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Potent and selective 2-oxoamide inhibitors of phospholipases A_2 as novel medicinal agents for the treatment of inflammatory diseases*

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Abstract: Phospholipases A_2 (PLA_2s) are enzymes that are capable of catalyzing the hydrolysis of the *sn*-2 ester bond of glycerophospholipids, releasing free fatty acids, including arachidonic acid (AA), and lysophospholipids. Both products are precursor signaling molecules involved in inflammation. Among the various PLA_2s, cytosolic GIVA $cPLA_2$ is considered a major target for inflammatory diseases, while secreted GIIA $sPLA_2$ is involved in cardiovascular diseases. We have developed lipophilic 2-oxoamides based on (*S*)-γ- or δ-amino acids as potent and selective inhibitors of GIVA $cPLA_2$, which present interesting in vivo anti-inflammatory activity. 2-Oxoamides based on natural α-amino acids are selective inhibitors of GIIA $sPLA_2$. The mode of binding of 2-oxoamides with either GIVA $cPLA_2$ or GIIA $sPLA_2$ has been studied by various techniques.

Keywords: enzyme inhibitors; inflammation; inhibitors; medicinal chemistry; organic synthesis; 2-oxoamides; phospholipase A_2; structure–activity.

INTRODUCTION

Phospholipases A_2 (PLA_2s) constitute one of the largest families of lipolytic enzymes defined by their ability to catalyze the hydrolysis of the ester bond at the *sn*-2 position of glycerophospholipids, yielding free fatty acids and lysophospholipids [1]. Free fatty acids released, mainly arachidonic acid (AA), may be metabolized to form various eicosanoids and related bioactive proinflammatory lipids [2]. Hence, inhibiting AA release is of great relevance for the development of new anti-inflammatory drugs. Lysophospholipids also have various important roles in biological processes [3]. The PLA_2 superfamily currently consists of 15 groups and many subgroups, which differ in primary sequence, structure, and catalytic mechanism [1]. There are four predominant types of PLA_2: the secreted PLA_2 ($sPLA_2$), the cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), the cytosolic Ca^{2+}-independent PLA_2 ($iPLA_2$), and the lipoprotein-associated PLA_2 ($LpPLA_2$). Each of them plays key roles in cells and organisms being involved in diverse kinds of lipid biology and producing a variety of bioactive lipids. Potent and selective inhibitors of lipolytic enzymes could help to shed light on their physiological functions and associated metabolic pathways [4]. This is the reason for tremendous interest in discovering PLA_2 inhibitors as novel potential therapeutic agents of inflammatory diseases.

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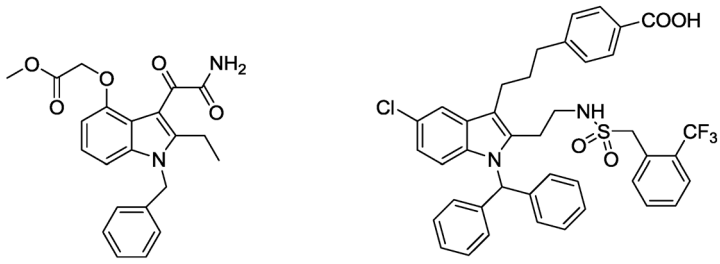
The various classes of intracellular and extracellular PLA_2 inhibitors have been summarized in recent review articles [1,5–8]. Among them, varespladib methyl (Fig. 1), a human GIIA $sPLA_2$ inhibitor, was entered into clinical trials for the treatment of severe sepsis. However, the trials terminated at phase II because the results were not robust. Years later, Anthera Pharmaceuticals pursued the same inhibitor for the treatment of cardiovascular diseases, which is currently in phase III trials. Concerning GIVA $cPLA_2$, giripladib (Fig. 1) was advanced into a phase II clinical trial for osteoarthritis, but the trial was terminated owing to a failure to differentiate from the standard of care with naproxen because of gastroenterologic effects.



Varespladib methyl **Giripladib**

Fig. 1

Our laboratory has developed a new class of inhibitors of PLA_2, containing the 2-oxoamide functionality, which have been studied for their activity and selectivity on three human PLA_2 classes: GIVA $cPLA_2$, GVIA $iPLA_2$ and GV $sPLA_2$. To understand the role and specificity of 2-oxoamide inhibitors, experiments in cells and in vivo have also been performed. This review article summarizes synthetic methods for the preparation of 2-oxoamides, in vitro and in vivo activities of 2-oxoamides and studies on their mode of interaction with GIVA $cPLA_2$ and GIIA $sPLA_2$.

PHOSPHOLIPASE A_2 ENZYMES

Cytosolic phospholipase A_2

The cytosolic PLA_2s are large proteins with variable sizes (61–114 kDa). Groups IVA, IVB, IVC, IVD, IVE, and IVF are included in this category. The first group IVA cytosolic enzyme was identified in human platelets and was cloned and sequenced in 1991 [9,10]. The most well characterized enzyme of this category is GIVA $cPLA_2$, which selectively releases AA from the *sn*-2 position of phospholipids. This enzyme also possesses lysophospholipase and transacylase activity.

The properties of the group IVA PLA_2 have been reviewed [11]. The structure of GIVA $cPLA_2$ shows that it is composed of a Ca^{2+}-dependent lipid binding C2 domain and a catalytic α/β hydrolase domain [12]. The catalytic domain is composed of a core α/β hydrolase region, a typical motif for many different lipases, as well as a cap region. Ca^{2+} binding in this enzyme is not required for catalysis, as in the secreted $sPLA_2$ enzymes, but for translocation to the membrane surface. The enzyme possesses an unusual Ser-Asp catalytic dyad located in a deep cleft at the center of a hydrophobic funnel. The catalytic mechanism of GIVA $cPLA_2$ proceeds through a serine-acyl intermediate using Ser-228 as the nucleophilic residue [13,14].

Ca^{2+}-independent phospholipase A_2

GVIA PLA_2 is actually a group of cytosolic enzymes ranging from 85 to 88 kDa and expressed as several distinct splice variants of the same gene. Among them, only two have been shown catalytically active (groups VIA-1 and VIA-2 PLA_2) [15]. Six different types, GVIA, GVIB, GVIC, GVID, GVIE, and GVIF, are included in this category. GVIA $iPLA_2$ was purified from macrophages in 1994 [16] and is the best characterized enzyme of this category. The GVIA $iPLA_2$ enzyme contains a consensus lipase motif, Gly-Thr-Ser*-Thr-Gly, with the catalytic serine confirmed by site-directed mutagenesis [17]. Although it shares the same catalytic mechanism with GIVA PLA_2, it does not show specificity for AA at the *sn*-2 position of phospholipids.

