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Inhibition of the complete set of mammalian secreted phospholipases A_2 by indole analogues: a structure-guided study^{\uparrow}

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Abstract—Structure-guided design was employed in a search for potent and selective inhibitors of mammalian secreted phospholipases A₂ (sPLA₂s). Using the X-ray structures of human groups IIA and X sPLA₂s (hGIIA and hGX) as templates, homology structural models were made for the other human and mouse sPLA₂s (hGIB, mGIB, mGIIA, mGIIC, hGIID, mGIID, hGIIE, mGIIE, hGIIF, mGIIF, hGV, mGV, and mGX). Me-Indoxam is a previously discovered indole analogue that binds tightly to many sPLA₂s, and the X-ray structure of the hGX-Me-Indoxam complex was determined at a resolution of 2.0 Å. Modeling suggests that the residues near the N₁-substituent of Me-Indoxam vary significantly among the mammalian sPLA₂s, and therefore a library of 83 N₁-variants was prepared by parallel synthesis. Several Me-Indoxam analogues bearing a 4-(2-oxy-ethanoic acid) side chain were potent inhibitors (IC₅₀ < 0.05 μ M) of hGIIA, mGIIA, mGIIC, hGIIE, mGIIE, hGV, and mGV, while they displayed intermediate potency (0.05–5 μ M) against hGIB, mGIB, hGX, and mGX, and poorly inhibited (> 5 μ M) hGIID, mGIID, hGIIF, and mGIIF. Me-Indoxam analogues bearing a 5-(4-oxy-butanoic acid) side chain were generally less potent inhibitors. Although no compounds were found to be highly specific for a single human or mouse sPLA₂s in cellular events. For example, Me-Indoxam analogues were discovered that could be used to distinguish the action of various sPLA₂s in cellular events. For example, Me-Indoxam and compound **5** are approximately 5-fold more potent on hGIIA than on hGV, and compound **21** is 10-fold more potent on hGV versus hGIIA.

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1. Introduction

The phospholipase A_2 class of enzymes catalyze the hydrolysis of the *sn*-2 ester of glycero-phospholipids to release fatty acids and lysophospholipids.¹ One or more of these enzymes is thought to initiate pro-inflammatory cascades by liberating arachidonic acid from membrane phospholipids for the biosynthesis of the eicosanoids (prostaglandins, leukotrienes, and others). The current thinking is that arachidonic acid liberation from the membranes of mammalian cells is catalyzed by the 87-kDa cytosolic phospholipase A_2 (group IVA) and by one or more low molecular weight, secreted phospholipases A_2 (sPLA₂s).^{2,3} Understanding the role of sPLA₂s

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in this process is complicated by the fact that mammals contain a large number of these enzymes. Humans contain the groups IB, IIA, IID, IIE, IIF, III, V, X, and XIIA sPLA₂s (designated hGIB, hGIIA, etc.), whereas the mouse contains all of these sPLA₂s (designated mGIB, mGIIA, etc.) plus the group IIC enzyme (which is present in the human genome as a pseudogene).^{4,5} Deciphering the role played by sPLA₂s in arachidonic acid release as well as in other physiological events would be greatly aided by the availability of potent and specific inhibitors of each mammalian homologue.

Of the numerous known sPLA₂ inhibitors, many are non-specific agents that act by perturbing the physical structure of the membrane substrate.⁶ sPLA₂s are water-soluble enzymes that must bind to the membrane surface to access their water-insoluble substrates, (interfacial enzymes).⁷ Methods have been developed for the proper quantitative analysis of inhibitors of

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interfacial enzymes that allow 'true', active site-directed, competitive inhibitors to be investigated.^{6,8,9} Inhibitors that have been convincingly established to be potent and active site-directed competitive inhibitors of sPLA₂s either by proper kinetic analysis or by determination of the enzyme-inhibitor X-ray structure include phospholipid analogues (i.e., *sn*-2 amides and phosphonates)^{10–14} as well as non-phospholipid analogues including the plant-derived alkaloid aristolochic acid,¹⁵ 1,3-dioxane-4,6-dione-5-carboxamides,^{16,17} fatty acid amides,¹⁸ aromatic sulfonamides (i.e., SB-203347),¹⁹ plant-derived sterols,²⁰ bis-carboxylates,^{21,22} complex poly-*p*-hydroxy benzoate natural products,^{23–25} highly substituted pyrazoles,²⁶ short peptides,^{27,28} vitamin E analogues,²⁹ and indole analogues.^{30–33} The X-ray structure of some of these compounds bound to hGIIA, porcine and bovine pancreatic sPLA₂s, and venom sPLA₂s have been determined.^{15,17,27–30,34–41}

