA C kit (Wako Chemicals).

^gColorimetric assay using a pH indicator.

demonstrate efficacy and development was discontinued (Springer, 2001). Modification of the BMS-181162 structure led to the development of a series of biaryl dicarboxylic acids (Springer et al., 2000). Compounds **10** and **11** from this series demonstrated improved sPLA₂-IIA activity in vitro compared to BMS-181162. Both compounds also had significant in vivo anti-inflammatory activity. Roche developed another potent inhibitor of this class (Ro 23-9358) which demonstrated in vivo anti-inflammatory activity in two animal models (LeMahieu et al., 1993).

Oinuma et al. (1991) studied the role of intracellular membrane-bound PLA₂ in ischemic diseases. They tested a series of benzenesulfonamides against intracellular membrane-bound PLA₂ from rabbit hearts as well as their protective effects against myocardial injury in an animal model. Compounds **12** (ER-3826) and **13** both demonstrated potent in vitro and in vivo activity.

To achieve competitive PLA₂ inhibition, studies have focused on developing various phospholipid analogs (Dennis et al., 2011; Jain et al., 1992). Jain et al. (1992) studied the inhibitory effect of primary amides of long-chain unsaturated fatty acids on sPLA₂-IIA. Synthetic γ -linolenoyl amide and arachidonyl amide showed potent in vitro activity. Several studies have also investigated various nonphospholipid amide inhibitors (Antonopoulou et al., 2008; Beaton et al., 1994; Hansford et al., 2003). Beaton et al. (1994) replaced the phosphocholine moiety of a previously developed substrate analog (Bennion et al., 1992), with a carboxylic acid group. FPL67047XX was the most potent compound of this series, with an IC₅₀ value of 0.021 μM against human platelet sPLA₂. Hansford et al. (2003) developed a series of amide inhibitors derived from D-tyrosine with in vitro sPLA₂-IIA activity. KH064 was identified in this study and demonstrated a variety of in vivo effects in different rat models such as protection

against colitis (Woodruff et al., 2005), reperfusion injury of the small intestine (Arumugam et al., 2003), fibrosis due to hypertension (Levick et al., 2006) and ovariectomy-induced bone loss (Gregory et al., 2006). Compound **14** is a (R)- γ -norleucine-based amide developed by Antonopoulou et al. (2008). When tested against human intracellular PLA₂s (cPLA₂-IVA and iPLA₂-VIA) and sPLA₂-V, this compound demonstrated potent and selective activity for sPLA₂-V.

Compounds **15** (PMS1062), **16** and **17** belong to the oxadiazolone class of inhibitors. These compounds exhibit varying degrees of activity towards human sPLA₂-IIA, with **17** being particularly potent (IC₅₀=0.03 μM) (Assogba et al., 2005; Boukli et al., 2008; Meddad-Belhabich et al., 2010). Despite its moderate in vitro activity (IC₅₀=9 μM), Compound **16** demonstrated in vivo anti-inflammatory activity similar to the anti-inflammatory agent, indomethacin (Boukli et al., 2008).

Knerr et al. (2018) focused their research efforts on human sPLA₂-X as this enzyme has been identified as a potential therapeutic target for atherosclerosis. They designed and synthesized a series of indole-2-carboxamide derivatives based on a lead structure identified during high-throughput screening. Compound **18** demonstrated potent (IC₅₀=0.026 μM) and selective activity towards sPLA₂-X (compared to sPLA₂-IIA and -V; IC₅₀=0.31 and 2.23 μM, respectively), but required further optimization in terms of its metabolic properties. Further optimization of the indole-2-carboxamide series, led to the development of compound **19** (Giordanetto et al., 2018). This compound was a potent and selective inhibitor of sPLA₂-X (sPLA₂-X: IC₅₀=0.014 μM; sPLA₂-IIA and -V: IC₅₀=0.63 and 5.5 μM, respectively) and demonstrated good pharmacokinetic properties. However, treatment with compound **19** did not prevent atherosclerosis in an animal model.

The biphenyl derivative, AZD2716, demonstrated potent in vitro activity against sPLA₂-IIA, -V and -X isoforms (IC₅₀=0.01, 0.04 and 0.4 μM, respectively) (Giordanetto et al., 2016). In addition to excellent preclinical pharmacokinetic properties and minimal toxicity in several species, AZD2716 also demonstrated preclinical efficacy as an in vivo plasma sPLA₂ inhibitor. This compound was selected as a clinical candidate for the treatment of coronary artery disease.

2-Oxoamides, which are cytosolic PLA₂ inhibitors, were also investigated for activity against sPLA₂ (Mouchlis et al., 2011; Vasilakaki et al., 2016). Compound GK126, based on (S)-leucine, inhibited human sPLA₂-IIA and -V with similar potency, but did not inhibit sPLA₂-X. In an attempt to improve the potency of GK126, molecular docking was used to design a new series of 2-oxoamides (Vasilakaki et al., 2016). The (S)-valine derivative from this series, GK241, demonstrated good sPLA₂-IIA potency and selectivity.

Alasmary et al. (2017) developed a series of quinoxalinone inhibitors for sPLA₂, α-glucosidase and α-amylase due to their involvement in diabetic cardiovascular complications. Several of these compounds (**20** and **21**) demonstrated promising sPLA₂ (IIA, V, X and XIA) as well as α-glucosidase activity.

Pedada et al. (2016) developed indole containing isoxazoles with potential inhibition activity against sPLA₂-IIA. Compound **22** had the best sPLA₂-IIA activity of the series (IC₅₀=10.23 μM), which were comparable to that of the positive control (urosolic acid IC₅₀=12.59 μM). This compound also demonstrated in vitro antiproliferative effects in cancer cells and in vivo anti-inflammatory activity in a rat model.

Various phytochemicals, known for their anti-inflammatory effects, have also demonstrated sPLA₂-IIA inhibition. Celastrol, ursolic acid and elemolic acid inhibited human sPLA₂-IIA in vitro (IC₅₀=6, 12 and 5.7 μM, respectively) and reduced sPLA₂-IIA mouse paw edema in vivo (Giresha et al., 2022; Joshi et al., 2016; Nataraju et al., 2007). In addition, elemolic acid also reduced the synergistic hemorrhagic effect of sPLA₂-IIA and *Vipera russelii* neurotoxic non-enzymatic peptide (VNTx-II) (Giresha et al., 2022). In vitro studies with maslinic acid demonstrated inhibition of monocyte migration and differentiation which is responsible for inflammation in atherosclerotic lesions (Yap et al., 2016).