Secreted phospholipase A_2

The $sPLA_2$s were the first type of PLA_2 enzymes discovered. PLA_2 was known to be a major component of snake venoms from the end of the 19th century, and it was recognized as a component of pancreatic juice. $sPLA_2$s from various sources belong to groups IA, IB, IIA, IIB, IIC, IID, IIE, IIF, III, V, IX, X, XIA, XIB, XII, XIII, and XIV [1]. GIIA PLA_2 was found in high concentrations in synovial fluid from patients suffering from rheumatoid arthritis [18]. Thus, great attention was initially focused on mammalian GIIA $sPLA_2$ in the sense that it plays an important role in inflammation. Later, it was reported that various secreted PLA_2 enzymes are expressed in rheumatoid arthritis and augment prostaglandin production in cultured synovial cells [19]. The $sPLA_2$s are small secreted proteins (14–18 kDa) usually containing five to eight disulfide bonds. They require millimolar concentrations of Ca^{2+} for hydrolysis of phospholipids and do not exhibit preference for specific fatty acids at the *sn*-2 position of the substrate. This group of enzymes utilizes an active-site histidine together with an aspartate residue for the catalytic mechanism. Many of the $sPLA_2$s share the property of exhibiting an increase in activity when the substrate is presented as a large lipid aggregate, rather than a monomeric form, a phenomenon called "interfacial activation" [20].

Lipoprotein-associated phospholipase A_2

Several secreted and intracellular platelet-activating factor (PAF) acetylhydrolases have been identified [1]. Plasma PAF-acetylhydrolase (PAF-AH), also known as lipoprotein-associated phospholipase A_2 ($LpPLA_2$), catalyzes the hydrolysis, and therefore the inactivation, of PAF and certain oxidized phospholipids. Owing to its PLA_2 catalytic activity, it has been classified as GVIIA PLA_2 [1]. The plasma enzyme is tightly bound to low- and high-density lipoprotein particles and therefore acts on the lipid–aqueous interface and can be considered as a peripheral membrane protein. The PAF-AH crystal structure has been very recently resolved [21]. The enzyme has a typical lipase α/β hydrolase fold, with a Ser273/His351/Asp296 catalytic triad in its active site. Catalytic Ser273 is located at the *N*-terminus of an α-helix, within the typical GXSXG sequence found in other lipases. The amide nitrogen atoms of Phe274 and Leu153 stabilize the negative charge of the hydrolysis reaction tetrahedral intermediate, and these residues act as an oxyanion hole.

Biological function and involvement of PLA_2s in diseases

GIVA $cPLA_2$ is generally considered to be a central enzyme mediating generation of multiple lipid mediators, including eicosanoids and potentiating pain and inflammation. Thus, it plays a major role in inflammatory diseases. GIVA $cPLA_2$ deficiency in patients and knockout mice models has shown decreased eicosanoid production and mitigation in the effects of inflammatory diseases. These mice showed significant decrease in allergic response, damage from acute lung injury and postischemic brain injury [22–26]. Notable defects caused by the loss of GIVA $cPLA_2$ are found in the female knockout

mice reproduction system [25]. Most recently, the role of GIVA $cPLA_2$ in prostate cancer [27] and Alzheimer's disease [28] has been proposed, and the application of $cPLA_2$ inhibitors for the prevention and treatment of these diseases has been discussed.

Recent review articles discuss the role of GVIA $iPLA_2$ in signaling and pathological conditions, for example, cancer and ischemia [29–32]. GVIA $iPLA_2$ has been proposed to be a homeostatic enzyme involved in basal metabolism inside the cell. However, a number of studies suggest that GVIA $iPLA_2$ plays important though different roles in numerous cell types. It has been reported that $iPLA_2$ participates in ER stress-induced apoptosis, a pathway that promotes β-cell death in diabetes [33]. It was also demonstrated that $iPLA_2$-null male mice produce spermatozoa with impaired motility and have greatly reduced fertility [34]. In addition, the absence of $iPLA_2$ causes abnormalities in osteoblast function and bone marrow stromal cells differentiation, indicating the role of $iPLA_2$ in bone formation [35].

The biochemistry and physiology of mammalian $sPLA_2$s have been reviewed [36–38]. Numerous in vitro studies document the ability of GV and GX $sPLA_2$, and to a lesser extent GIIA $sPLA_2$, to generate AA for eicosanoid production in inflammatory cells, including macrophages, neutrophils, eosinophils, and mast cells [36]. In some cases, the activity of $sPLA_2$ has been shown to be dependent on or linked to the activity of GIVA $cPLA_2$. The $sPLA_2$s appear to play a role in several inflammatory diseases. The first evidence was that GIIA $sPLA_2$ was present at high concentrations in the synovial fluid from arthritic knee joints [18]. GIIA $sPLA_2$-deficient mice have shown reduced signs of arthritis when compared with wild-type mice. Accumulating evidence has indicated that at least three $sPLA_2$s, GIIA, GV, and GX, exert multiple proatherogenic properties in the vessel wall [39–45]. A recent article by Gelb et al. provides clear evidence that GX $sPLA_2$ is a novel therapeutic target for asthma [46].

2-OXOAMIDES: SYNTHESIS AND STRUCTURE–ACTIVITY RELATIONSHIP STUDIES

2-Oxoamide inhibitors of cytosolic phospholipase A_2

Design of 2-oxoamide inhibitors of GIVA $cPLA_2$

Novel inhibitors were initially designed taking into consideration the enzyme's catalytic mechanism and the structure of the substrate [47,48]. The catalytic mechanism of GIVA $cPLA_2$ proceeds through a serine-acyl intermediate after attack of the nucleophilic Ser-228. Our strategy for the design of novel GIVA $cPLA_2$ inhibitors was based on the principle that the inhibitor should consist of several parts: (a) an electrophilic functionality, such as an activated carbonyl group that may interact with the active-site serine, (b) a lipophilic segment, and (c) the appropriate chemical motifs necessary for specific interactions and proper orientation in the enzyme's active site (Fig. 2).

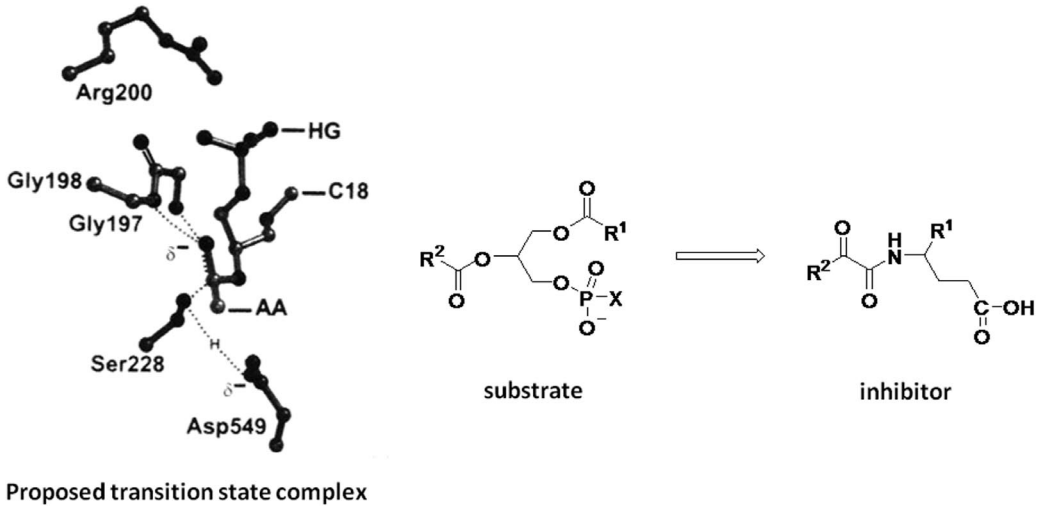


Fig. 2 Design of 2-oxoamide inhibitors.

