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Differential Inhibition of Group IVA and Group VIA Phospholipases A_2 by 2-Oxoamides

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Abstract

Inhibitors of the Group IVA phospholipase A_2 (GIVA cPLA$_2$) and GVIA iPLA$_2$ are useful tools for defining the roles of these enzymes in cellular signaling and inflammation. We have developed inhibitors of GVIA iPLA$_2$ based on the 2-oxoamide backbone that are uncharged, containing ester groups. While the most potent inhibitors of GVIA iPLA$_2$ also inhibited GIVA cPLA$_2$, there were three 2-oxoamide compounds that selectively and weakly inhibited GVIA iPLA$_2$. We further show that several potent 2-oxoamide inhibitors of GIVA cPLA$_2$, containing free carboxylic groups (Kokotos *et al. J. Med. Chem*. **2002**, *45*, 2891–2893), do not inhibit GVIA iPLA$_2$, and are therefore selective GIVA cPLA$_2$ inhibitors.

Introduction

Phospholipase A_2 (PLA$_2$) constitutes a superfamily of enzymes that catalyze the hydrolysis of the fatty acid ester from the *sn*-2 position of a membrane phospholipid, yielding a free fatty acid and a lysophospholipid. Among the intracellular PLA$_2$s are the cytosolic Group IVA PLA$_2$ (GIVA cPLA$_2$), which is generally considered a pro-inflammatory enzyme, and the calcium-independent Group VIA iPLA$_2$ (GVIA iPLA$_2$), which is typically referred to in the literature as iPLA$_2$. GVIA iPLA$_2$ is actually a group of cytosolic enzymes ranging from 85 to 88 kDa and expressed as several distinct splice variants of the same gene, only two of which have been shown to be catalytically active (Group VIA-1 and VIA-2 iPLA$_2$).1 The role of GVIA iPLA$_2$ in the inflammatory process is unclear, but this enzyme appears to be the primary PLA$_2$ for basal metabolic functions within the cell, reportedly including membrane homeostasis,[2–7] insulin receptor signaling[5,8] and calcium channel regulation.[9–11]

The GVIA iPLA$_2$ enzymes all contain a consensus lipase motif, Gly-Thr-Ser*-Thr-Gly, with the catalytic serine confirmed by site-directed mutagenesis.[1,12] More recently the homologous Group VIB iPLA$_2$ was confirmed to have an active site catalytic dyad consisting of the

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conserved Ser and an equally conserved Asp.[13] The first identification of the novel catalytic Ser/Asp dyad was for GIVA cPLA$_2$ based on exhaustive mutagenesis and a crystal structure, which confirmed that the catalytic dyad is present in a non-canonical α/β hydrolase and that the mechanism involves an acyl-enzyme intermediate on the serine.[14–19] A similar structure, topology, and conserved catalytic dyad were also found in patatin, a distant plant homolog of both GIV and GVI PLA$_2$.20 The growing family of lipid hydrolases ulilizing a catalytic Ser-Asp dyad now includes bacterial ExoU, fungal phospholipase B/Spo1, plant patatins, and the many mammalian enzymes in the GIV PLA$_2$, GVI PLA$_2$, and neuropathy target esterase groupings.[21]

Arachidonyl trifluoromethyl ketone (ATFK) has been shown to function as a tight binding, reversible inhibitor of both GIVA and GVIA PLA$_2$,22,23 while methyl arachidonyl fluorophosphonate (MAFP) functions as an irreversible inhibitor of both enzymes.[24] Variants of the trifluoromethyl ketones show differential potencies for GIVA and GVIA PLA$_2$; oleic acid- and phenyl-containing compounds are more potent than ATFK with GVIA iPLA$_2$ and less potent than ATFK with GIVA cPLA$_2$.25 Similar trends in potency are seen with the fluorophosphonate inhibitors; oleic acid and phenyl derivatives are more potent than MAFP towards GVIA iPLA$_2$.25 Interestingly, the trifluoromethylketone and fluorophosphonate inhibitors all show fast binding to GVIA iPLA$_2$ and slow binding to GIVA cPLA$_2$,22,25,26 suggesting subtle differences in the active sites of GIVA and GVIA PLA$_2$. Bromoenol lactone (BEL) is an irreversible, covalent inhibitor of GVIA iPLA$_2$ but does not inhibit GIVA cPLA$_2$. Because of this, BEL is commonly used to selectively inhibit GVIA iPLA$_2$ in cellular systems.[3,5,7,9,22] However, it has been shown that in addition to inhibiting GVIA iPLA$_2$, BEL inhibits numerous cellular enzymes including the magnesium-dependent phosphatidate phosphohydrolase 1.[27]

We have recently reported that 2-oxoamides containing a free carboxyl group are potent inhibitors of human GIVA cPLA$_2$.28,29 The aim of the present work was to develop inhibitors based on the 2-oxoamide backbone that are selective for GIVA or GVIA PLA$_2$. Based upon the similarity of substrates, classes of common inhibitors, and the homologous Ser-Asp catalytic dyad, it is very likely that the active sites of GIVA and GVIA PLA$_2$ are similar such that inhibitors of GIVA cPLA$_2$ may show cross-reactivity with GVIA iPLA$_2$. There are, however, significant differences in substrate preference, known inhibitor profiles, and the primary sequence between GIVA and GVIA PLA$_2$ that could be exploited in designing selective inhibitors.

Design and Synthesis of 2-Oxoamide Inhibitors

We have developed a strategy for the design of inhibitors of serine-containing lipolytic enzymes, which is based on the principle that the inhibitor should consist of two components: (a) an electrophilic group that is able to react with the active-site serine residue, and (b) a lipophilic segment that contains chemical motifs necessary for both specific interactions and a proper orientation in the substrate binding cleft of the enzyme.[30] This strategy has been successfully applied in the development of lipophilic 2-oxoamides,[31,32] 2-oxoamide and bis-2-oxoamide triacylglycerol analogues,[33,34] as well as lipophilic aldehydes[35] and trifluoromethyl ketones[36] as effective inhibitors of pancreatic and gastric lipases. Accordingly, we have recently developed a novel class of 2-oxoamides that inhibit GIVA cPLA$_2$.28,29 The noted homology of GVIA iPLA$_2$ to GVIB PLA$_2$, patatin and GIVA cPLA$_2$ (lipases known to possess a catalytic Ser-Asp dyad) and the confirmation of its catalytic serine strongly suggest that GVIA iPLA$_2$ would be susceptible to inhibition by 2-oxoamides.[12] Thus, we studied a number of 2-oxoamides of the generic structure shown in Scheme 1 in an effort to understand the effect of R^1 and R^2 groups on GVIA iPLA$_2$ inhibition.

2-Oxoamide inhibitors containing either a free carboxyl group or a carboxymethyl ester group and 2-oxoacyl residues based on oleic acid or phenyl groups were synthesized using methods previously developed,[29] as depicted in Scheme 2. In Scheme 3, the synthesis of inhibitors based on a γ-amino-α,β-unsaturated acid is shown. It should be noted that the oxidation of the unsaturated 2-hydroxyamides **2c**, **6** and **7** was carried out using Dess-Martin periodinane,[37] instead of NaOCl/TEMPO, to avoid oxidation of the double bonds.

Selective Inhibition of GIVA and GVIA PLA_2 by 2-Oxoamide Inhibitors

Fourteen 2-oxoamides were tested for inhibition of GVIA $iPLA_2$ in our *in vitro* assay system[27,28] and compared with GIVA $cPLA_2$ inhibition. The data, summarized in Table 1, are represented as $X_I(50)$ values. $X_I(50)$ is defined as the inhibitor concentration in a two-dimensional micellar surface that produces 50% inhibition. The surface concentration (mole fraction units) is calculated as the moles of inhibitor divided by the total moles of inhibitor, detergent, and phospholipid in the micelle surface. $X_I(50)$ is utilized as opposed to the more common IC_{50} because GIVA and GVIA PLA_2 are active at a two-imensional lipid interface containing the substrate phospholipids rather than in three-dimensional solution with soluble, monomeric substrates.[22,25,38,42] Because the 2-oxoamide inhibitors also partition to the micelle interface, the relevant concentration of inhibitor for membrane-bound enzymes is the surface concentration (mole fraction) not the bulk concentration (molar units).[22,25,28,38,39,42] Of the fourteen compounds listed in Table 1, five show at least partial inhibition of GVIA $iPLA_2$ at the highest concentrations tested.