8 | INTERACTIONS OF SMALL MOLECULES WITH sPLA₂s

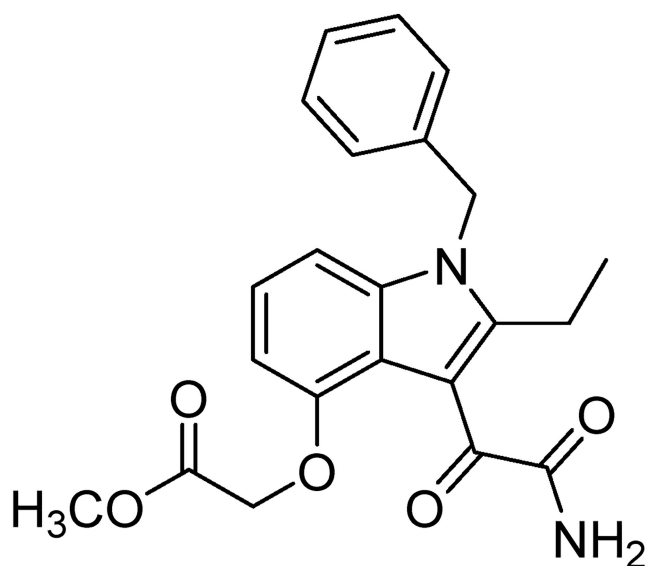
As mentioned, to date 11 sPLA₂ enzymes (group IB–XIIB) have been identified in mammals (Dennis et al., 2011). The

sPLA₂-IIA and sPLA₂-X isoforms are of great medicinal importance and inhibitors of these enzymes may act as potential treatment for atherosclerosis (Kimura-Matsumoto et al., 2008; Rosenson & Gelb, 2009). Furthermore, sPLA₂-IIA is the most widely expressed isoform in humans (Giordanetto et al., 2016). Varespladib and varespladib methyl represent model sPLA₂ inhibitors and inhibit the IIA, V and X isoforms of sPLA₂ (Figure 5) (Fraser et al., 2009). Varespladib exhibit IC₅₀ values for the inhibition of group IIA sPLA₂ of 9–14 nM, while for group V and group X sPLA₂s, IC₅₀ values of 77 and 15 nM have been recorded (Fraser et al., 2009). Another compound, AZD2716, has been developed and displays excellent preclinical pharmacokinetic properties and in vivo efficacy as a plasma sPLA₂ inhibitor. This compound was selected as a clinical candidate for the treatment of coronary artery disease (Giordanetto et al., 2016). Other noteworthy compounds include the indolizine, indoxam, and the indole, Me-indoxam (Hagishita et al., 1996; Singer et al., 2002). As discussed above, a variety of compounds have been described as human sPLA₂ inhibitors with differing selectivities for the sPLA₂ isoforms (Dennis et al., 2011). A search of the Protein Data Bank shows that 33 X-ray crystal structures of human sPLA₂s with ligands bound have been deposited. Among these, 20 structures are deemed suitable for molecular docking experiments, a selection based on the resolution of the structure, the placement of the ligand in the active site and drug-likeness of the bound ligand (Table 3). This review selected four representative structures to illustrate the important molecular interactions of inhibitor compounds with the sPLA₂s. From the three-dimensional structures of sPLA₂ complexed with inhibitors such as those listed in Table 3, much information regarding the structural requirements of good potency inhibitors may be obtained. The selected structures are 3U8D, 5WZV and 5OWC, which are three-dimensional models of human groups IIA, IIE and X, respectively (Hou et al., 2017; Knerr et al., 2018; Lee et al., 2013). The three sPLA₂ isoforms (groups IIA, IIE and X) for which three-dimensional structures have been recorded (with ligands bound to the active sites) are thus represented in this review. A further consideration for the selection of these structures is that 3U8D and 5WZV are complexed with well known inhibitors LY311727 and Me-indoxam, respectively, while the selected complexes represent both phosphonic acid and carboxylate type inhibitors, the two groups that interact with the calcium ion and which are present in all potent sPLA₂ inhibitors. The structure of human sPLA₂-IIA (5G3N) complexed to a structural analog (**23**) of AZD2716 will also be presented (Giordanetto et al., 2016). This structure was selected since AZD2716 has been identified as a clinical candidate for further development, and the structure provides much information regarding the

possible interactions of AZD2716 with human sPLA₂-IIA. Unfortunately, the structures of varepladib and AZD2716 complexed with human sPLA₂s have not been reported and deposited in the Protein Data Bank. The structure of varepladib complexed to a Lys49-PLA₂-like snake venom toxin (MjTX-II) from a South American snake, *Bothrops moojeni*, has however been reported (Salvador, Gomes, et al., 2019). This study yielded much insight into the interactions of varepladib with PLA₂-like toxins, as well as the different mechanisms by which inhibitors function, and will also be presented here. Structures such as these would greatly facilitate the discovery of small molecule inhibitors of sPLA₂s for the treatment of snakebite envenoming. In this section key interactions between the inhibitors and the sPLA₂ will be presented. These interactions are those that have been reported in the relevant publications as well as those predicted by the NGL Viewer and Mol* Viewer, tools available via the RCSB Protein Data Bank (Berman et al., 2000; Rose et al., 2018; Sehnal et al., 2021). The illustrations were prepared with the PyMOL molecular graphics system (DeLano, 2002).

8.1 | LY311727 complexed to human sPLA₂-IIA

Figure 6 shows the structure of LY311727 bound to human sPLA₂-IIA (Lee et al., 2013). The important binding groups that most inhibitors have in common are the amide and phosphonic acid (may also be replaced by a carboxylate). The amide is a key hydrogen bond donor and acceptor



Varespladib methyl

FIGURE 5 The structure of varepladib methyl.

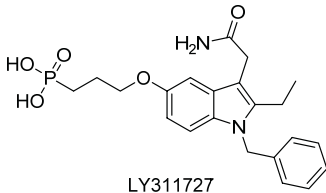
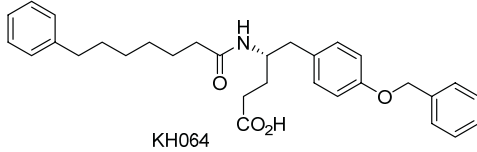
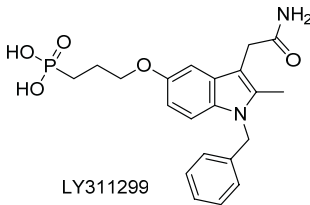
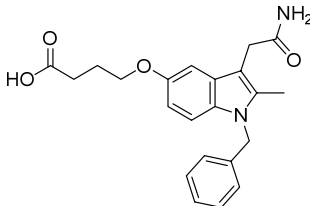
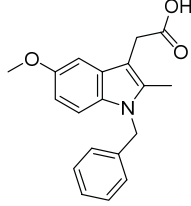
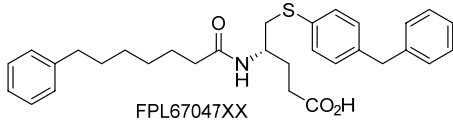
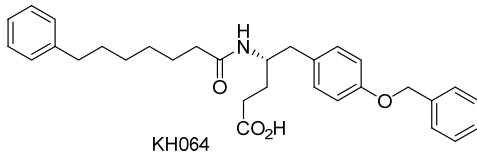
for Asp48 and His47, and possibly also Gly29 as shown in some structures (e.g. 5G3N). The carbonyl further exhibits an electrostatic interaction with the calcium ion. The phosphonic acid (or carboxylate for some inhibitors) also undergoes an electrostatic interaction with the calcium ion, and in some structures hydrogen bonding with Gly31. An aromatic moiety (e.g. 1-benzyl) is also present in the structures of potent inhibitors and forms hydrophobic interactions with Leu2, Ala17 and Ala18. The five-membered ring of the indole undergoes π -stacking interactions with Phe-5. As exemplified by indoxam and Me-indoxam, larger substituents and additional rings may be accommodated. For LY311727, the terminal methyl of the ethyl substituent at C2 of the indole is bound in a hydrophobic cavity and forms contacts to Phe-5, Phe-98 and Ile-9. These interactions may enhance the affinity of the inhibitor for the enzyme compared to similar compounds for which the ethyl is truncated to a methyl group (Lee et al., 2013). Visual inspection of a surface rendering of the active site of human sPLA₂-IIA with the inhibitor LY311727 bound, shows that the ligand fills the binding pocket with limited space available for additional substitution and extension of the structure. Space is available for larger substituents (e.g. small aromatic ring systems) on the phenyl ring as exemplified by the structures of indoxam and Me-indoxam. Other substitutions and chain extension, should, however, be limited to halogens and smaller alkyl groups.