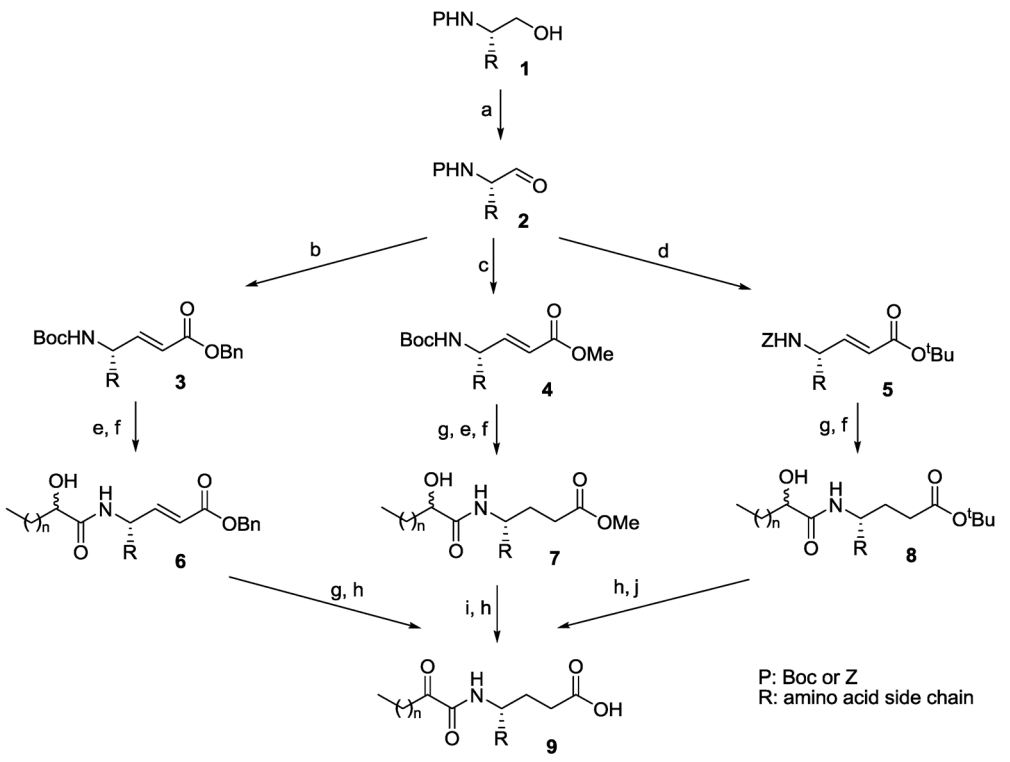
Synthesis of 2-oxoamide inhibitors based on natural and non-natural amino acids

According to the initial design, first-generation 2-oxoamides inhibitors synthesized by the Kokotos group, were based on γ-amino acid residues, such as γ-aminobutyric acid and γ-norleucine [47–50]. Therefore, three routes were developed for their synthesis, depending on the *C*-protecting group of the ylide and the *N*-protecting group used (Scheme 1). Wittig reactions of Boc-protected amino aldehydes **2** with benzyl or methyl (triphenylphosphoranylidene)acetate led to α,β-unsaturated esters **3** and **4**. The Boc group was removed, and the amino component was coupled with 2-hydroxyalkanoic acid. Catalytic hydrogenation and saponification, followed by oxidation led to the target compounds **9**. The Z-protected amino aldehydes **2** were treated with *tert*-butyl (triphenylphosphoranylidene)-acetate, to give compounds **5**. After catalytic hydrogenation, the amino component was coupled with 2-hydroxyalkanoic acid. Oxidation of the latter, followed by the removal of the *tert*-butyl group, led to the desired products **9**.

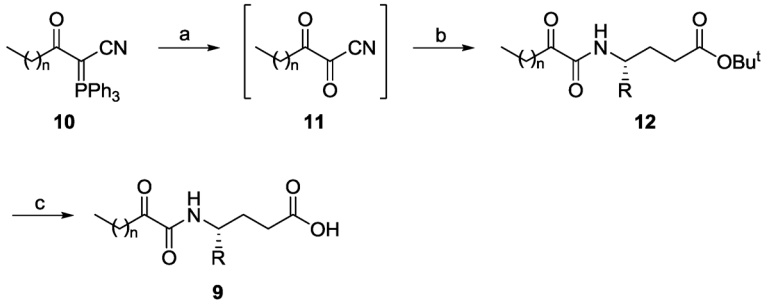
Another synthetic approach for the preparation of the 2-oxoamide functionality has been applied via direct coupling reaction between labile α,β-diketo-nitriles **11** and γ-aminobutyric acid *tert*-butyl ester derivatives, as depicted in Scheme 1 [51].

To obtain β- and δ-norleucine-based inhibitors **16** and **19**, the oxidative conversion of a phenyl group to a carboxylic acid was the key reaction, as shown in Scheme 2. Amino components may be prepared via a Wittig-type olefination reaction of a Boc-protected aminoaldehyde with the suitable ylide [52,53]. Alcohols **13** and **1** were used as starting materials.

For the synthesis of the ε-norleucine-based inhibitors **22**, the Boc-protected norleucinol **1** was oxidized to the aldehyde and directly reacted with triethyl phosphonocrotonate (Scheme 3). Catalytic hydrogenation of **20**, removal of the Boc group, and coupling with 2-hydroxy acids produced the corresponding 2-hydroxyamides **21**. Saponification, followed by oxidation, led to the desired inhibitors **22** [50,52]. To obtain α- through δ-amino acid-based 2-oxoamide derivatives without any side chain, 2-hydroxy acids were coupled with glycine, β-alanine, γ-aminobutyric, and δ-aminovaleric acid esters [48–50]. In all the above-mentioned cases, to obtain the corresponding ester derivatives, oxidation of the 2-hydroxy-amide moiety took place without deprotection of the carboxyl group.