Among the primary 2-oxoamides **13** (AX001)[29] and **14** (AX015),[29] neither exhibits significant inhibition of GIVA or GVIA PLA_2. The secondary 2-oxoamides, **15** (AX002)[29] and **16** (AX009),[29] with long carbon chains either at the R^1 or at the R^2 position present limited inhibition of GVIA $iPLA_2$, but Four 2-oxoamides containing a substituted phenyl chain at the no detectable inhibition of GIVA $cPLA_2$. R^1 position (**4a,b**, **5a,b**) (AX035-AX038) did not inhibit GVIA $iPLA_2$. This is somewhat unexpected given previous reports of the selectivity of phenyl-containing fluoroketones or fluorophosphonates. None of the phenyl-containing 2-oxoamides inhibits GIVA $cPLA_2$.

The 2-oxoamides containing a free carboxyl group **17** (AX006),[29] **12** (AX040), and **10** (AX074) inhibit GIVA $cPLA_2$ but do not inhibit GVIA $iPLA_2$. In fact, in all cases these compounds enhance GVIA $iPLA_2$ enzymatic activity. The increased GVIA $iPLA_2$ activity may be due to increased negative charge at the micelle surface due to addition of inhibitors with a free carboxyl group. Unlike the inhibitors of GIVA $cPLA_2$, the inhibitors of GVIA $iPLA_2$ **18** (AX010),[29] **4c** (AX041), and **11** (AX073) are uncharged. The effect of charge is highlighted when comparing **17** to **18**, where **18** possesses a carboxymethyl ester in place of the free carboxyl found in **17**. Compound **18** exhibits limited inhibition of GVIA $iPLA_2$ but does not significantly inhibit GIVA $cPLA_2$. Compound **17** does not significantly inhibit GVIA $iPLA_2$ at concentrations up to 0.091 mole fraction but is a potent inhibitor of GIVA $cPLA_2$ with an $X_I(50)$ value of 0.017 mole fraction.28 Compound **4c** is an inhibitor of GVIA $iPLA_2$ with an $X_I(50)$ value of 0.067 mole fraction. Interestingly, it also inhibits GIVA $cPLA_2$ with an $X_I(50)$ value of 0.012 mole fraction. Compound **12**, the charged variant of **4c**, does not inhibit GVIA $iPLA_2$ but is an inhibitor of GIVA $cPLA_2$ with an $X_I(50)$ value of 0.011 mole fraction. Consistent results were seen with compounds **11** and **10**. These compounds are also variants that contain either a carboxymethyl ester (**11**) or a free carboxyl (**10**). Compound **10** is the most potent 2-oxoamide inhibitor of GIVA $cPLA_2$ reported to date with an $X_I(50)$ of 0.003 mole fraction. By observing the trend of inhibition of GVIA $iPLA_2$ by **18**, **4c**, and **11**, it appears that an unsaturated chain at R^1 or R^2 is preferable to a saturated one. This is consistent with the presence of unsaturated fatty acids at the *sn*-2 position of many phospholipids. The inhibition dose-response curve for **18** appears to plateau at the higher mole fractions tested. The *in*

vitro assay contains detergent and phospholipid that should readily form mixed micelles with **18**, which has a similar hydrophobicity (ClogP) to many other compounds that behave normally. Most other lower potency 2-oxoamide inhibitors possess a linear dose-response. Compound **18** is unique as a lower potency inhibitor with a logarithmic dose-response.

A known reference inhibitor (non-covalent and readily reversible) for GIVA cPLA$_2$ is not commercially available, but a patented inhibitor of GIVA cPLA$_2$, pyrrophenone, is described in the literature[40,41]. Comprehensive analysis of pyrrophenone demonstrated that it inhibits GIVA cPLA$_2$ with an X_I(50) of 0.002 mole fraction under a variety of assay conditions.42 This level of potency is similar to the most potent GIVA cPLA$_2$ 2-oxoamide inhibitors, (4*S*)-4-[(2-oxododecanoyl)amino]octanoic acid (AX007)[29] and **10** (this work). Pyrrophenone was reported to have no effect on the activity of GVIA iPLA$_2$.42 A known reference inhibitor (non-covalent and readily reversible) for GVIA iPLA$_2$ is palmitoyl trifuoromethyl ketone (PATK). Previous tests of this compound in our lab have confirmed the X_I(50) of PATK for GVIA iPLA$_2$ is 0.0075 mole fraction.[22] A further study tested an expanded panel of hydrophobic trifluoromethyl ketones and found that most are slow, tight-binding inhibitors of GIVA cPLA$_2$ and fast, reversible inhibitors of GVIA iPLA$_2$, so the inhibition of the two enzymes by these compounds are not readily comparable.[25]

Mechanism of GVIA PLA$_2$ Inhibition by 2-Oxoamide Inhibitors

We tested **18** and **11** to determine if these inhibitors showed either time-dependent or irreversible inhibition of GVIA iPLA$_2$. GVIA iPLA$_2$ (25 ng) was preincubated with either **18** or **11** (5 μM) for 0, 5, 15 or 30 minutes and then assayed in the standard GVIA iPLA$_2$ assay mix containing 5 μM inhibitor. The final concentration of the inhibitors in the assay mix was 0.01 mole fraction, and the samples were incubated for 30 minutes at 40°C. Both **18** and **11** showed no increased potency with prolonged incubation, demonstrating a fast-binding and reversible mode of inhibition (Figure 2A). We next preincubated 25 ng of GVIA iPLA$_2$ with 10 μM **18** or **11** for 10 minutes before diluting the enzyme 1:50 into the standard GVIA iPLA$_2$ assay mix lacking inhibitor, and incubating for 30 minutes at 40 °C. The final inhibitor concentration in these assays was 0.0004 mole fraction, well below surface concentrations that either **18** or **11** inhibit the enzyme. GVIA iPLA$_2$ showed full activity in this system, demonstrating that both **18** and **11** are freely reversible inhibitors (Figure 2B).

Inhibition of PGE$_2$ Production by 2-Oxoamide Inhibitors

We tested several 2-oxoamides in the long-term lipopolysaccharide (LPS) stimulation pathway in the murine RAW 264.7 macrophage-like cell line.[43,44] This pathway requires GIVA cPLA$_2$ activity for maximal extracellular release of many eicosanoid compounds, including the prostaglandin PGE$_2$.45 Compound **18**, which does not significantly inhibit GIVA cPLA$_2$ *in vitro*, also did not inhibit PGE$_2$ release from the RAW cells (data not shown). In the low μM range, **4c** and **11** reduced PGE$_2$ release by roughly 40% (Figure 3). Based on previous work, this is the fraction of PGE$_2$ release attributable to GIVA cPLA$_2$.44,45 At 1 μM and 5 μM concentrations, small activations were often seen, suggesting minor stimulation of the cells from membrane perturbing compounds.

In conclusion, based on the 2-oxoamide backbone structure we have developed inhibitors that selectively inhibit GIVA cPLA$_2$ or inhibit both GIVA and GVIA PLA$_2$. The selective 2-oxoamide inhibitors of GIVA cPLA$_2$ were found to be charged, containing a free carboxyl group. Interestingly some non-charged 2-oxoamides showed dual specificity in inhibiting both GIVA cPLA$_2$ and GVIA iPLA$_2$. Inhibitors selective for GIVA cPLA$_2$ or dual specificity inhibitors reduced PGE$_2$ levels in cellular assays that test for inhibition of GIVA cPLA$_2$. Several 2-oxoamide compounds that significantly inhibit GVIA iPLA$_2$ are promising leads for selective inhibitors of GVIA iPLA$_2$ that would significantly improve investigations into the

role of GVIA iPLA$_2$ in cellular systems. As we have previously demonstrated for 2-oxoamide inhibitors of GIVA cPLA$_2$, the inhibitors of GVIA iPLA$_2$ are also fast-binding and freely reversible. Such selective inhibitors of GIVA and GVIA enzymes will be a significant asset in examining the role of these enzymes in cellular signaling and inflammation.