A series of potent sPLA₂-IIA inhibitors were designed using glycerophospholipid substrates as basis (Hansford et al., 2003). KH064 was identified and a potent inhibitor with an IC₅₀ value of 0.029 μ M and the crystal structure of the inhibitor bound to group IIA sPLA₂ yielded much insight, not only into inhibitor interactions, but also the binding orientation that a glycerophospholipid substrate might adopt. The amide carbonyl and the carboxylate coordinate with the calcium ion while the amide NH is hydrogen bonded to the catalytic His48 residue (Figure 6). Further coordination with the calcium ion is provided by Asp49, His48 and Gly30 to produce the octahedral 6-coordinate environment. The two hydrophobic side chains of the inhibitor are analogous to the *sn*-1 and *sn*-2 aliphatic chains of the glycerophospholipid substrate and interact with hydrophobic residues. Interestingly, the phenyl ring of the heptanoyl moiety displaces the imidazole ring of His6 and forms an edge-to-face T-shaped interaction with the His6 and the phenolic ring of the *sn*-1 side chain.

8.2 | Me-indoxam complexed to human sPLA₂-IIE

The structure of Me-indoxam bound to the human sPLA₂-IIE enzyme has been reported (Figure 7) (Hou

TABLE 3 Native human sPLA₂ structures that have been reported and which may be suitable for molecular docking experiments.

PDB code	sPLA ₂ subtype	Resolution (Å)	Inhibitor	Reference
3U8D ^a	IIA	1.80	 LY311727	Lee et al. (2013)
3U8H ^a	IIA	2.30	 KH064	
3U8B	IIA	2.30	Free enzyme (Ca ²⁺ bound)	Schevitz et al. (1995)
3U8I	IIA	1.10	Alkylated enzyme (His47)	
1DB4 ^a	IIA	2.20	 LY311299	
1DB5 ^a	IIA	2.80		
1DCY ^a	IIA	2.70		
1KVO ^a	IIA	2.00	 FPL67047XX	Cha et al. (1996)
1J1A ^a	IIA	2.20	 KH064	Hansford et al. (2003)

(Continues)

TABLE 3 (Continued)

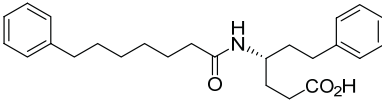
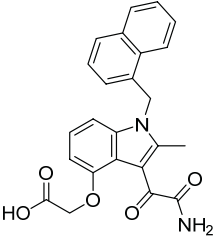
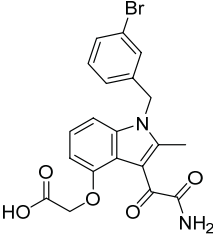
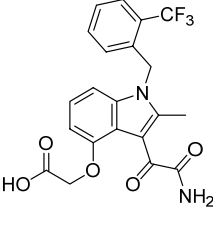
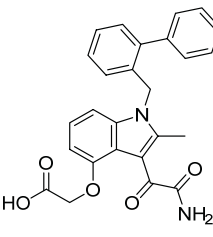
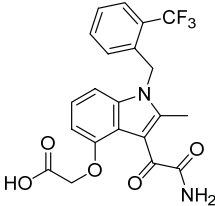
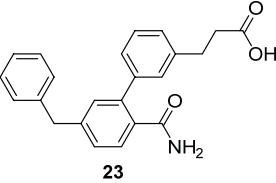
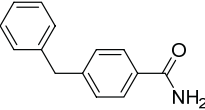
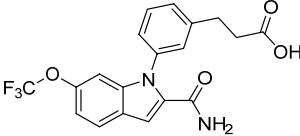
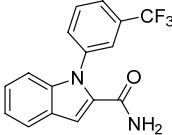
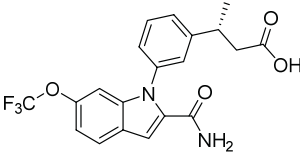
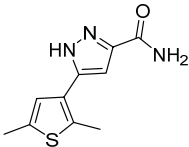
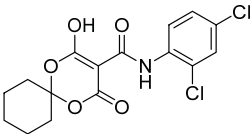
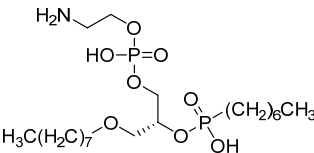
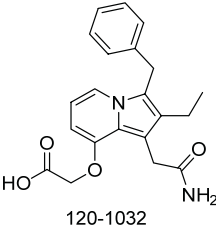
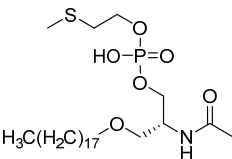
PDB code	sPLA ₂ subtype	Resolution (Å)	Inhibitor	Reference
1KQU ^d	IIA	2.10		
5WZS ^d	IIE	2.30		Hou et al. (2017)
5WZT ^d	IIE	2.40		
5WZU ^d	IIE	2.20		
5WZV ^d	IIE	2.20	 Me-indoxam	
5WZW ^d	IIE	1.95	LY311727	

TABLE 3 (Continued)

PDB code	sPLA ₂ subtype	Resolution (Å)	Inhibitor	Reference
5Y5E ^a	IIE	1.80	LY311727	
				
5WZM	IIE	2.00	Free enzyme (Ca ²⁺ bound)	
5WZO	IIE	1.90	Free enzyme (Ca ²⁺ bound)	
5G3N ^a	IIA	1.80		Giordanetto et al. (2016)
3G3M ^a	X	1.85		
5OWC ^a	X	1.75		Knerr et al. (2018)
5OW8 ^a	X	1.90		
6G5J ^a	X	1.85		Giordanetto et al. (2018)