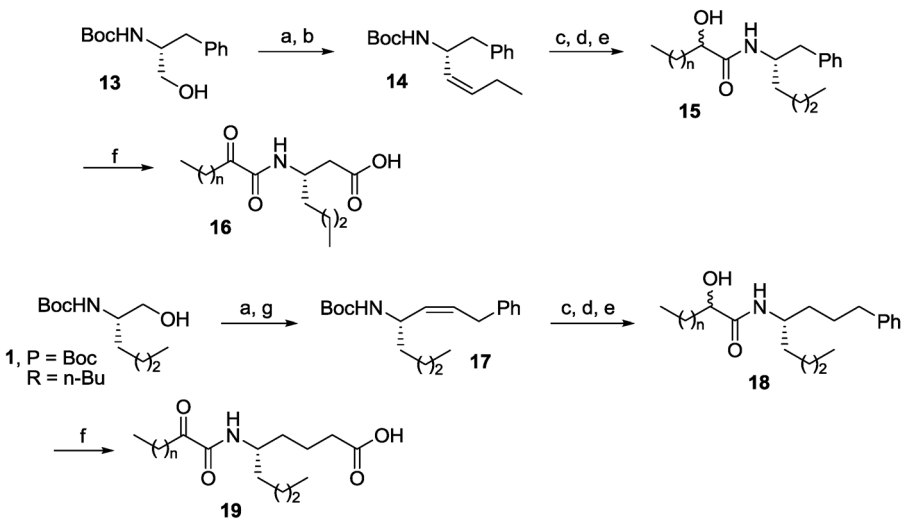


Reagents and conditions: (a) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, -5 °C; (b) Ph_3P=CHCOOBn, THF, reflux; (c) Ph_3P=CHCOOMe, THF, reflux; (d) Ph_3P=CHCOOBu^t, THF, reflux; (e) 4N HCl/THF; (f) $CH_3(CH_2)_nCHOHCOOH$, WSCI, HOBt, Et_3N, CH_2Cl_2; (g) H_2, 10 % Pd/C; (h) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, 0 °C; (i) 1N NaOH, dioxane/H_2O 9:1; (j) 50 % CF_3COOH, CH_2Cl_2.



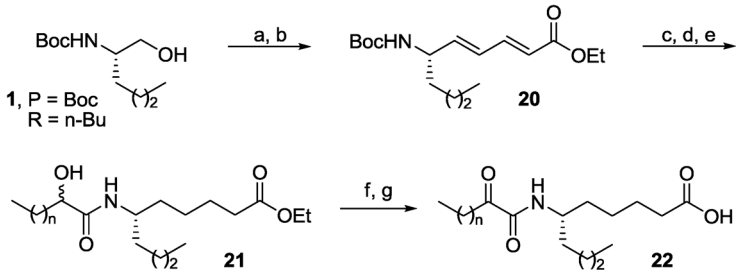
Reagents and conditions: (a) O_3-bubbling, CH_2Cl_2, -78 °C, then Ar-purging, -78 °C; (b) $H_2NCH(R)(CH_2)_2COOBu^t$, CH_2Cl_2, -78 °C; (c) 50 % CF_3COOH, CH_2Cl_2.

Scheme 1 Routes for the synthesis of γ-amino acid-based inhibitors.



Reagents and conditions: (a) NaOCl, TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, -5 °C; (b) $Ph_3P^+(CH_2)_2CH_3Br^-$, KHMDS, -15 °C, $PhCH_3$; (c) H_2, 10 % Pd/C; (d) 4N HCl/Et_2O; (e) $CH_3(CH_2)_nCHOHCOOH$, WSCI, HOBt, Et_3N, CH_2Cl_2; (f) $NaIO_4$, $RuCl_3$, EtOAc/MeCN/H_2O 1:1:8; (g) $Ph_3P^+(CH_2)_2PhBr^-$, KHMDS, -15 °C, $PhCH_3$.

Scheme 2 Routes for the synthesis of β- and δ-norleucine-based inhibitors.



Reagents and conditions: (a) NaOCl, TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, -5 °C; (b) $(EtO)_2P(=O)CH_2CH=CHCOOEt$, LiOH, $PhCH_3$, r.t.; (c) H_2, 10 % Pd/C, EtOH; (d) 4N HCl/THF; (e) $CH_3(CH_2)_nCHOHCOOH$, WSCI, HOBt, Et_3N, CH_2Cl_2; (f) 1N NaOH, dioxane/H_2O 9:1; (g) NaOCl, TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, 0 °C.

Scheme 3 Synthesis of ε-norleucine-based inhibitors.

Inhibitory activity of amino acid-based 2-oxoamides toward GIVA cPLA$_2$

Among derivatives based on a γ-amino acid residue, three potent inhibitors of GIVA $cPLA_2$ were recognized, bearing a free carboxyl group and a long aliphatic alkyl chain, namely, AX006, AX007, and AX012 (Fig. 3), based on γ-aminobutyric acid, γ-norleucine, and γ-leucine [48], with $X_I(50)$ values of 0.017, 0.009, and 0.017, respectively. The role of the free carboxy group was shown to be very important. The need for the negative charge by testing a variant of AX006 that contained a carboxymethyl ester (AX010) in place of the free carboxy group was further confirmed. AX010 had no measurable inhibition up to 0.08 mol fraction, whereas AX006 almost completely inhibited GIVA $cPLA_2$ at this concentration. Moreover, AX006, AX007, and AX012 were tested on the murine P388D1 macrophage-like cell line for inhibition of the GIVA $cPLA_2$ in the long-term lipopolysaccharide (LPS) stimulation pathway. GIVA $cPLA_2$ is the central enzyme for the long-term LPS pathway through its ability to pro-

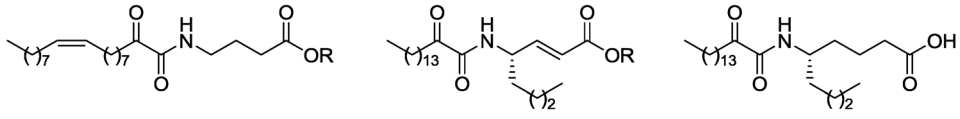
AX006, n = 13, R = H
AX007, n = 9, R = n-Bu
AX012, n = 12, R = i-Bu

AX010, R^1 = H, R^2 = Me
AX048, R^1 = H, R^2 = Et

Fig. 3

duce AA, which subsequently can be converted to prostaglandin E_2 (PGE_2). AA can also be used to upregulate the message and protein levels of GV PLA_2, which in turn produces its own AA that goes on to form PGE_2. Both AX006 and AX007 were able to completely block PGE_2 release with the addition of 50 μM inhibitor, and neither of them showed cytotoxicity at this dose. The IC_{50} values for the inhibition of PGE_2 release were 7.8 μM for AX006 and 5.8 μM for AX007 [48]. To evaluate the anti-inflammatory activity of 2-oxoamide inhibitors, the rat paw carrageenan-induced edema assay was employed as a model for acute inflammation. Indomethacin was used in these experiments as a reference drug and gave 47 % inhibition of inflammation at a dose of 0.01 mmol/kg. AX006 and AX012 exhibited in vivo anti-inflammatory activity comparable to that of indomethacin, whereas AX007 exhibited a weaker effect [48].