Of these sPLA₂ inhibitors, the indole analogues developed by workers at Lilly and Shionogi laboratories are particularly appealing for further study as they appear to have properties most suitable for use in studies with cultured mammalian cells and are the most generally potent among the full set of human and mouse sPLA₂s.⁴² We have tested the indole analogue Me-Indoxam (Fig. 1) on all of the human and mouse sPLA₂s except the human and mouse group III and mouse group XIIA enzymes, and found it to be a potent inhibitor of mGIIA, hGIIA, mGIIC, mGIIE, hGIIE, mGV, and hGV (IC₅₀ \sim 0.01–0.02 μ M), and a modestly potent inhibitor of mGIB, hGIB, mGX, and hGX (IC₅₀ $\sim 0.1-1 \ \mu M$).⁴² On the other hand Me-Indoxam is a poor inhibitor of mGIID, hGIID, mGIIF, hGIIF, and hGXIIA (IC₅₀ > 10 μ M).⁴²

The groups V and X sPLA₂s are particularly appealing for further study because these enzymes display high enzymatic activity when added exogenously to a variety of mammalian cells,^{43–48} whereas the other mammalian sPLA₂s are poorly active in this assay⁴² (the human and mouse group III enzymes remain to be analyzed on mammalian cells). These two sPLA₂s display the unique ability to bind to phosphatidylcholine-rich vesicles in



Figure 1. Structure of Me-Indoxam. Atom labels are as given in the pdb file (except for carbons which are labeled C1, C2, etc., whereas they are labeled 1, 2, 3, etc. in the figure). The atom numbering of the indole ring is different than the conventional IUPAC ring numbering. The latter is used to name the compounds synthesized in this study.

vitro, which accounts for their ability to act on the phosphatidylcholine-rich outer leaflet of the mammalian cell plasma membrane.^{42–45} Thus, among the sPLA₂ family members, group V and X enzymes seem to be the strongest candidates for playing a role in arachidonic acid liberation for eicosanoid production. For example, a role for mGV in lipopolysaccharide-induced arachidonic acid release from a murine macrophage-like cell line has been reported.⁴⁹ The group V and X sPLA₂s also show relatively high activity on serum lipoproteins, and may play a role in the development of atherosclerosis.^{50,51}

In the present study, we extend our structural studies of hGX^{52} by reporting the 2.0 Å X-ray crystal structure of this sPLA₂ complexed with Me-Indoxam. This structure was then used as a guide for preparing analogues of the lead compound Me-Indoxam with the hope of identifying compounds with improved potency and specificity for one or more of the various human and mouse sPLA₂s. We report the preparation, by small-scale, parallel organic synthesis, of a library of 83 Me-Indoxam analogues, and provide inhibition values of many of these compounds toward the full set of human and mouse sPLA₂s.

2. Results

2.1. Crystal structure of the complex of hGX with Me-Indoxam

The X-ray crystal structure of hGX-Me-Indoxam was solved in a protein crystal form with two independent subunits in the asymmetric unit. The overall structure is well refined with good geometry with an R_{working} of 0.219 and an R_{free} of 0.267. These statistics are similar to the converged $R_{factors}$ for the ligand free form of hGX that was previously reported from a protein crystal also with symmetry of the C222₁ space group and similar unit cell lengths.⁵² Furthermore, there are parallels between the two structures with one of the two subunits in each structure exhibiting more disorder than the other, with a notable increase in the individual B-factors. Importantly, the 2-phenyl-benzyl group of the Me-Indoxam inhibitor is well ordered in subunit A and completely disordered in subunit B. The probable cause of this phenomenon is a difference in proteinprotein crystal contacts between the two subunits, particularly near the 2-phenyl of the 2-phenyl-benzyl group of the inhibitor. This observed disorder in subunit B indicates the lack of multiple contacts between the protein and the 2-phenyl group of the N₁-substituent of the inhibitor bound to the same subunit. In our design of inhibitors, we hoped to take advantage of the N₁-substituent with a goal to achieve inhibitors that are specific for the various sPLA₂ enzymes. For the remainder of the structure description, the more ordered subunit A will be used for analyses and figures.