(Continues)

TABLE 3 (Continued)

PDB code	sPLA ₂ subtype	Resolution (Å)	Inhibitor	Reference
4UY1 ^a	X	2.20		Chen et al. (2014)
NI	IIA			Bryant et al. (1999)
1POE	IIA	2.10		Scott et al. (1991)
1POD NI	IIA	2.10	Free enzyme (Ca ²⁺ bound) 	Kitadokoro et al. (1998)
1AYP	IIA	2.57		Oh (1995)
1BBC	IIA	2.20	Free enzyme	Wery et al. (1991)
1LE6	X	1.97	Free enzyme (Ca ²⁺ bound)	Pan et al. (2002)
1LE7	X	2.09	Free enzyme (Ca ²⁺ bound)	
3ELO	1B	1.55	Free pro-enzyme	Xu et al. (2009)
6KQU	IIE	2.00	Free enzyme (Ca ²⁺ bound)	Hou et al. (2020)
6Q42	1B	1.90	Free enzyme	Pothlichet et al. (2020)

^aStructures complexed with small molecule ligands that are most suitable for molecular docking experiments.

et al., 2017). Similar to human sPLA₂-IIA, the sPLA₂-IIE enzyme has seven disulphide bonds. The calcium ion is coordinated to the carbonyl oxygens of Tyr26, Gly28 and Gly30 as well as to the side chain carboxylate of Asp47.

Interestingly, a second calcium-binding site exists for both human sPLA₂-IIA and sPLA₂-IIE. Residues Asp22, Gly24, Tyr111, Asn113 and water molecules are involved in the coordination of the second calcium. The second calcium

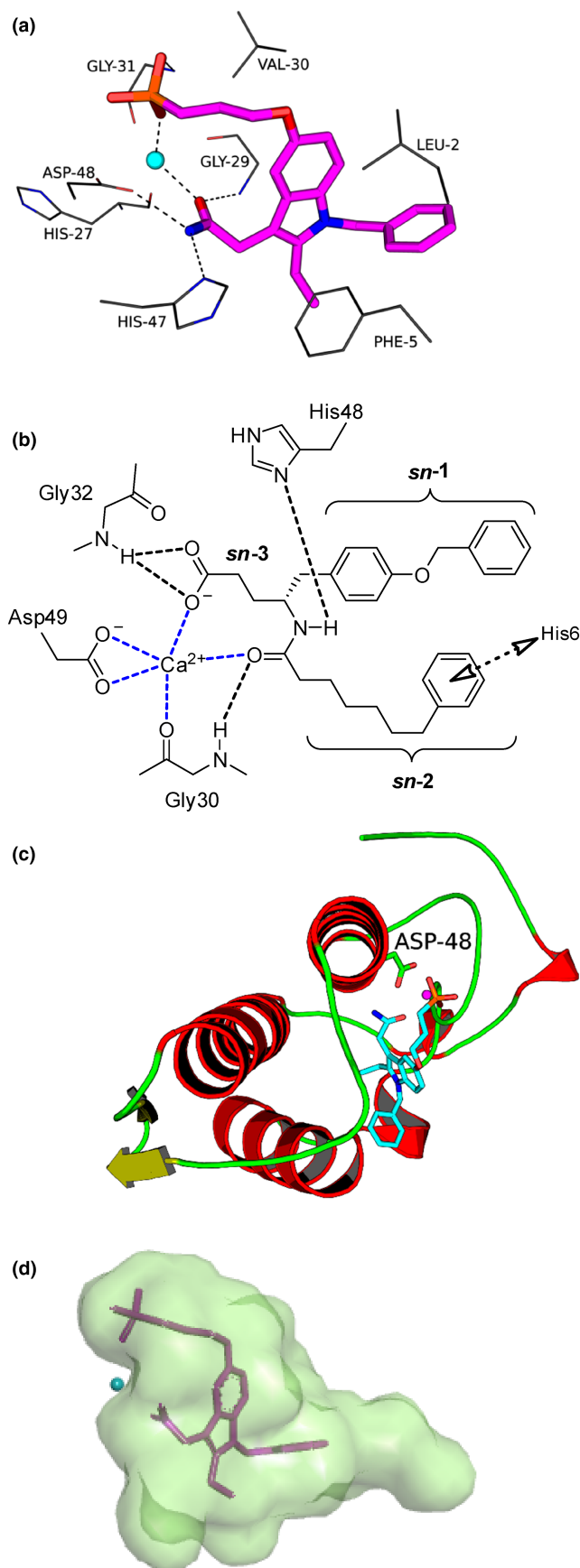


FIGURE 6 (a) The three-dimensional structure of the complex between LY311727 (magenta) and human sPLA₂-IIA (PDB code: 3U8D). Extensive hydrogen bond interactions (dashed lines) between LY311727 and the active site of the enzyme. These include the following interactions: (i) hydrogen bonding between the amide nitrogen and the side chains of Asp48 and His47; (ii) the amide carbonyl coordinates with the calcium ion (and possibly forms a hydrogen bond interaction with the main chain NH of Gly29); (iii) the phosphonic acid coordinates with the calcium ion and is (iv) hydrogen bonded to numerous water molecules and the main chain NH of Gly31 (Lee et al., 2013); (b) The interactions of KH064 with sPLA₂-IIA (PDB code: 1J1A) (Hansford et al., 2003); (c) The overall fold of the human sPLA₂-IIA protein (PDB code: 3U8D); (d) A surface rendering of the shape of the active site.

ion is not present in inhibitor-bound complexes of sPLA₂-IIE. For Me-indoxam, the amide of the glyoxamide group acts as a hydrogen bond donor and acceptor for Asp47 and His46, while the carbonyl of the glyoxamide group may also be hydrogen bonded to His46. The carboxylate forms an ionic bond to the side chain ammonium of Lys61, while both the carboxylate and amide coordinate to the calcium ion. Asn21 is an important residue for inhibitor recognition and an amide- π interaction is formed between the side chain amide of this residue and the terminal phenyl ring of the inhibitor (Hou et al., 2017).