To further elucidate the structure–activity relationships, additional 2-oxoamide derivatives based on α- through ε-amino acids, were synthesized and tested for their ability to inhibit GIVA $cPLA_2$ (Fig. 4). Compounds AX040 and AX074, based on γ-aminobutyric acid and α,β-unsaturated γ-norleucine, bearing an oleyl-like and a tetradecanyl chain, strongly inhibited GIVA $cPLA_2$, with $X_I(50)$ values of 0.011 and 0.003 mol fraction. The latter is the most potent in vitro 2-oxoamide inhibitor of GIVA $cPLA_2$ reported to date [49]. A δ-amino acid derivative, (*S*)-5-(2-oxohexadecanamido)nonanoic acid (AX109, Fig. 4) proved also to be a very potent GIVA $cPLA_2$ inhibitor, with a $X_I(50)$ value of 0.005, presenting anti-inflammatory activity at very low doses [52].



AX040, R = H
AX041, R = Me

AX074, R = H
AX073, R = Me

AX109

Fig. 4

2-Oxoamide AX059, based on γ-leucine, is also a potent and selective inhibitor of GIVA $cPLA_2$ with a $X_I(50)$ value of 0.008 mol fraction (Fig. 5). Mice treated with AX059 showed a significant reduction in the severity of the early course of the experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis [54]. Moreover, it prevented the hydrolysis of all fatty acids from phospholipids that were increased in EAE, and the production of PGE_2, thromboxane B2 (TXB2), and other proinflammatory agents [54]. In contrast to neuroinflammatory conditions, such as EAE, mice treated with AX059 showed greater locomotor deficits and tissue loss after spinal cord injury (SCI) [55]. It was found that GIVA $cPLA_2$ was expressed in neurons and oligodendrocytes in the spinal cord after injury, and $cPLA_2$-null mice and wild-type mice treated with AX059 displayed greater neuronal and myelin loss after injury, suggesting that inhibition or deletion of $cPLA_2$ make these cells more vulnerable. Thus, contrary to other models of CNS disorders in which $cPLA_2$ seems to contribute to tissue damage [23,56–58], our very recent data indicated a protective role for $cPLA_2$ after SCI [55].

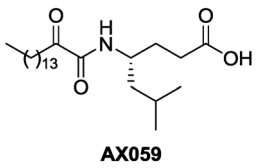


Fig. 5

The ethyl ester variant of compound AX006, AX048 (Fig. 3), inhibits both GIVA cPLA$_2$ and GVIA iPLA$_2$ to almost the same extent [59]. Very interestingly, it exerted a significant effect upon centrally and peripherally initiated hyperalgesia in rats, induced by spinally delivered substance P and carrageenan (Fig. 6). The selective in vitro GIVA cPLA$_2$ inhibitor AX006 did not seem to get access to the intracellular PLA$_2$. This fact maybe indicates that this molecule's properties allow penetration of cellular membranes and it maybe acts as a prodrug of AX006 [59].

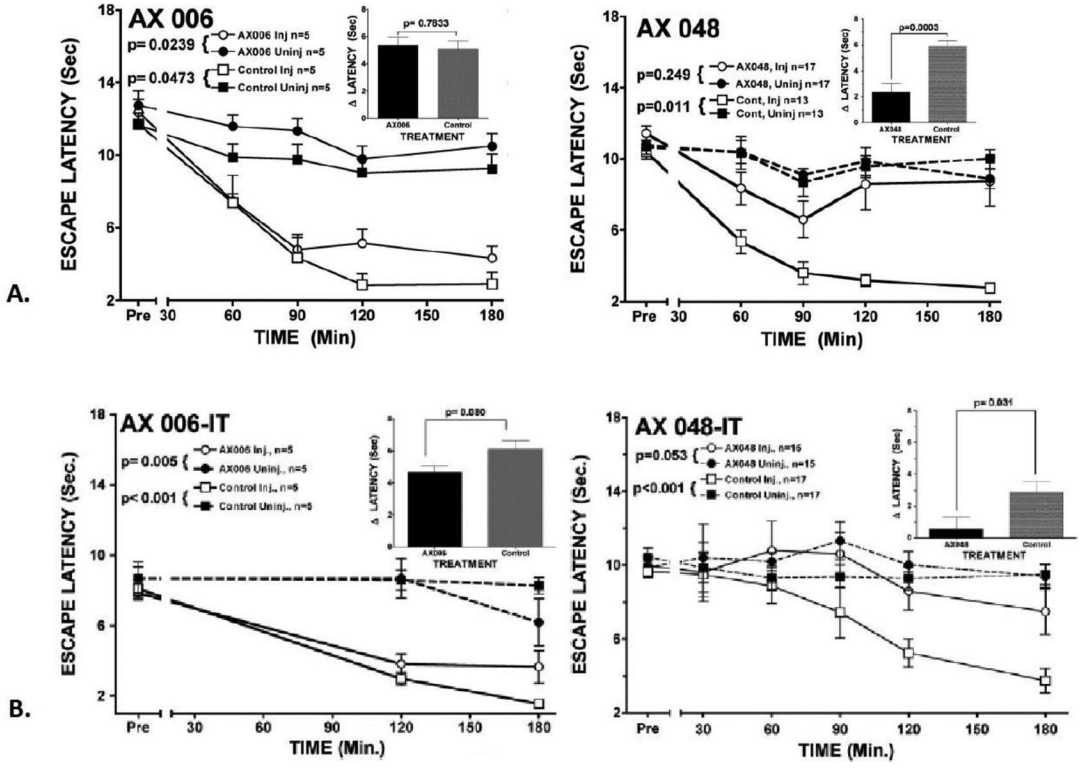


Fig. 6 (A) Effects of AX006 and AX048 (3 mg/kg i.p.) on thermal hyperalgesia evoked by unilateral hind paw injection of carrageenan; (B) Effects of AX006 and AX048 (i.t. 30 μg/10 μl) on thermal hyperalgesia evoked by unilateral hind paw injection of carrageenan [59].

Design and synthesis of 2-oxoamide inhibitors based on dipeptides/pseudodipeptides

To extend our studies on the inhibition of PLA$_2$s by 2-oxoamides, a variety of 2-oxoamides based on dipeptides and pseudodipeptides were synthesized and their in vitro activity on three human PLA$_2$ classes: GIVA cPLA$_2$, GVIA iPLA$_2$, and GV sPLA$_2$ were studied [60]. Dipeptides are considered to be δ-amino acid analogs, because of the distance between the *N*- and *C*-terminal groups. Ether pseudo-