Experimental Section

Synthesis of 2-Oxoamide Inhibitors

Melting points were determined on a Buchi 530 apparatus and are uncorrected. Specific rotations were measured at 25 °C on a Perkin-Elmer 343 polarimeter using a 10 cm cell. NMR spectra were recorded on a Varian Mercury (200 MHz) spectrometer. Fast atom bombardment (FAB) mass spectra were recorded using a VG analytical ZAB-SE instrument. Electron spray ionization (ESI) mass spectra were recorded on a Finnigan, Surveyor MSQ Plus spectrometer. TLC plates (silica gel 60 F_{254}) and silica gel 60 (70–230 or 230–400 mesh) for column chromatography were purchased from Merck.

Coupling of 2-hydroxy acids with amino components

To a stirred solution of 2-hydroxy acid (2.0 mmol) and hydrochloride amino component (2.0 mmol) in CH_2Cl_2 (20 mL), Et_3N (0.61 mL, 4.4 mmol) and subsequently 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (WSCI) (0.42 g, 2.2 mmol) and 1-hydroxybenzotriazole (HOBt) (0.27 g, 2.0 mmol) were added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature. The solvent was evaporated under reduced pressure and EtOAc (20 mL) was added. The organic layer was washed consecutively with brine, 1N HCl, brine, 5% $NaHCO_3$, and brine, dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column-chromatography using $CHCl_3$ as eluent.

4-(2-Hydroxy-5-phenyl-pentanoylamino)-butyric acid methyl ester (2a)

yield 82%; white solid; m.p. 34–35 °C; ^{1}H NMR: δ 7.24-7.11 (5H, m, C_6H_5), 6.82 (1H, m, NHCO), 4.06 (1H, m, CH), 3.62 (3H, s, CH_3O), 3.53 (1H, d, J = 5.2 Hz, OH), 3.26 (2H, m, CH_2NH), 2.59 (2H, t, J = 7.8 Hz, C$H_2C_6H_5$), 2.30 (2H, t, J = 6.8 Hz, CH_2COO), 1.82-1.70 (6H, m, 3×CH_2); 13C NMR: δ 174.2, 173.8 142.0, 128.3, 128.2, 125.7, 71.7, 51.7, 38.3, 35.5, 34.3, 31.3, 26.8, 24.6; MS (ESI): m/z (%): 316 (100) $[M + Na]^+$. Anal. ($C_{16}H_{23}NO_4$) C, H, N.

4-(2-Hydroxy-6-phenyl-hexanoylamino)-butyric acid methyl ester (2b)

yield 85%; white solid; m.p. 50–51 °C; ^{1}H NMR: δ 7.31-7.15 (5H, m, C_6H_5), 6.76 (1H, m, NHCO), 4.08 (1H, m, CH), 3.68 (3H, s, CH_3O), 3.32 (2H, m, CH_2NH), 3.10 (1H, d, J = 4.8 Hz, OH), 2.62 (2H, t, J = 7.8 Hz, C$H_2C_6H_5$), 2.36 (2H, t, J = 7.4 Hz, CH_2COO), 1.91-1.49 (8H, m, 4×CH_2); 13C NMR: δ 174.0, 142.3, 128.3, 128.2, 125.7, 72.0, 51.7, 38.4, 35.7, 34.7, 31.4, 31.1, 24.6; MS (ESI): m/z (%): 330 (88) $[M + Na]^+$, 308 (100) $[M + H]^+$. Anal. ($C_{17}H_{25}NO_4$) C, H, N.

4-(2-Hydroxy-nonadec-10-enoylamino)-butyric acid methyl ester (2c)

yield 82%; white solid; m.p. 55–57 °C; ^{1}H NMR: δ 6.80 (1H, m, NHCO), 5.33 (2H, m, CH=CH), 4.07 (1H, m, CH), 3.67 (3H, s, CH_3O), 3.30 (2H, m, CH_2NH), 2.37 (2H, t, J = 7.2 Hz, CH_2COO), 1.98 (4H, m, 2×CH_2CH=CH), 1.85 (2H, m, CH_2CH_2NH), 1.26 (24H, br s, 12×CH_2), 0.87 (3H, t, J = 6.6 Hz, CH_3); ^{13}C NMR: δ 174.2, 173.8, 129.9, 129.7, 72.1, 51.7, 38.4, 34.8, 31.8, 31.3, 29.7, 29.5, 29.4, 29.3, 29.2, 27.2, 25.0, 24.6, 22.6, 14.1. Anal. ($C_{24}H_{45}NO_4$) C, H, N.

4-(2-Hydroxy-hexadecanoylamino)-oct-2-enoic acid methyl ester (9)

The oxidation of compound **4** follows method A. The Wittig reaction of the resulting N-protected α-aminoaldehyde with a stabilized ylide and the general method for the removal of the Boc group was carried out as described previously.[29] The coupling reaction to yield compound **9** is as described above. The overall yield 52%; white solid; m.p 40–42 °C; ^{1}H NMR: δ 6.85 (1H, dd, J_1 = 5.2 Hz, J_2 = 15.4 Hz, CHC*H* =CH), 6.60 (1H, d, J = 9.2 Hz, NHCO), 5.87 (1H, d, J = 15.4 Hz, CH=C*H*COOCH$_3$), 4.62 (1H, m, CH), 4.14 (1H, m, CH), 3.73 (3H, s, COOCH$_3$), 2.77 (1H, m, OH), 1.98-1.01 (32H, m, 16xCH$_2$), 0.86 (6H, t, J = 7 Hz, 2×CH$_3$); ^{13}C NMR: δ 173.3, 166.7, 148.0, 120.5, 72.3, 51.6, 49.6, 37.0, 34.9, 34.0, 31.9, 29.7, 29.5, 29.3, 27.7, 25.0, 24.9, 22.7, 22.3, 14.1, 13.8; MS (ESI): m/z (%): 448 (100) [M + Na]$^+$. Anal. ($C_{25}H_{47}NO_4$) C, H, N.

Oxidation of 2-hydroxy-amides

Method A—To a solution of 2-hydroxy-amide (5.00 mmol) in a mixture of toluene-EtOAc 1:1 (30 mL), a solution of NaBr (0.54 g, 5.25 mmol) in water (2.5 mL) was added followed by TEMPO (11 mg, 0.050 mmol). To the resulting biphasic system, which was cooled at −5 °C, an aqueous solution of 0.35 M NaOCl (15.7 mL, 5.50 mmol) containing NaHCO$_3$ (1.26 g, 15 mmol) was added dropwise under vigorous stirring, at −5 °C over a period of 1 h. After the mixture had been stirred for a further 15 min at 0 °C, EtOAc (30 mL) and H$_2$O (10 mL) were added. The aqueous layer was separated and washed with EtOAc (20 mL). The combined organic layers were washed consecutively with 5% aqueous citric acid (30 mL) containing KI (0.18 g), 10% aqueous $Na_2S_2O_3$ (30 mL), and brine and dried over Na_2SO_4. The solvents were evaporated under reduced pressure and the residue was purified by column chromatography [EtOAc-petroleum ether (bp 40–60 °C), 1:9].