8.3 | An indole-2 carboxamide (18) complexed to human sPLA₂-X

Among a series of experimental indole-2 carboxamides, a potent inhibitor (18) of human sPLA₂-X was discovered with good isoform selectivity over sPLA₂-IIA and sPLA₂-V (Knerr et al., 2018). The crystal structure of this compound complexed to human sPLA₂-X was reported and it was found that the amide and carboxylate coordinates to the calcium ion while the carboxylate may also form a hydrogen bond interaction with the main chain NH of Gly30 (Figure 8). The amide nitrogen further establishes hydrogen bonding with Asp47 and His46 while the carbonyl oxygen may be hydrogen bonded to the main chain NH of Gly28. The N-phenyl of the inhibitor may interact with the calcium ion by a cation- π interaction. The trifluoromethoxy group binds within a hydrophobic pocket and interacts via van der Waals interactions with Ile2, Ala6, Pro17, Ile18 and Met21. Residues Leu5, Val9 and Leu98 in sPLA₂-X are replaced by Phe, Ile and Phe in sPLA₂-IIA, and the resulting difference in pocket sizes could potentially be used to design inhibitors with isoform selectivity. In this regard, a suitable substituent on the six-membered

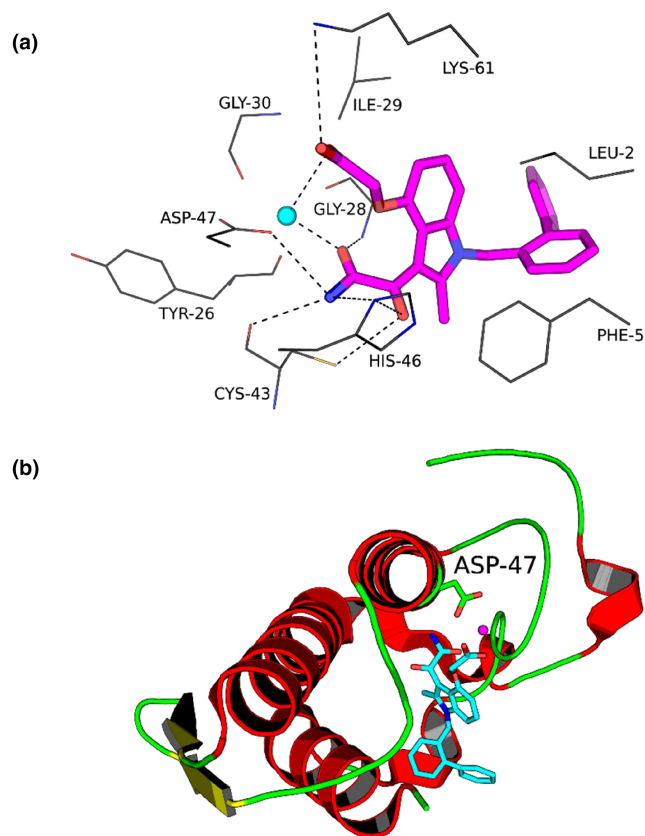


FIGURE 7 (a) The three-dimensional structure of the complex between Me-indoxam (magenta) and human sPLA₂-IIE (PDB code: 5WZV). Extensive hydrogen bond interactions (dashed lines) between Me-indoxam and the active site of the enzyme. These include the following interactions: (i) hydrogen bonding between the amide nitrogen and the side chains of Asp47 and His46 and the main chain carbonyl of Cys43; (ii) hydrogen bonding between the glyoxamide carbonyl and the side chain of His46, while a polar interaction with the thiol of Cys43 is also possible; (iii) the amide carbonyl and the (iv) carboxylate coordinate with the calcium ion; (v) the amide carbonyl forms a hydrogen bond with the NH of Gly28; (vi) the carboxylate may also undergo an electrostatic interaction to the Lys61 (Hou et al., 2017); (b) The overall fold of the human sPLA₂-IIE protein (PDB code: 5WZV).

ring of the indole could interact differently with these pockets in sPLA₂-IIA and sPLA₂-V (Knerr et al., 2018).

8.4 | Compound 23 complexed to human sPLA₂-IIA

Structure-based optimization of 4-benzylbenzamide, a lead identified by high-throughput screening, yielded AZD2716, a potent inhibitor of human sPLA₂-IIA, sPLA₂-V and sPLA₂-X (Giordanetto et al., 2016). This compound also inhibited human plasma sPLA₂ and sPLA₂ activity in atherosclerotic plaque homogenates. Furthermore, AZD2716 displayed good preclinical pharmacokinetic

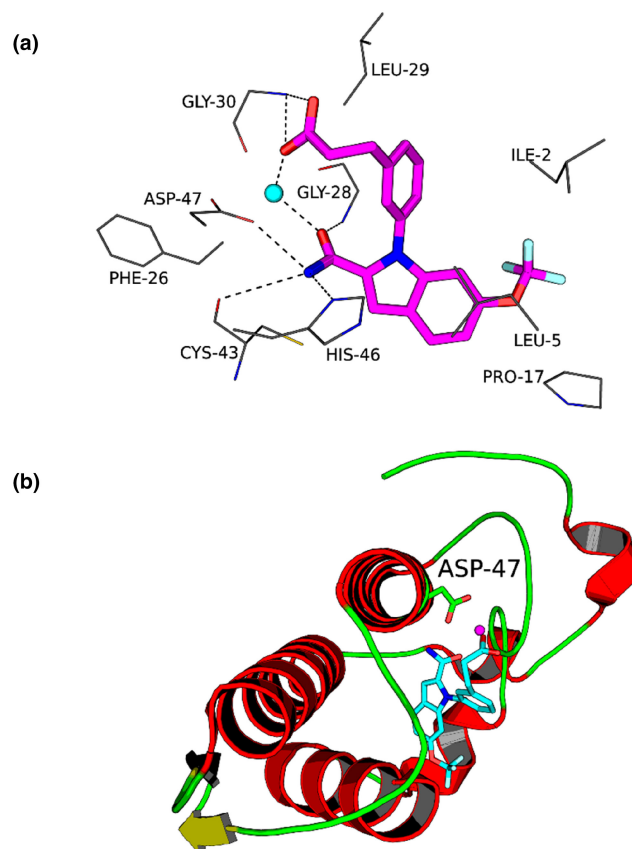


FIGURE 8 (a) The three-dimensional structure of the complex between an indole-2 carboxamide (**18**; magenta) and human sPLA₂-X (PDB code: 5OWC). Extensive hydrogen bond interactions (dashed lines) between the inhibitor and the active site of the enzyme. These include the following interactions: (i) hydrogen bonding between the amide nitrogen and the side chains of Asp47 and His46 as well as the main chain carbonyl of Cys43; (ii) the carbonyl oxygen of the amide may be hydrogen bonded to the main chain NH of Gly28; (iii) the amide carbonyl and the (iv) carboxylate coordinate with the calcium ion; (v) the carboxylate forms a hydrogen bond to the main chain NH of Gly30 (Knerr et al., 2018); (b) The overall fold of the human sPLA₂-X protein (PDB code: 5OWC).

profiles in several species, with in vivo efficacy in cynomolgus monkeys. Although the crystal structure of AZD2716 was not reported, the complex between human sPLA₂-IIA and a close structural analog, compound **23**, was determined (Figure 9). The amide of the inhibitor establishes hydrogen bonds with Gly29, His47 and Asp48 while the carbonyl oxygen of the amide coordinates with the calcium. The carboxylate provides the second coordination bond to the calcium ion and forms hydrogen bonds to the backbone amide NH of Gly31. The phenyl of the phenylpropanoic acid moiety establishes van der Waals contacts with Leu2, Gly29 and Val30. The benzyl substituent is located in a pocket defined by Leu2, Phe5, His6, Ile9, Ala17, Ala18 and Gly22. For human sPLA₂-IIA, the