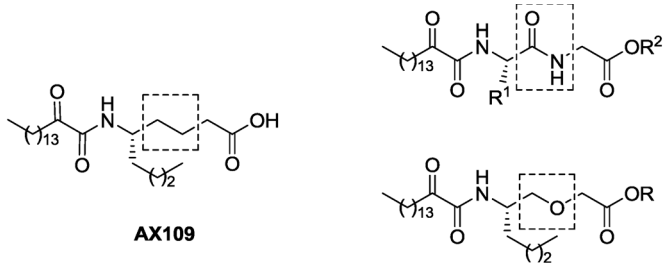
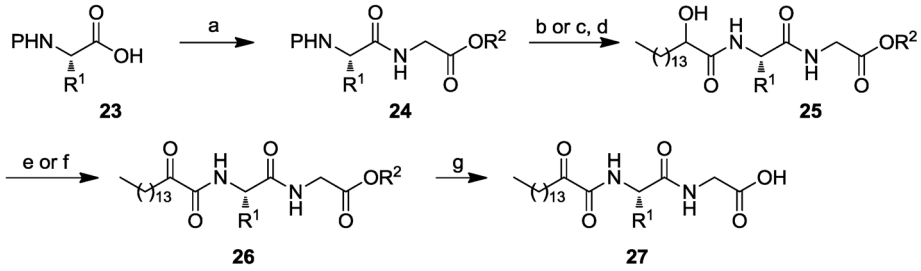


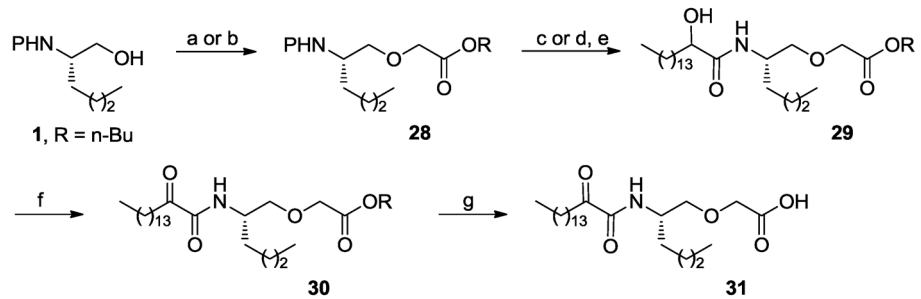
Fig. 7

peptide derivatives may be also considered δ-amino acid derivatives. Thus, L-norleucine and L-valine were chosen as starting materials to synthesize a series of derivatives that contain the 2-oxoamide functionality and an amide group or ether group to replace the two methylenes of the δ-amino acid derivative (Fig. 7).

For the synthesis of 2-oxoamide derivatives based on dipeptides, *N*-protected amino acids **23** were coupled with methyl, ethyl or *tert*-butyl glycinate (Scheme 4). Removal of the protecting group, followed by coupling with 2-hydroxyhexadecanoic acid yielded the 2-hydroxyamides **25**. Oxidation of the latter led to the target compounds **26**. Following similar reactions, *tert*-butyl ester derivatives were prepared as depicted in Scheme 4, starting from *Z*-protected amino acids. For the synthesis of pseudodipeptide derivatives, the key reaction step was the treatment of *N*-protected norleucinol with ethyl or



Reagents and conditions: (a) HCl.H-Gly-OR^2, Et_3N, WSCl, HOBt, CH_2Cl_2; (b) 4N HCl/Et_2O; (c) H_2, 10 % Pd/C, EtOH; (d) $CH_3(CH_2)_{13}CHOHCOOH$, Et_3N, WSCl, HOBt, CH_2Cl_2; (e) Dess-Martin reagent, CH_2Cl_2; (f) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, 0 °C; (g) 50 % TFA/CH_2Cl_2.



Reagents and conditions: (a) $BrCH_2COOEt$, NaH, 18-crown-6, THF; (b) $BrCH_2COOBu^t$, Bu_4NHSO_4, 50 % NaOH, C_6H_6; (c) 4N HCl/Et_2O; (d) H_2, 10 % Pd/C, EtOH; (e) $CH_3(CH_2)_{13}CHOHCOOH$, Et_3N, WSCl, HOBt, CH_2Cl_2; (f) Dess-Martin reagent, CH_2Cl_2; (g) 50 % TFA/CH_2Cl_2.

Scheme 4 Routes for the synthesis of dipeptide- and pseudodipeptide-based inhibitors.

tert-butyl bromoacetate to give ether derivatives **28** (Scheme 4). The corresponding free carboxylic acid compounds **27** and **31** were obtained after removal of the *tert*-butyl ester group of **26** and **30** [60].

Inhibition of cPLA$_2$ by dipeptide- and pseudodipeptide-based 2-oxoamides

2-Oxoamide derivatives based on dipeptides Nle-Gly and Val-Gly (**27**, Scheme 4), bearing a free carboxylic acid, were moderate, though selective inhibitors of GIVA cPLA$_2$. The bioisosteric replacement of the Nle-Gly derivative amide bond with an ether moiety (**31**, Scheme 4), led to significantly higher inhibition of [X_I(50) 0.017]. Very interestingly, 2-oxoamides ester variants **26** based on Nle-Gly-OMe and Val-Gly-OBu^t, showed a higher inhibition potency in comparison with the corresponding free acid derivatives. However, both inhibited GVIA iPLA$_2$ as well. Moreover, selective GIVA cPLA$_2$ inhibitors blocked AA release in RAW 264.7 macrophages. Particularly, ether pseudodipeptide derivative **31** inhibited AA release with an IC$_{50}$ value of 2 μM, whereas under the same conditions, the most potent in vitro inhibitors of GIVA cPLA$_2$ AX074, and AX109 had IC$_{50}$ values of 25 and 7 μM, respectively [60].

2-Oxoamide inhibitors containing carboxylate bioisosteric groups

A variety of lipophilic 2-oxoamides containing sulfonamide groups bioisosteric to the carboxyl group, as analogs of γ- and δ-amino acids, as well as acyl sulfonamides of γ-aminobutyric acid were synthesized (Fig. 8), and their activity on three human PLA$_2$ classes was evaluated [61]. Three of them inhibited GIVA cPLA$_2$ at a level similar to that of the lead compounds AX006 and AX048. Thus, the bioisosteric sulfonamide group seems a group suitable to replace the carboxyl group in lipophilic 2-oxoamide inhibitors of GIVA cPLA$_2$. Other bioisosteric analogs synthesized and tested were based on the replacement of the carboxyl or the carboxyl ester group of AX006 and AX048 by a tetrazole or phosphonate group (Fig. 8) [62]. All compounds of this category proved to be weak inhibitors of GIVA cPLA$_2$.