4-(2-Oxo-5-phenyl-pentanoylamino)butyric acid methyl ester (4a)

yield 67%; white solid; m.p. 30–31 °C; ^{1}H NMR: δ 7.19-7.15 (6H, m, C$_6$H$_5$, NHCO), 3.67 (3H, s, CH$_3$O), 3.35 (2H, m, C*H*$_2$NH), 2.94 (2H, t, J = 7.4 Hz, CH$_2$COCO), 2.65 (2H, t, J = 7.8 Hz, C*H*$_2$C$_6$H$_5$), 2.36 (2H, t, J = 7.0 Hz, CH$_2$COO), 1.91 (4H, m, 2×CH$_2$); ^{13}C NMR: δ 198.7, 173.2, 160.0, 141.1, 128.3, 128.2, 125.8, 51.6, 38.5, 35.9, 34.8, 31.1, 24.6, 24.1; MS (ESI): m/z (%): 314 (63) [M + Na]$^+$. Anal. ($C_{16}H_{21}NO_4$) C, H, N.

4-(2-Oxo-6-phenyl-hexanoylamino)butyric acid methyl ester (4b)

yield 75%; white solid; m.p. 52–54 °C; ^{1}H NMR: δ 7.29-7.16 (6H, m, C$_6$H$_5$, NHCO), 3.69 (3H, s, CH$_3$O), 3.37 (2H, m, C*H*$_2$NH), 2.95 (2H, t, J = 7.0 Hz, CH$_2$COCO), 2.64 (2H, t, J = 7.0 Hz, C*H*$_2$C$_6$H$_5$), 2.38 (2H, t, J = 7.0 Hz, CH$_2$COO), 1.89-1.66 (6H, m, 3×CH$_2$); 13C NMR: δ 198.8, 173.2, 160.1, 141.9, 128.21, 128.15, 125.6, 51.6, 38.5, 36.4, 35.4, 31.1, 30.6, 24.2, 22.6; MS (ESI): m/z (%): 328 (75) [M + Na]$^+$. Anal. ($C_{17}H_{23}NO_4$) C, H, N.

4-(2-Oxo-5-phenyl-pentanoylamino)butyric acid (5a)

The procedure is the same as that followed in method A described above, with the difference that in this case the aqueous layer was acidified before the work-up, and then extracted with EtOAc, and the combined organic layers were washed with 5% aqueous citric acid containing KI, and 10% aqueous $Na_2S_2O_3$ (30 mL). The residue was purified by column chromatography [EtOAc-petroleum ether (bp 40–60 °C)]. Yield 48%; white solid; m.p. 65–67 °C; ^{1}H NMR: δ 7.25-7.11 (6H, m, C$_6$H$_5$, NHCOCO), 3.33 (2H, m, C*H*$_2$NH), 2.86 (2H, t, J = 7.4 Hz, CH$_2$COCO), 2.60 (2H, m, CH$_2$), 2.36 (2H, m, CH$_2$), 1.86 (4H, m, 2×CH$_2$); ^{13}C NMR: δ 198.8, 178.5, 160.3, 141.2, 128.41, 128.37, 126.0, 38.5, 36.1, 34.9, 31.2, 24.7, 24.0; MS (ESI) : m/z (%): 276 (100) [M – H]$^-$. Anal. ($C_{15}H_{19}NO_4$) C, H, N.

4-(2-Oxo-6-phenyl-hexanoylamino)-butyric acid (5b)

The procedure is the same as that followed for **5a**. Yield 47%; white solid; m.p. 60–62 °C; ^{1}H NMR: δ 7.27-7.15 (6H, m, C_6H_5, NHCOCO), 3.35 (2H, m, C*H*$_2$NH), 2.94 (2H, t, J = 7.4 Hz, CH_2COCO), 2.60 (2H, m, CH_2), 2.38 (2H, m, CH_2), 1.86 (2H, m, CH_2), 1.64 (4H, m, 2×CH_2); 13C NMR: δ 198.8, 178.8, 160.3, 142.0, 128.33, 128.27, 125.7, 38.6, 36.5, 35.5, 31.4, 30.7, 24.2, 22.6; MS (FAB) : m/z (%): 292 (100) $[M + H]^+$. Anal. ($C_{16}H_{21}NO_4$) C, H, N.

Oxidation of 2-hydroxyamides

Method B—To a solution of 2-hydroxyamide (1 mmol) in dry CH_2Cl_2 (20 mL) Dess-Martin periodinane was added (0.64 g, 1.5 mmol) and the mixture was stirred for 2 h at room temperature. The organic solution was washed with 10% aqueous $NaHCO_3$, dried over Na_2SO_4 and the organic solvent was evaporated under reduced pressure. The residue was purified by recrystallization [EtOAc/petroleum ether (bp 40–60 °C)].

4-(2-Oxononadec-10-enoylamino)butyric acid methyl ester (4c)

yield 82%; oily solid; ^{1}H NMR: δ 7.13 (1H, m, NHCOCO), 5.33 (2H, m, CH=CH), 3.67 (3H, s, CH_3O), 3.33 (2H, m, C*H*$_2$NH), 2.91 (2H, t, J = 7.2 Hz, CH_2COCO), 2.38 (2H, t, J = 7.4 Hz, CH_2COO), 1.98 (4H, m, 2×C*H*$_2$CH=CH), 1.88 (2H, m, C*H*$_2$CH$_2$NH), 1.59 (2H, m, C*H*$_2$CH$_2$COCO), 1.26 (20H, br s, 10×CH_2), 0.87 (3H, t, J = 6.6 Hz, CH_3); 13C NMR: δ 199.2, 173.3, 160.3, 129.9, 129.7, 51.7, 38.0, 36.7, 31.8, 31.3, 29.7, 29.6, 29.5, 29.3, 29.2, 29.0, 28.98, 27.2, 27.1, 24.3, 23.1, 22.6, 14.1; MS (FAB): m/z (%): 410 (100) $[M + H]^+$. Anal. ($C_{24}H_{43}NO_4$) C, H, N.

4-(2-Oxohexadecanoylamino)oct-2-enoic acid methyl ester (11)

yield 81%**,** white solid; m.p. 48–50 °C; $[\alpha]_D$ −19.7 (*c* 0.95 $CHCl_3$); ^{1}H NMR: δ 6.93 (1H, d, J = 8 Hz, NHCOCO), 6.85 (1H, dd, J_1 = 6 Hz, J_2 = 16 Hz, CHC*H*=CH), 5.87 (1H, d, J = 16 Hz, CH=C*H*$COOCH_3$), 4.58 (1H, m, CH), 3.73 (3H, s, $COOCH_3$), 2.91 (2H, t, J = 7 Hz, CH_2COCO), 1.61 (4H, m, 2×CH_2), 1.30 (26H, m, 13×CH_2), 0.88 (6H, t, J = 7 Hz, 2×CH_3); 13C NMR: δ 199.3, 166.7, 159.8, 146.9, 121.4, 51.9, 50.4, 37.0, 34.1, 32.1, 29.9, 29.8, 29.6, 29.5, 29.3, 27.9, 23.4, 22.9, 22.5, 14.3, 14.0; MS (ESI): m/z (%): 446 (85) $[M + Na]^+$. Anal. ($C_{25}H_{45}NO_4$) C, H, N.

4-(2-Oxohexadecanoylamino)oct-2-enoic acid (10)

The procedure is the same as that followed in method B with the difference that the organic layer was not washed with 10% aqueous $NaHCO_3$. Yield 69%, white solid; m.p. 65–67 °C; $[\alpha]_D$ −7.7 (*c* 0.84 $CHCl_3$); ^{1}H NMR: δ 7.0 (1H, m, NHCOCO), 6.82 (1H, dd, J_1 = 6 Hz, J_2 = 16 Hz, CHC*H*=CH), 5.87 (1H, d, J = 16 Hz, CH=C*H*$COOCH_3$), 4.62 (1H, m, CH), 2.91 (2H, t, J = 7 Hz, CH_2COCO), 1.61 (4H, m, 2×CH_2), 1.44-1.25 (26H, m, 13×CH_2), 0.88 (6H, t, J = 7 Hz, 2×CH_3); 13C NMR: δ 199.0, 170.8, 159.6, 149.0, 120.8, 50.2, 36.7, 33.7, 31.9, 29.6, 29.4, 29.3, 29.0, 27.7, 23.1, 22.7, 22.3, 14.1, 13.8; MS (ESI): m/z (%): 408 (100) $[M - H]^-$. Anal. ($C_{24}H_{43}NO_4$) C, H, N.