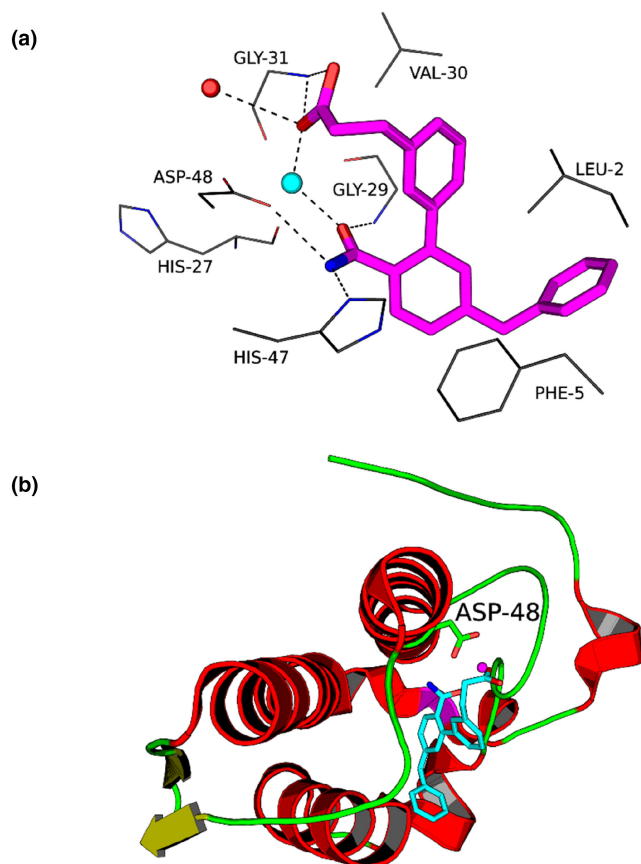


FIGURE 9 (a) The three-dimensional structure of the complex between **23** (magenta) and human sPLA₂-IIA (PDB code: 5G3N). Extensive hydrogen bond interactions (dashed lines) between the inhibitor and the active site of the enzyme. These include the following interactions: (i) hydrogen bonding between the amide nitrogen and the side chains of Asp48 and His47; (ii) the carbonyl oxygen of the amide may be hydrogen bonded to the main chain NH of Gly29; (iii) the amide carbonyl and the (iv) carboxylate coordinate with the calcium ion; (v) the carboxylate oxygens form hydrogen bonds to the main chain NH of Gly31 (Giordanetto et al., 2016); (b) The overall fold of the human sPLA₂-IIA protein (PDB code: 5G3N).

benzamide phenyl forms an edge-to-face π interaction with Phe5, which was proposed to significantly improve affinity. For human sPLA₂-X this interaction is not possible, resulting in lower inhibition potency.

8.5 | Varespladib complexed to a Lys49-PLA₂-like snake venom toxin (MjTX-II) from a South American snake, *Bothrops moojeni*

Several variants of sPLA₂-like proteins are found in snake venom. Asp49-PLA₂-like proteins display catalytic activity while Lys49-PLA₂-like proteins lack catalytic activity, although both toxins induce myonecrosis. Asp49-PLA₂

toxins are small proteins (~13kDa) and consist of three α -helices, one anti-parallel β -sheet and a calcium-binding loop. These are calcium-dependant enzymes that hydrolyze membrane phospholipids as a key step in their myotoxic action with the catalytic His48 residue being required for catalysis. Interestingly, catalytic-inactive Lys49-PLA₂-like proteins are also myotoxic by disrupting membrane integrity. Fatty acids bind to the hydrophobic channel of the enzyme, leading to interaction of the toxin with the cell membrane via the *membrane docking site* (MDoS) and disruption of the membrane via the action of the *membrane disrupting site* (MDiS). The three-dimensional structure of a typical sPLA₂ (from the snake venom of *Bothrop*) has been reported and is illustrated in Figure 10 (Salvador, Gomes, et al., 2019). This structure represents the complex between varespladib and a Lys49-PLA₂-like snake venom toxin (MjTX-II) from a South American snake, *Bothrops moojeni* (Salvador, Gomes, et al., 2019). The MjTX-II/varespladib structure, similar to other group II PLA₂ proteins, crystallizes as a dimer with seven disulphide bridges in each monomer. Two molecules of the inhibitor bind to the dimer, displaying different orientations and interactions with the two monomers. The interactions with monomer A will be presented here. The crystal structure shows that varespladib binds in the hydrophobic channel of the toxin, and interacts in particular with the His48 and Lys49 residues as well as with Leu2 and Leu5 (Helix I) and Phe126 (MDiS). The binding of varespladib likely prevents the binding of the membrane fatty acids, resulting in reduction of toxicity. Furthermore, varespladib induces distortion of the dimeric assembly of the protein that may impede the function of the toxin, particularly at the MDiS of the protein, thereby preventing the exposure of the MDiS to the solvent. This distortion has also been observed in crystal structures of MjTX-II with rosmarinic acid and acetylsalicylic acid (Salvador, Cardoso, et al., 2019).

9 | THE PHARMACOPHORE FOR THE INHIBITION OF sPLA₂

A relatively large amount of sPLA₂ inhibitors have been reported and have been summarized in review papers (Batsika et al., 2021; Dennis et al., 2011). These inhibitors include early phospholipid analogs, dicarboxylic acid analogs and compounds containing the amide functional group. The more recently discovered inhibitors are, however, of interest to this paper and are the indole and indolizine compounds (e.g. varespladib, indoxam and Me-indoxam, LY311727) and AZD2716. The structures of these compounds (or close analogs thereof) in complex with human sPLA₂ enzymes have been reported. Based on the structural data discussed above, these sPLA₂ inhibitors