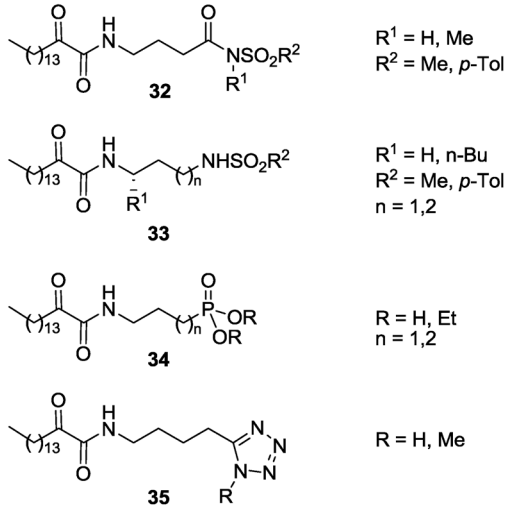


Fig. 8

2-Oxoamide inhibitors of Ca^{2+}-independent phospholipase A$_2$

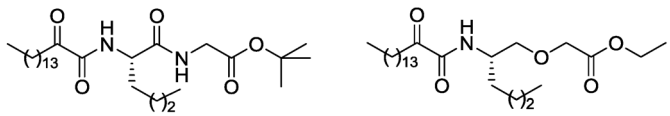
To understand the selectivity of 2-oxoamides toward each type of PLA$_2$s, all 2-oxoamide derivatives were tested for their ability to inhibit the three classes of PLA$_2$. As mentioned above, GVIA iPLA$_2$ shares the same catalytic mechanism with GIVA cPLA$_2$. Thus, it is very likely that inhibitors of GIVA cPLA$_2$ may show cross-reactivity with GVIA iPLA$_2$.

Natural and non-natural amino acid-based 2-oxoamide inhibitors

The first observation that occurred from inhibition studies was that derivatives with a free carboxylic acid functionality do not inhibit GVIA iPLA$_2$. On the other hand, corresponding esters may inhibit both GIVA cPLA$_2$ and GVIA iPLA$_2$. More specifically, uncharged variants of AX040 and AX074, namely, AX041 and AX073 (Fig. 4), showed high activity against both cytosolic enzymes, with preference for GIVA cPLA$_2$ [49]. As previously noted, 2-oxoamide AX048, based on ethyl γ-aminobutyrate, inhibited both GIVA cPLA$_2$ and GVIA iPLA$_2$ and exhibited very interesting antihyperalgesic activity [59]. Other 2-oxoamide esters with varying distance between the 2-oxoamide functionality and the ester group (one to five carbon atoms) and varying amino acid side chain, presented moderate inhibition of GVIA iPLA$_2$ at 0.091 mol fraction, and none of them was selective for this enzyme [50].

Dipeptide- and pseudodipeptide-based 2-oxoamide inhibitors

As mentioned above, 2-oxoamides based on dipeptides and ether pseudodipeptides were tested also for their ability to inhibit GVIA iPLA$_2$. Two interesting inhibitors were identified, based on Nle-Gly *tert*-butyl ester **26**, and the ether pseudodipeptide ethyl ester **30** (Fig. 9). Up to now, these are the most effective in vitro 2-oxoamides that preferentially inhibit GVIA iPLA$_2$ [60].

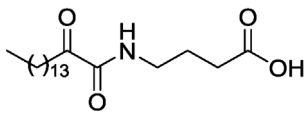
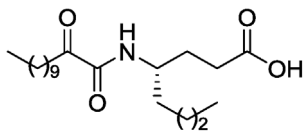
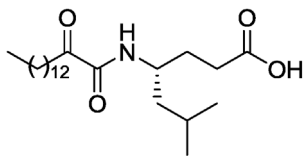


26, R^1 = n-Bu, R^2 = Bu^t **30**, R = Et

Fig. 9

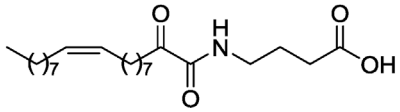
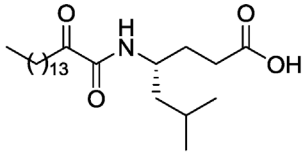
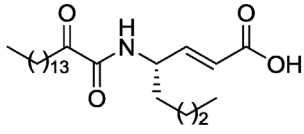
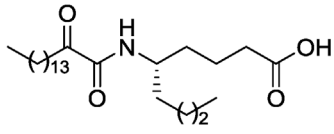
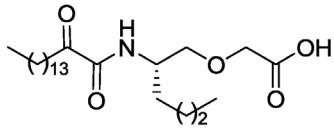
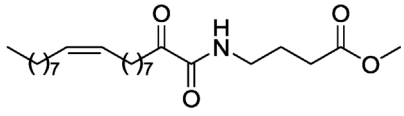
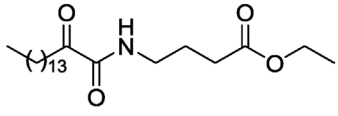
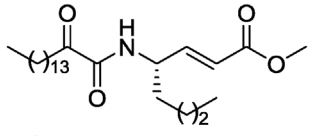
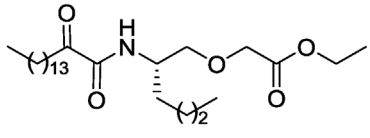
The results of GIVA cPLA$_2$ and GVIA iPLA$_2$ inhibition caused by some 2-oxoamides are summarized in Table 1. Initially, the percent inhibition of each PLA$_2$ at 0.091 mol fraction of inhibitor was determined. $X_I(50)$ values were estimated when the percent inhibition was higher than 90 %. The $X_I(50)$ is the mol fraction of the inhibitor in the total substrate interface required to inhibit the enzyme by 50 %.

Table 1 Differential inhibition of groups IVA and VIA phospholipases A$_2$ by 2-oxoamides.

Number	Structure	$X_I(50)$	
		Inhibition of GIVA cPLA$_2$	Inhibition of GVIA iPLA$_2$
AX006		0.017 ± 0.009 [48]	ND[a]
AX007		0.009 ± 0.004 [48]	ND
AX012		0.017 ± 0.006 [48]	ND

(*continues on next page*)

Table 1 (*Continued*).

Number	Structure	$X_I(50)$	
		Inhibition of GIVA $cPLA_2$	Inhibition of GVIA $iPLA_2$
AX040		0.011 ± 0.003 [49]	ND
AX059		0.008 ± 0.002 [54]	ND
AX074		0.003 ± 0.001 [49]	ND
AX109		0.005 ± 0.002 [52]	ND
31		0.017 ± 0.002 [60]	ND
AX041		0.012 ± 0.014 [49]	0.067 ± 0.003 [49]
AX048		0.022 ± 0.009 [59]	0.027 ± 0.009 [59]
AX073		0.018 ± 0.010 [49]	0.032 ± 0.010 [49]
30		52 % (0.091) [60]	0.017 ± 0.002 [60]

[a]N.D. signifies compounds with less than 25 % inhibition (or no detectable inhibition) at 0.091 mol fraction of inhibitor.

2-Oxoamide inhibitors of secreted phospholipase A_2

Although there is no serine nucleophile in $sPLA_2$s, 2-oxoamides may resemble the substrate phospholipids or the transition state in such way that they would bind to the $sPLA_2$s active site and inhibit the enzyme.

Amino acid-based 2-oxoamide inhibitors

Numerous 2-oxoamides based on α- through ε-amino acids with varying side chains were tested for their ability to cause inhibition of GV $sPLA_2$. In general, derivatives with a free carboxylic acid group, that are potent inhibitors of GIVA $cPLA_2$, poorly inhibit GV $sPLA_2$, or cause no inhibition at all [52,60]. The glutamic acid derivative **36** (Fig. 10) inhibited all three PLA_2 enzymes with no statistical preference for GV $sPLA_2$ [X_I(50) 0.035] or GIVA $cPLA_2$ [X_I(50) 0.020] [52].