Saponification of methyl esters

To a stirred solution of methyl ester (2.00 mmol) in a mixture of dioxane-H_2O (9:1, 20 mL), 1N NaOH (2.2 mL, 2.2 mmol) was added and the mixture was stirred for 12 h at room temperature. The organic solvent was evaporated under reduced pressure and H_2O (10 mL) was added. The aqueous layer was washed with EtOAc, acidified with 1N HCl, and extracted with EtOAc (3 × 12 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified after recrystallization [EtOAc-petroleum ether (bp 40–60 °C)].

4-(2-Hydroxy-5-phenylpentanoylamino)butyric acid (3a)

yield 79%; white solid; m.p. 63–65 °C; ^{1}H NMR: δ 7.26-7.12 (6H, m, C_6H_5, NHCO), 4.09 (1H, m, CH), 3.27 (2H, m, CH_2NH), 2.59 (2H, t, J = 6.6 Hz, C$H_2C_6H_5$), 2.31 (2H, t, J = 6.6 Hz, CH_2COOH), 1.78 (6H, m, 3×CH_2); 13C NMR: δ 177.3, 175.5, 142.0, 128.3, 125.8, 71.8, 38.4, 35.5, 34.1, 31.3, 26.8, 24.3. Anal. ($C_{15}H_{21}NO_4$) C, H, N.

4-(2-Hydroxy-6-phenylhexanoylamino)butyric acid (3b)

yield 86%; white solid; m.p. 78–80 °C; ^{1}H NMR: δ 7.30-7.13 (6H, m, C_6H_5, NHCO), 4.11 (1H, m, CH), 3.30 (2H, m, CH_2NH), 2.60 (2H, t, J = 7.8 Hz, C$H_2C_6H_5$), 2.35 (2H, t, J = 6.6 Hz, CH_2COOH), 1.81-1.47 (8H, m, 4×CH_2); ^{13}C NMR: δ 177.4, 175.5, 142.4, 128.3, 128.2, 125.7, 71.9, 38.4, 35.7, 34.3, 31.4, 31.1, 24.7, 24.4; MS (ESI): m/z (%): 316 (54) $[M + Na]^+$, 294 (100) $[M + H]^+$. Anal. ($C_{16}H_{23}NO_4$) C, H, N.

4-(2-Hydroxyhexadecanoylamino)oct-2-enoic acid (8)

yield 62%; white solid; m.p 46–48 °C; ^{1}H NMR: δ 6.92 (1H, m, NHCO), 6.76 (1H, dd, J_1 = 6 Hz, J_2 = 16 Hz, CHCH =CH), 5.87 (1H, d, J = 16 Hz, CH=CHCOOH), 4.64 (1H, m, CH), 4.20 (1H, m, CH), 3.42 (1H, br, OH), 1.95-1.25 (32H, m, 16xCH_2), 0.88 (6H, t, J = 7 Hz, 2xCH_3); ^{13}C NMR: δ 172.3, 170.5, 150.0, 120.5, 72.6, 49.9, 35.1, 34.2, 32.1, 29.9, 29.6, 28.0, 25.3, 22.9, 22.7, 22.5, 14.3, 14.1; MS (ESI): m/z (%): 434 (100) $[M + Na]^+$. Anal. ($C_{24}H_{45}NO_4$) C, H, N.

Inhibitor **12** was prepared by similar procedures.

4-(2-Oxononadec-10-enoylamino)butyric acid (12)

yield 69%; white solid; m.p. 57–59 °C; ^{1}H NMR: δ 10.05 (1H, br, COOH), 7.23 (1H, m, NHCOCO), 5.33 (2H, m, CH=CH), 3.38 (2H, m, CH_2NH), 2.90 (2H, t, J = 7.2 Hz, CH_2COCO), 2.41 (2H, t, J = 6.8 Hz, CH_2COOH), 1.98 (4H, m, 2×CH_2CH=CH), 1.89 (2H, m, CH_2CH_2NH), 1.58 (2H, m, CH_2CH_2COCO), 1.26 (20H, br s, 10×CH_2), 0.87 (3H, t, J = 6.6 Hz, CH_3); 13C NMR: δ 199.1, 178.4, 160.4, 129.9, 129.7, 38.5, 36.7, 32.7, 31.8, 31.2, 29.7, 29.6, 29.5, 29.3, 29.2, 29.02, 28.96, 27.1, 24.1, 23.1, 22.6, 14.1; MS (ESI): m/z (%): 418 (95) $[M + Na]^+$. Anal. ($C_{23}H_{41}NO_4$) C, H, N.

Inhibitors **13–18** were prepared as described previously.[28,29]

Expression and Purification of Recombinant Group VIA PLA_2

Protein was produced in Sf9 insect cells using a recombinant baculovirus. The virus had been constructed using the cDNA coding for human Group VIA-2 $iPLA_2$, kindly provided by Dr. Brian Kennedy at Merck-Frost, modified with a six residue histidine tag added three amino acids from the amino terminus using PCR with oligonucleotides 5′-ATGCAGTTCCACCATCACCATCACCATTTTGGAGCGCTGGTCAATACC-3′ and 5′-CCTCAGGGTGAGAGCAGCAGCTG-3′. Gateway cloning ends were added to the histidine-tagged Group VIA-2 cDNA followed by insertion into pDONOR201 (Invitrogen) to produce a Gateway entry clone. The gene construct was then transferred to pDEST8 using Gateway cloning technology and used to make recombinant baculovirus using the Bac-to-Bac system (Invitrogen).

A suspension culture of Sf9 insect cells at a density of 1.1 to 1.5 million cells per mL was infected with the recombinant baculovirus with an MOI of approximately 0.1. Infections were carried out for 72 hours and the cells were harvested by centrifugation at 3,000 × g for 10 minutes and stored at −80 °C. The frozen cell pellets from 200 mL of suspension culture Sf9 cells were resuspended in 25 mL resuspension buffer (25 mM Tris pH 8.0, 150 mM NaCl, 10

mM DTT, 5 mM EDTA, 2 mM ATP, 0.2% methyl-β-cyclodextrin (Sigma-Aldrich) and 1X protease inhibitor cocktail). The cells were lysed by repeated sonication, and the lysate was allowed to sit on ice for 10 minutes and then clarified by centrifugation at 15,000 × g for 30 minutes at 4 °C. The resulting pellet was resuspended in solubilization buffer (25 mM Tris pH 8.0, 150 mM NaCl, 10 mM β-mercaptoethanol, 2 mM ATP, 1 M urea and 1X protease inhibitor cocktail) by 20 passes of a Dounce homogenizer with the tight pestle. The resuspended pellet was then stirred at 4 °C for one hour followed by centrifugation at 15,000 × g for 30 minutes at 4 °C to remove insoluble material. At this point, 2.5 mL of Fast-flow Ni-NTA resin per 200 mL cell pellet was mixed with the soluble protein fraction and allowed to incubate at 4 °C for 30 minutes for batch binding. The protein/resin slurry was poured into a column and allowed to settle. The column was washed with 15 column volumes of Ni-wash buffer (25 mM $NaHPO_4$ pH 7.4, 250 mM NaCl, 2 mM ATP, 0.2% dodecyl maltoside (Anatrace) and 1X protease inhibitor cocktail) and eluted with 10 column volumes of Ni-elution buffer (25 mM $NaHPO_4$ pH 7.4, 100 mM NaCl, 50 mM urea, 2 mM ATP and 200 mM imidazole, 30% v/v glycerol). Eluate was collected as 1.5 mL fractions into tubes containing 15 μL 500 mM DTT (5 mM DTT final). Fractions containing protein were pooled, measured for activity and protein concentration, and stored as 200 μL aliquots at −80 °C.