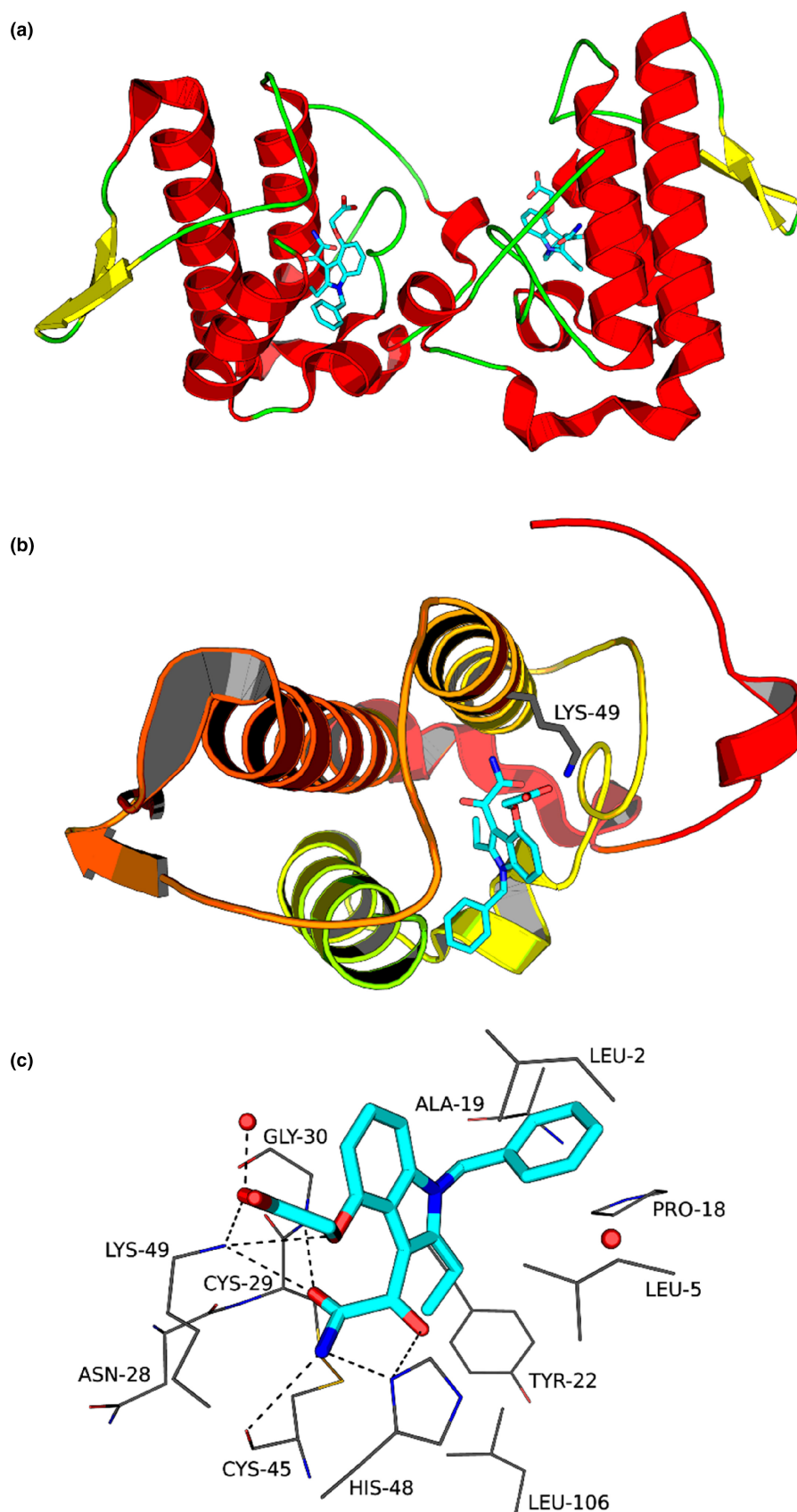


FIGURE 10 (a) The three-dimensional structure of the complex between varespladib (cyan) and a Lys49-PLA₂ (MjTX-II) from snake venom (PDB code: 6PWH); (b) Varespladib located in the hydrophobic channel of the toxin; (c) Extensive hydrogen bond interactions (dashed lines) between varespladib and the active site of the toxin. These include hydrogen bonding between: (i) amide nitrogen and the backbone carbonyl of Cys45 and the side chain of His48; (ii) glyoxamide ketone and His48; (iii) amide carbonyl and the ϵ -amino group of Lys49; (iv) amide carbonyl and backbone nitrogen of Gly30; (v) ether oxygen and the ϵ -amino group of Lys49; (vi) carboxylate and the ϵ -amino group of Lys49 (electrostatic interaction); (vii) carboxylate and an active site water molecule (Salvador, Gomes, et al., 2019).

possess some of the following structural features and functional groups (Figure 11): (a) An amide, which acts as a key hydrogen bond donor and acceptor, and forms

a coordination bond to the calcium ion. This functional group is considered privileged for potent sPLA₂ inhibition (Giordanetto et al., 2016); (b) a negatively charged

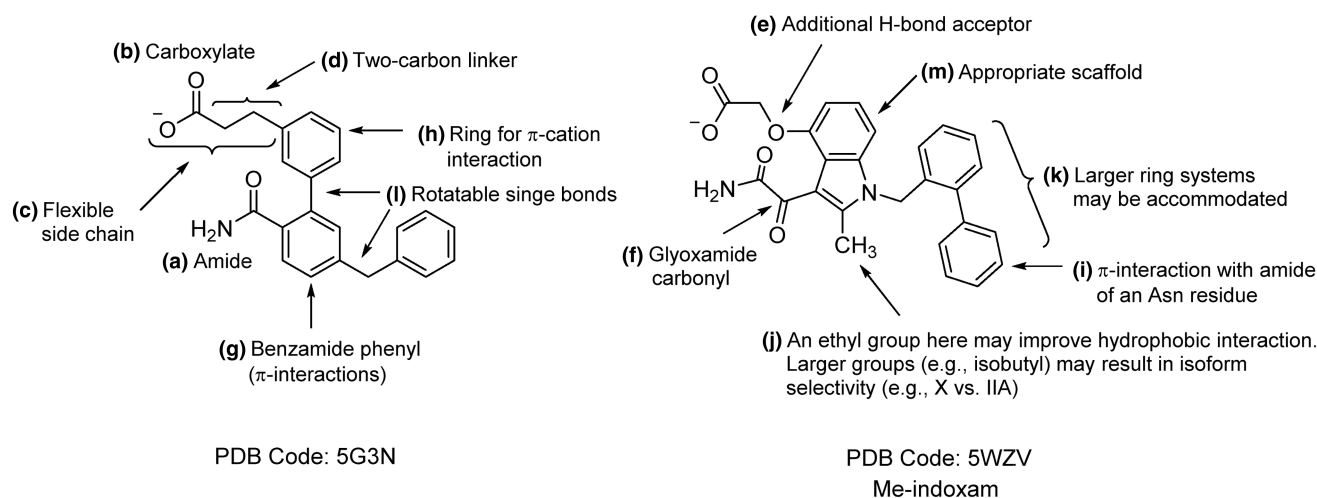


FIGURE 11 The binding groups and structural features of typical sPLA₂ inhibitors illustrated with the structures of ligands in two sPLA₂ crystal structures. The numbering (a–m) of the structural features corresponds to the numbering system used in the text.