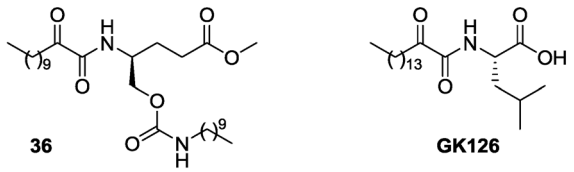


Fig. 10

Most recently, 2-oxoamide derivatives based on α-amino acids have been synthesized and tested for their in vitro inhibitory activity against three human $sPLA_2$s (GIIA, GV, and GX) [63]. Compound GK126 (Fig. 10), which is based on (*S*)-leucine, displayed inhibition of human and mouse GIIA $sPLA_2$ (IC_{50} 300 and 180 nM, respectively). It also inhibited the human GV $sPLA_2$ with similar potency, while it did not display any measurable inhibition of GX $sPLA_2$.

STUDIES ON THE MODE OF BINDING OF 2-OXOAMIDES WITH PLA_2s

The identification of new PLA_2 inhibitors can be assisted by the so-called computer-aided drug design (CADD). Through several approaches, including ligand-based design, structure-based design, and quantitative structure–activity relationships, researches apply computational techniques in an effort to streamline the discovery, design, development, and optimization of inhibitors against a target receptor. The application of rational design on the PLA_2 enzymes area and specifically the contribution of CADD in the identification of new PLA_2 inhibitors has been recently reviewed [64].

Binding of 2-oxoamides with GIVA $cPLA_2$

A detailed analysis of the binding mode of two potent, but structurally different GIVA $cPLA_2$ inhibitors, namely, pyrrophenone and the 2-oxoamide AX007, has been conducted using for the first time a combination of deuterium exchange mass spectrometry (DXMS) and molecular dynamics (MD) simulations [65]. This study allowed key residues contacting both inhibitors to be modeled. It was found that the carboxylic acid moiety of AX007 interacted with Arg200, as postulated in the initial design, and the carbonyl group of the 2-oxoamide functionality was in contact with the oxyanion hole composed of the residues Gly197 and Gly198 (Fig. 11). It was also observed that the 2-oxoamide mainly occupied the active-site area, while the inhibitor pyrrophenone was bound in the cap region near the interfacial surface of the enzyme. This methodology certainly provides a new exciting tool to develop inhibitors with improved properties.

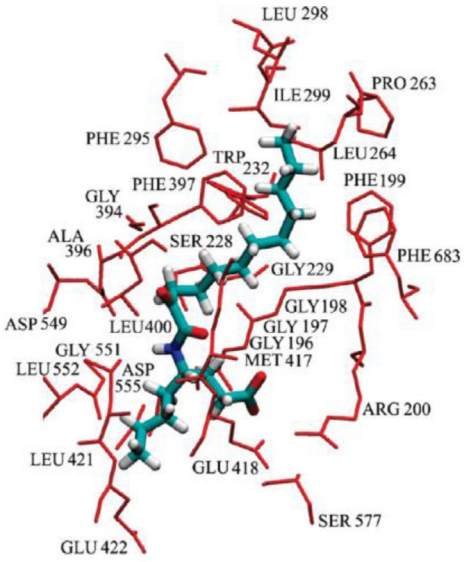


Fig. 11 Location of inhibitor AX007 bound to $cPLA_2$ determined by DEMS and MD [65].

Binding of 2-oxoamides with GIIA $sPLA_2$

Assisted by molecular docking studies, it was possible to understand the structural characteristics that contribute to the inhibitory activity of the 2-oxoamide GK126 against GIIA $sPLA_2$ [63]. The 2-oxoamide group is essential for the binding because it participates in hydrogen bonds with Gly29 and interacts with the calcium ion. The carboxylate group is also essential for binding because it interacts with the calcium ion and participates in a hydrogen bond with Lys62 through a water molecule placed near the hydrophilic region of the active site. The (*S*)-leucine side chain contributes to the tight binding of GK126 by interacting with the side chain of the active-site Leu2 (Fig. 12).

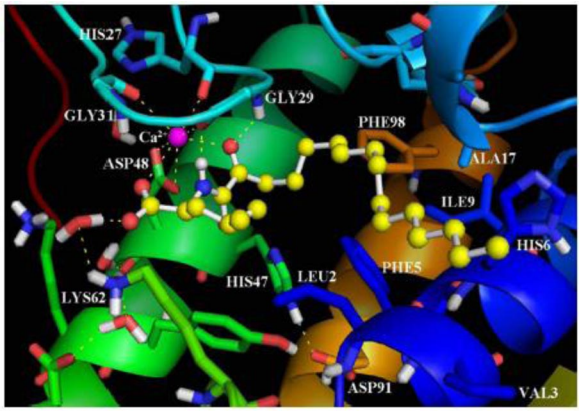


Fig. 12 The binding mode of the inhibitor GK126 in the GIIA $sPLA_2$ active site as calculated using GOLD [63].

CONCLUSIONS

PLA_2 has been widely recognized as a target for the development of novel anti-inflammatory medicinal agents. Although the area of research on PLA_2 inhibitors has been very active during the last decade, no inhibitor has reached the market. 2-Oxoamides constitute one of the promising classes of PLA_2 inhibitors. Lipophilic 2-oxoamides based on (*S*)-γ- or δ-amino acids are potent and selective inhibitors of GIVA $cPLA_2$, which present interesting in vivo anti-inflammatory activity. However, 2-oxoamides based on natural α-amino acids are selective inhibitors of GIIA $sPLA_2$ (Fig. 13).

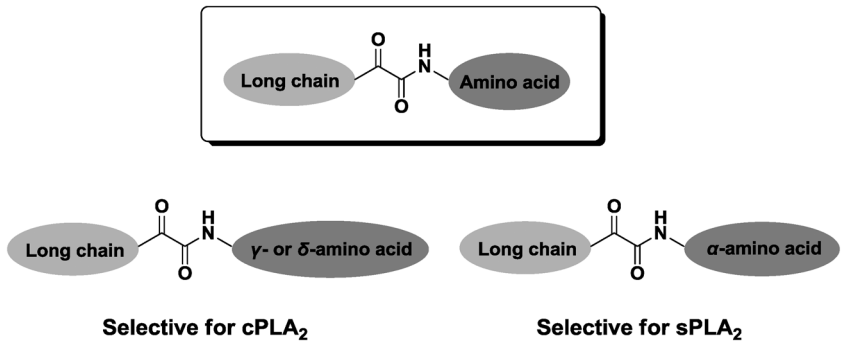


Fig. 13

The application of various techniques unraveled the mode of binding of 2-oxoamides with the cytosolic GIVA $cPLA_2$ and the secreted GIIA $sPLA_2$. Further modifications on the 2-oxoamide structure are expected to lead to new improved inhibitors with therapeutic potential.

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