Group VIA iPLA$_2$ Activity Assays

The standard Group VIA iPLA$_2$ activity assay utilizes DPPC/Trition X-100 mixed micelles at a ratio of 1:4 as previously described.[46,47] A stock solution of lipid was generated by drying down 50 nmoles of dipalmitoyl phosphatidylcholine (DPPC) mixed with 1×10^5 cpm of 1-palmitoyl, 2-[1-^{14}C]-palmitoyl PC per assay tube under a stream of nitrogen gas. The dried lipids were solubilized in 50 μL of 10X assay buffer (100 mM HEPES pH 7.5, 50 mM EDTA, 20 mM DTT, 10 mM ATP, 4 mM Triton X-100) per assay tube by repeated vortexing and heating to 40 °C. The resulting 10X substrate mixture was combined with 100 mM HEPES pH 7.5 to give a final volume of 500 μL upon addition of enzyme and inhibitor. Inhibitors were dissolved in DMSO to a stock concentration of 5 mM and diluted with DMSO prior to addition of 5 μL to the reaction tube, yielding a final DMSO concentration of 1%. The final substrate concentration in this mixed-micelle assay is 100 μM DPPC and 400 μM Triton X-100. Purified enzyme (190 ng) was added to start the reaction followed by incubation for 30 minutes at 40 °C. The reaction was quenched, extracted and analyzed using the modified Dole assay.[48]

Group IVA cPLA$_2$ Activity Assays

The GIVA cPLA$_2$ assays have been described previously.[28,29,46] Pure, native, human GIVA cPLA$_2$ was a generous gift from Dr. Ruth Kramer of Lilly Research Laboratories. Briefly, the final assay conditions were 10 ng GIVA cPLA$_2$ in 100 mM HEPES (pH 7.5), 80 μM $CaCl_2$, 0.1 mg/mL fatty acid free bovine serum albumin, 2 mM DTT, 97 μM 1-palmitoyl-2-[^{14}C]-arachidonoyl phosphatidylcholine (100,000 cpm), 3 μM phosphatidylinositol 4,5-bisphosphate, and 400 μM Triton X-100 in 500 μL. The reaction contained 1% DMSO with varying amounts of inhibitors added as described above. The assays were incubated at 40 °C for 30 min. Reactions were quenched, extracted and analyzed using the modified Dole assay as above.[48]

Cell Culture and PGE$_2$ Assay

The RAW 264.7 macrophage-like cell line was maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (HyClone Labs, Provo Utah), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad California). Prior to stimulation, cells were plated at a density of 5×10^5 cells per well in standard 12 well tissue culture plates and were allowed to adhere for 24 hours. They were then washed with serum-free medium and allowed to adjust

for 18 hours. Cells were then exposed to 100 ng/mL LPS (Sigma L4130 from *E. coli* 0111:B4) for 24 hours. Following stimulation, the media was removed and the cells were scraped into 1 mL PBS and counted. Deuterated PGE_2 internal standard (10 ng) was added to the media of each sample, and the media was cleared of cellular debris by centrifugation (3000 × g, 10 min). Methanol and acetic acid were added to the cleared supernate to a final concentration of 10% and 2% respectively. Prostaglandins were extracted using 60 mg/3 mL Strata-X columns (Phenomenex). The columns were preconditioned with 2 mL methanol followed by 2 mL water. The sample was loaded and the column washed with 2 mL 0.5% methanol. The sample was eluted from the column with 1 mL 100% methanol.

Inhibitors, when included, were dissolved in DMSO and diluted into serum-free medium prior to addition to cells. The DMSO concentration was kept below 0.5% v/v in all studies. All inhibitors were added 30 minutes prior to stimulation.

PGE_2 released by the cells was quantitated by the following LCMS procedure. The chromatography was performed on a Grace-Vydac reverse phase C18 column (2.1 mm X 250 mm) run with a gradient beginning with 100% Buffer A (63:37:0.02 water:acetonitrile:formic acid) and ending with 100% Buffer B (50:50 acetonitrile:isopropanol). PGE_2 was detected on an ABI 4000 Qtrap mass spectrometer in MRM mode with the electrospray ion source operating in negative ion mode using the following settings: curtain gas = 10, spray voltage = −4.5kV, source temperature = 525 °C, source gas 1 = 60, source gas 2 = 60, declustering potential = −50V. The PGE_2 was detected via CID with a precursor ion of 351 and a product ion of 189 amu and a collision energy = −27V and Q2 collision gas = high.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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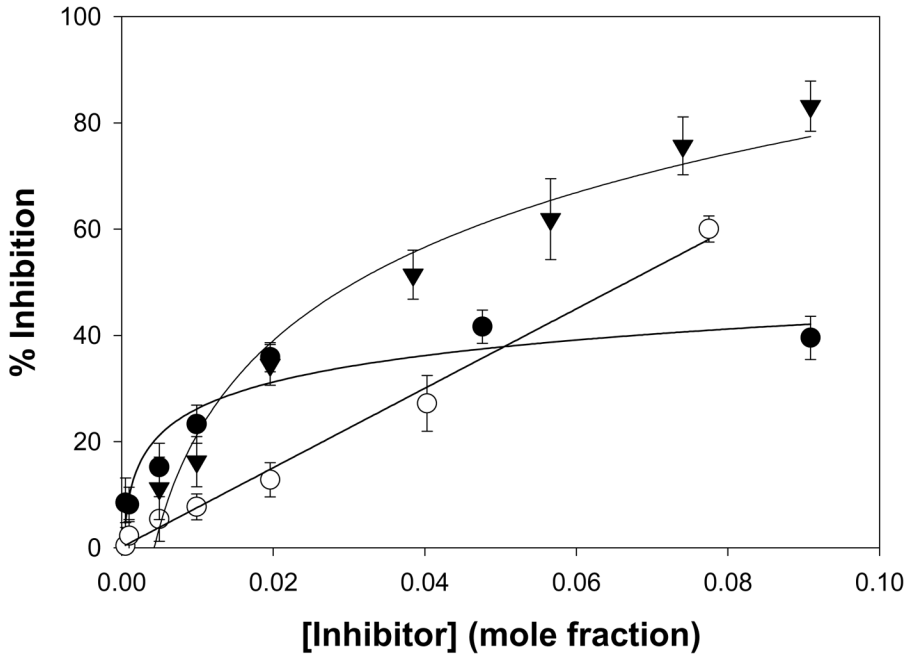


Figure 1.

Dose-response curves for 2-oxoamide inhibitors of GVIA iPLA$_2$. The activity of human GVIA iPLA$_2$ was tested on mixed-micelles containing 100 μM DPPC and 400 μM Triton X-100. The surface concentration of **18** (●), **4c** (○), and **11** (▼) was increased as shown. A logarithmic or linear fit function was used to calculate the $X_I(50)$ values shown in Table 1.