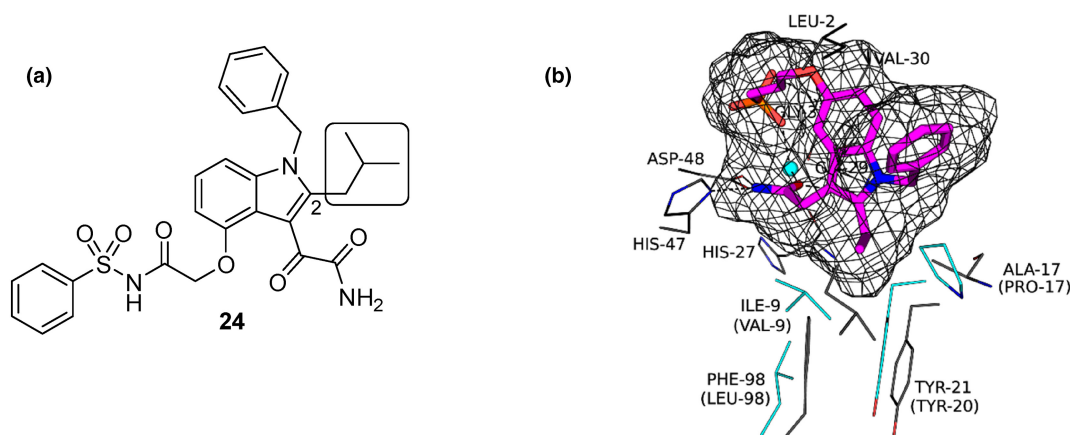


FIGURE 12 (a) The structure of compound **24**, a specific inhibitor of human group X sPLA₂. (b) An overlay of the cavities of human groups IIA (with LY311727 bound) and X sPLA₂s illustrates the corresponding positions of Ile-9 in sPLA₂-IIA (grey) and Val-9 in sPLA₂-X (cyan). This amino acid substitution results in additional space available for binding of larger substituents (e.g. the isobutyl substituent on the two-position of compound **24**) in the active site of group X sPLA₂ that is not available in group IIA.

carboxylate or phosphonic acid with interacts electrostatically with the calcium ion is present in the structures of all inhibitors; (c) flexible side chain to place the carboxylate in the correct position to form a coordination bond with the calcium; (d) a two-carbon alkyl linker between the carboxylate and the scaffold provide structural flexibility for the correct positioning of the carboxylate, (e) while an ether oxygen in this linker may act as an additional hydrogen bond acceptor; (f) a glyoxamide carbonyl group is present in some inhibitors for additional polar interaction; (g) a benzamide phenyl to interact with aromatic residues (e.g. Phe5 in sPLA₂-IIA) via π -interactions improves affinity and may provide selectivity (e.g. for sPLA₂-IIA over sPLA₂-X); (h) an aromatic ring that may interact via cation- π interaction with the calcium; (i) a phenyl to form an amide- π interaction with an asparagine residue

(e.g. sPLA₂-IIE) (Hou et al., 2017); (j) an ethyl group that may improve surface complementarity by binding is a hydrophobic pocket (Lee et al., 2013); (k) larger substituents and additional rings may be accommodated in a larger hydrophobic region; (l) rotatable single bonds at appropriate positions allow for the correct conformation of the inhibitor to optimize interactions of the binding groups; (m) a phenyl ring (e.g. AZD2716) or bicyclic aromatic ring system (e.g. indole derivatives such as varespladib, indoxam, Me-indoxam and LY311727) may be used as scaffold for the attachment of the required functional groups and moieties.

The following interactions between sPLA₂ and the inhibitors are observed in most structures: (a) Coordination bonds to the calcium ion by an amide as well as a carboxylate (which could be replaced by phosphonic acid); (b) the

amide NH_2 as well as carbonyl are a key hydrogen bonding groups with several active site residues; (c) the carboxylate (or phosphonic acid) undergoes hydrogen bonding (or electrostatic interactions). Certain interactions, however, are specific to the type of inhibitor, and depending on the structure of the inhibitor, additional polar groups (e.g. glyoxamide carbonyl, ether oxygen atoms) may be present that undergo hydrogen bonding with active site residues, while aromatic ring systems are placed in specific regions in the active site where different π -interactions are possible. Furthermore, inhibitors may fill different hydrophobic pockets and thus form dissimilar van der Waals interactions.

An excellent example of a structure-based approach for the design of an sPLA₂ inhibitors was the discovery of AZD2716 (Giordanetto et al., 2016). The general structural features and functional groups above may be considered in similar design projects. For the discovery of AZD2716, a fragment containing aromatic rings and the amide functional group (e.g. benzamide) was used as starting point. As exemplified by many other sPLA₂ inhibitors, the amide should be retained while the phenyl rings may be replaced by various other aromatic or heteroaromatic ring systems, depending on the need for novelty and the potential to further develop into a clinical drug. In the second step, a carboxylic acid group is added. The objective is to place the amide and carboxylate in the correct positions with respect to each other to establish coordination bonds to the calcium ion. This may be done by expansion of the aromatic ring system and/or addition of a suitable alkyl linker which may be progressively elongated to search for the optimal placement of the carboxylate. Additional alkyl groups and halogens may now be introduced to increase affinity and obtain isoform specificity, as well as to optimize physicochemical and pharmacokinetic properties.

One of the challenges of the design of sPLA₂ inhibitors is obtaining isoform specificity. Many sPLA₂ inhibitors act against various isoforms. For example, Me-indoxam inhibits human groups IIA, IIE and V sPLA₂s with nanomolar potencies (Singer et al., 2002; Smart et al., 2006), while varespladib potently inhibits the human groups IIA, V and X sPLA₂s enzymes (Fraser et al., 2009). In a medicinal chemistry study, the structures of human groups IIA and X sPLA₂s were overlayed to reveal an additional binding region in the human group X active site that is not present in human group IIA (Oslund et al., 2008). This region is a hydrophobic space and is the result of the substitution of an isoleucine residue in group IIA for a valine in group X. For a series of synthetic indole derivatives, this region interacts with the substituent on the two-position and larger substituents on this position would be less

well accommodated in the active site of group IIA than in group X (Figure 12). As exemplified by compound 24 with an isobutyl substituent on the two-position, such compounds would therefore act as specific inhibitors of human group X sPLA₂.

10 | CONCLUSION

Due to their roles in inflammation, the sPLA₂ enzymes are of much medicinal interest. sPLA₂ inhibitors have been developed for the treatment of inflammatory and other conditions such as cardiovascular disease, arteriosclerosis and rheumatoid arthritis. Although no sPLA₂ inhibitor has been approved for use in the clinic, these compounds remain of interest, and varespladib has been granted orphan drug status by the Food and Drug Administration (FDA) for its potential to treat snakebite envenomation. From a medicinal chemistry point of view, the crystal structures of human sPLA₂ enzymes complexed with sPLA₂ inhibitors are valuable to drug discovery projects, not only to define the structural requirements for potent interaction, but possibly to also aid in the design of isoform specific inhibitors. This review selected four human sPLA₂s and presented the characteristics of the bound inhibitors that are considered key for high affinity interaction. A summary of the pharmacophoric groups highlight the carboxylate (or phosphonic acid) and amide as being privileged for sPLA₂ inhibition while several other groups (e.g. glyoxamide carbonyl) and structural features (e.g. linker length and alkyl groups of differing sizes) will contribute to binding affinity and specificity.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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