The Me-Indoxam inhibitor is complexed to the catalytic calcium through interactions between the amide carbonyl oxygen and the carboxyl oxygen atoms. A simu-



Figure 2. Stereo ball and stick depiction of the hGX-Me-Indoxam complex. The active site is shown including residues H46 and D47, the Ca^{2+} -binding loop and the competitive inhibitor Me-Indoxam. The Ca^{2+} is seven coordinate with three carbonyl backbone ligands (F26, G28, and G30), a bidentate coordination from D47, and coordination to the amide carbonyl oxygen and carboxyl oxygen atoms of Me-Indoxam. Hydrogen bonding of the nitrogen and oxygen atoms of H46 and D47, respectively, are shown by dotted lines. Difference electron density from a composite simulated annealing omit map contoured at 1.0 sigma is displayed around the Me-Indoxam ligand (coefficients 2Fo–Fc). The figure was rendered using the programs MOLSCRIPT,⁶² POVSCRIPT (http://people/brandeis.edu/~fenn/povscript/), and POVRAY (http://www.povray.org).

lated annealing omit map of the active site (Fig. 2) demonstrates a well refined structure and provides convincing evidence of the proper modeling of each atom of the Me-Indoxam inhibitor in subunit A of the crystal structure. The resulting calcium coordination of hGX sPLA₂ shows the typical 7-coordinate geometry of a ligand bound form of the sPLA₂ enzyme³⁷ as shown in Figure 2. Additional hydrogen bonds exist between the hydrogen on the amide nitrogen of Me-Indoxam and the residues His-46 and Asp-47. The only other hydrogen bonding interactions come from three water molecules that are interacting with the carbonyl oxygen of the carboxyl group of Me-Indoxam. The balance of interactions come from hydrophobic interactions with side chains from residues IIe-2, Leu-5, Ala-6, Met-21,

Gly-28, Leu-29, Gly-30, Cys-43, Tyr-50, and Ile-94 as shown in Figure 3. These side chain interactions form the basis of predicted binding affinity differences of Me-Indoxam between the various sequences for the $sPLA_2$ enzymes (discussed below).

The surface of the hGX sPLA₂ that binds to lipid water interfaces (i-face) is composed of polar and non-polar residues and displays a preference to zwitterionic interfaces.⁵² The 2-phenyl group of the 2-phenyl-benzyl group of the Me-Indoxam inhibitor protrudes out of the enzyme active site opening. The minimal protein sidechain interactions with the 2-phenyl group of Me-Indoxam are consistent with an observation of a disordered N₁-substituent in subunit B of this structure. Furthermore, the hGX-Me-Indoxam inhibitor complex suggests that for the enzyme-inhibitor complex bound to the membrane surface, the 2-phenyl of the 2-phenylbenzyl group penetrates into the membrane interface, possibly the polar head group region or just below this region.

2.2. Design of the Me-Indoxam library

We desired to prepare Me-Indoxam analogues with improved potency and selectivity for the various mammalian sPLA₂s. The X-ray structures of groups I, II, and X sPLA₂s show that these enzymes are structurally related,52,53 and based on sequence homology we anticipate that the structure of group V sPLA2s will also be similar. We built homology structural models of several mammalian sPLA2s (hGIB, mGIB, mGIIA, mGIIC, hGIID, mGIID, hGIIE, mGIIE, hGIIF, mGIIF, hGV, mGV, and mGX) using the X-ray structures of hGIIA and hGX as templates. This was done with the Insight-II software (MSI) simply by replacing peptide segments of hGIIA and hGX with the homologous segments of the other sPLA₂s. Many of the active site residues are conserved among all the sPLA₂s, and no attempt was made to minimize the energy of the structural models using molecular dynamics. Addition-



Figure 3. hGX-Me-Indoxam contacts. Side chain residues of hGX that lie within 4 Å of Me-Indoxam are displayed in ball and stick format for residues Ile-2, Leu-5, Ala-6, Met-21, Leu-29, Cys-43, Tyr-50, and Ile-94. Coordination of Me-Indoxam to the Ca^{2+} is shown. The side chain contacts from His-46 and Asp-47 and main chain contacts within residues of the calcium binding loop (residues 26–30) are not shown for clarity and can be observed in Figure 2. Cys-43 is disulfide linked to another cysteine residue (not shown).

ally, no attempt to model the structure of hGIII and hGXIIA was made since these enzymes show amino acid sequence homology to the groups I, II, V and X sPLA₂s only in short stretches that include the catalytic residues. With notable exceptions (see below), the residues that contact the indole core, the glyoxamide side chain, and the 4-(2-oxy-ethanoic acid) side chain of Me-Indoxam are well conserved among the groups I, II, V, and X sPLA₂s. On the other hand, residues that are likely to contact the 2-phenyl-benzyl side chain attached to the N₁-position of Me-Indoxam differ significantly among the various group I, II, V, and X sPLA₂s. Thus, it seemed reasonable to prepare a library of Me-Indoxam analogues in which the N₁-substituent is varied. Since the analogue of Me-Indoxam in which the 4-(2-oxy-ethanoic acid) side chain is replaced with hydrogen and the longer 4-oxy-butanoic acid side chain is present at the 5-position of the indole core is also reported to be a potent inhibitor of hGIIA,⁵⁴ we also prepared a library of these compounds in which the N₁substituent was varied.