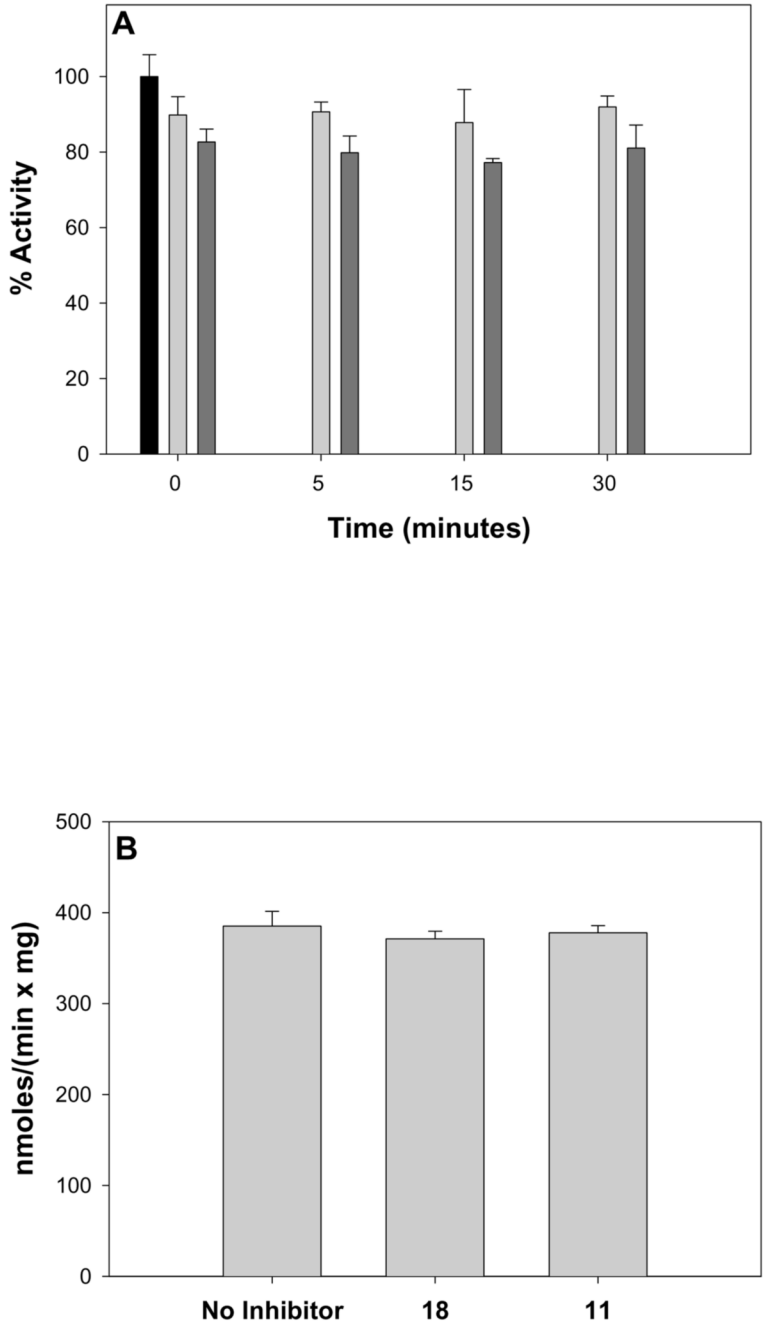


Figure 2.

Immediate and reversible inhibition of GVIA iPLA$_2$ by **18** and **11**. A. Time-dependent binding of **18** and **11** was tested by pre-incubating no inhibitor (black bar), 5 μM **18** (light bars), or 5 μM **11** (dark bars) with GVIA iPLA$_2$ prior to adding to mixed micelles consisting of 100 μM DPPC and 400 μM Triton X-100 containing 0.01 mole fraction inhibitor. B. Reversibility of **18** and **11** was tested by preincubating no inhibitor, 10 μM **18**, or 10 μM **11** with GVIA iPLA$_2$ for 10 minutes prior to diluting 1:50 into mixed micelles consisting of 100 μM DPPC and 400 μM Triton X-100 and assaying for activity.

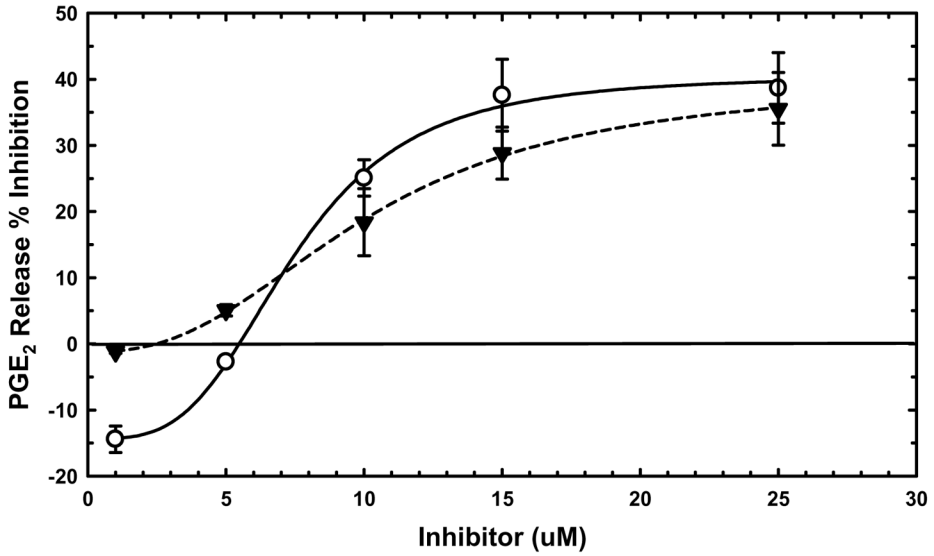
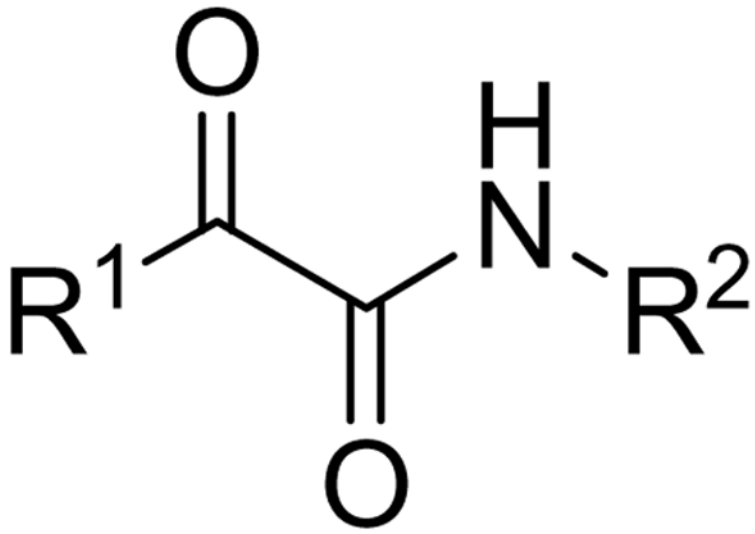
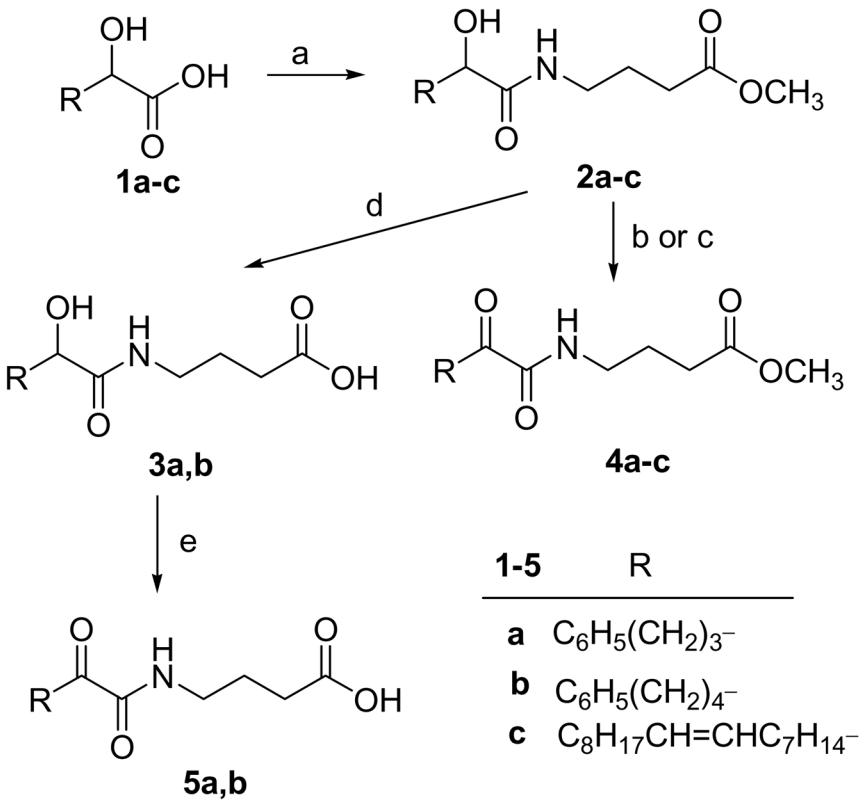


Figure 3.

Inhibition of PGE_2 production in RAW 264.7 cells by 2-oxoamides containing a methyl ester. Increasing concentrations of **4c** (○) or **11** (▼) were added to cells for 30 minutes prior to stimulation with 100 ng/ml LPS for 24 hours. Media was harvested and assayed for PGE_2 production as described in the experimental section.

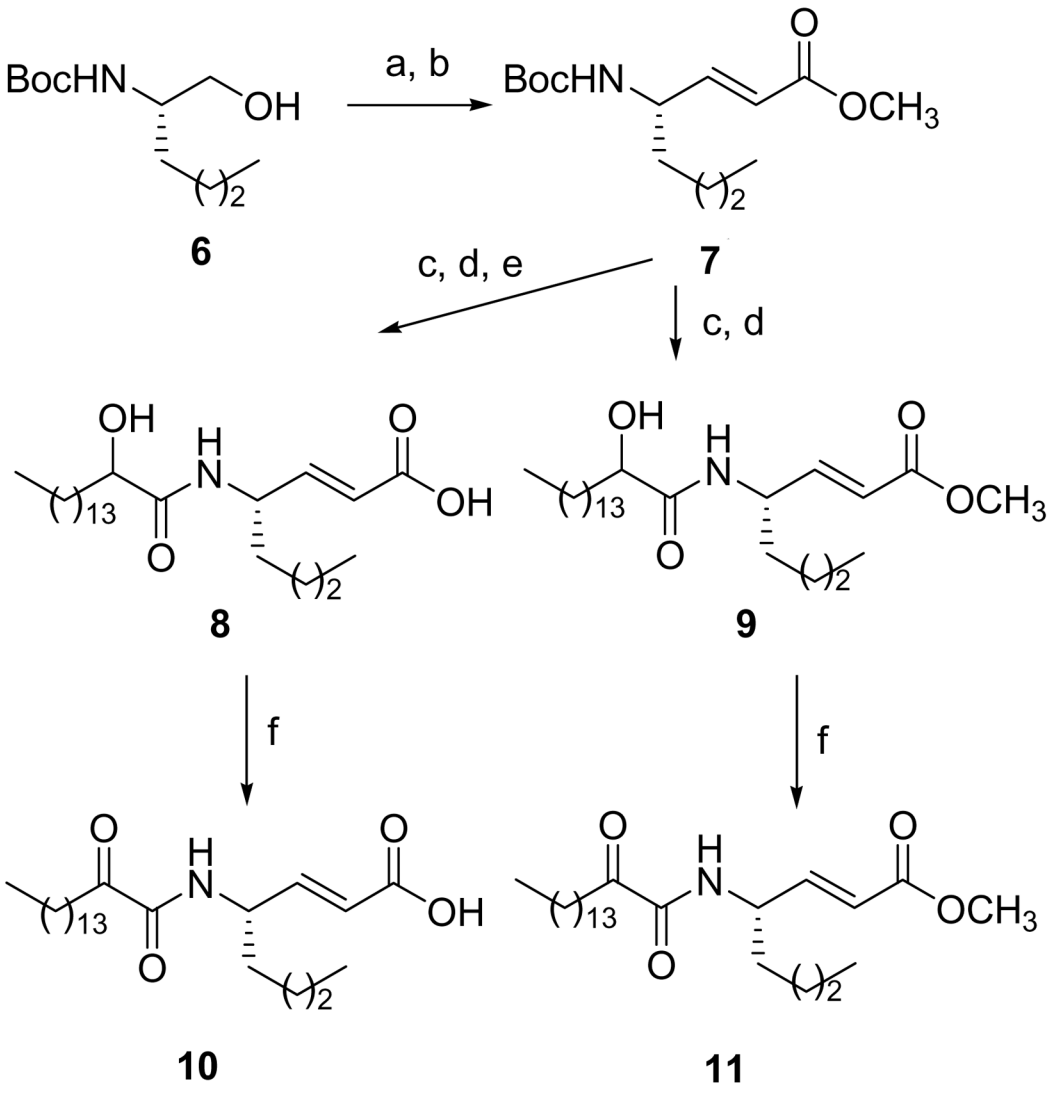


Scheme 1.



Scheme 2a.

[a]*Reagents and conditions:* (a) $H_2N(CH_2)_3COOCH_3$, Et_3N, WSCI, HOBt, CH_2Cl_2; (b) NaOCl, TEMPO, NaBr, $NaHCO_3$, EtOAc/toluene/H_2O, 0 ºC; (c) Dess-Martin periodinane, CH_2Cl_2; (d) 1N NaOH/MeOH; (e) NaOCl, TEMPO, NaBr, $NaHCO_3$, EtOAc/toluene/H_2O, 0 ºC, then HCl.

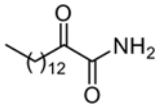
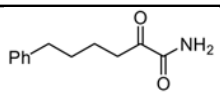
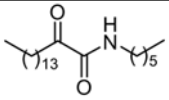
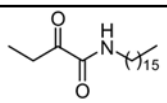
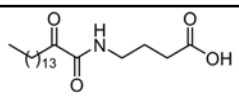
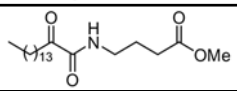
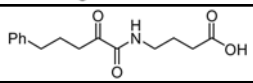
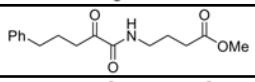
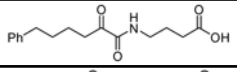
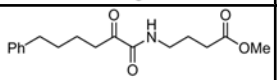
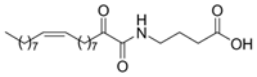
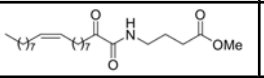
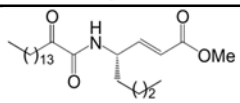
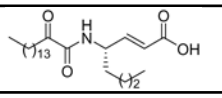


Scheme 3a.

[a]*Reagents and conditions* (a) NaOCl, TEMPO, NaBr, $NaHCO_3$, EtOAc/toluene/H_2O, −5 °C; (b) $Ph_3P{=}CHCOOCH_3$, THF, reflux; (c) 4 N HCl in THF; (d) $CH_3(CH_2)_{13}CHOHCOOH$, Et_3N, WSCI, HOBt, CH_2Cl_2; (e) 1N NaOH/MeOH; (f) Dess-Martin periodinane, CH_2Cl_2.

Table 1

Structures of 2-Oxoamide Inhibitors and their Effects on GIVA and GVIA PLA_2.

Number	Structure	Inhibition of GVIA iPLA$_2$	Inhibition of GIVA cPLA$_2$
13		ND[a,f]	ND[e]
14		ND	ND
15		LD[b,f]	ND[e]
16		LD[f]	ND[e]
17		ND	$X_I(50) = 0.017 \pm 0.009$[c,d]
18		LD	ND
5a		ND	ND
4a		ND	ND
5b		ND	ND
4b		ND	ND
12		ND	$X_I(50) = 0.011 \pm 0.003$
4c		$X_I(50) = 0.067 \pm 0.003$	$X_I(50) = 0.012 \pm 0.014$
11		$X_I(50) = 0.032 \pm 0.010$	$X_I(50) = 0.018 \pm 0.010$
10		ND	$X_I(50) = 0.003 \pm 0.001$

[a] ND: negligible inhibition (0–25%) at highest dose. Unless otherwise indicated the highest dose tested was 0.091 mole fraction.

[b] LD: limited inhibition (25–50%) at highest dose.

[c] Data taken from Ref. 28.

[d] $X_I(50)$ is the surface concentration of inhibitor at which there is 50% inhibition.

[e] 0.01 mole fraction.

[f] 0.02 mole fraction.