2.3. Chemistry

Figure 4 shows the synthetic scheme used to prepare a library of indole analogues containing the common functionalities, a 2-methyl group, a 3-glyoxamide side chain and either a 4-(2-oxy-ethanoic acid) or a 5-(4-oxybutanoic acid) side chain, and with varying substituents at the N₁-position. The N₁-substituent is introduced late in the synthesis, which facilitated the preparation of a large number of analogues in which this substituent was varied. Commercially available 4- and 5-hydroxy-2methyl indoles (A) and (E), respectively (Fig. 4), were treated with potassium carbonate in refluxing acetone, resulting in selective deprotonation of the hydroxyl group. Alkylation of the alkoxide with tert-butyl 2-bromoacetate or tert-butyl 4-bromobutanoate provided indoles (B) and (F), respectively. The 3-glyoxamide side chain was added to the indole in two steps. First, indole **(B)** or **(F)** was added to a dilute solution of oxalyl chloride in CH₂Cl₂. High dilution conditions were necessary to avoid the troublesome dimerization of the indole.



Figure 4. Synthesis of Me-Indoxam analogues. The reagents used are: (i) K_2CO_3 , $Br(CH_2)_nCO_2t$ -Bu (n=1 or 3), acetone, reflux; (ii) oxalyl chloride, CH_2Cl_2 followed by NH₃; (iii) NaH, RBr, THF followed by neat CF₃CO₂H.

Next, the acyl chloride intermediate was converted to the amide by bubbling ammonia gas into the solution. Common intermediates (C) and (G) were used to prepare the library of Me-Indoxam analogues (D) and (H), respectively. A single solution of (C) or (G) in THF was treated with NaH leading to deprotonation of the N₁hydrogen. This mixture was distributed into several small vials and allowed to react with various alkyl bromides to form the N₁-substituted indole analogues. Treatment with neat trifluoroacetic acid led to deprotection of the tert-butyl ester of the side chain at the 4or 5-position of the indole ring to give the desired library members (D) and (H). Each compound was purified by HPLC and confirmed to have the correct structure by electrospray ionization mass spectrometry. The structure of the 48 indole analogues with a 4-(2oxy-ethanoic acid) chain (compounds 1-48) are shown in Table 1, and the structures of the 35 indole analogues with a 5-(4-oxy-butanoic acid) chain (compounds 49-**83**) are shown in Table 3.

We desired an accurate and rapid method to quantify the amount of Me-Indoxam analogues that were prepared by multiple, small-scale, parallel organic synthesis. Thus, we used low specific activity, carbon-14-labeled oxalyl chloride to form the glyoxamide side chain, which permitted the concentration of Me-Indoxam analogues in stock solutions to be quantified by scintillation counting. Labeled oxalyl chloride was synthesized from commercially available [¹⁴C]oxalic acid by reaction with phosphorous pentachloride.

2.4. sPLA₂ inhibition studies

We developed a high throughput, fluorometric assay of sPLA₂s that can be carried out in multi-well plates.⁴³ The full set of 48 Me-Indoxam analogues with a 4-(2oxy-ethanoic acid) side chain were tested on the set of human sPLA₂s (hGIB, hGIIA, hGIID, hGIIE, hGIIF, hGV, hGX, and hGXIIA) (Table 1) and on the set of mouse sPLA₂s (mGIB, mGIIA, mGIIC, mGIID, mGIIE, mGIIF, mGV, and mGX) (Table 2). Indole analogues were tested at a single concentration and retested at a second concentration if inhibition approached 100% or if inhibition was <30%. Studies with mGXIIA were not carried out because the Me-Indoxam analogues were uniformly poor inhibitors of hGXIIA, and mGXIIA is 96% identical in amino acid sequence to hGXIIA (the active site residues of these two enzymes are identical). Studies with hGIII were limited to testing only analogue 21 because of limited amount of enzyme. It is anticipated that other indole analogues would not be potent against hGIII given that indole 21 is a weak inhibitor, and that the active site structure of hGIII is predicted to be different from that of groups I, II, V, and X sPLA₂s (based on the X-ray structure of the related group III sPLA₂ from honey bee venom³⁶).

Me-Indoxam analogues with a 5-(4-oxy-butanoic acid) side chain were tested (Table 3) and were found to be much less potent $sPLA_2$ inhibitors than the 4-(2-oxy-ethanoic acid)-containing analogues. Thus the former were tested only on hGIIA and hGX $sPLA_2s$.

Table 1. Inhibition of human sPLA₂s by indoles bearing a 4-(2-oxy-ethanoic acid) side chain



R Group	Compd	Compd % Inhibition against human sPLA ₂											
		IB 0.5 μΜ	ΠΑ 0.33 μΜ	IIA 0.025 μΜ	IID 5 μM	IIE 0.5 μM	IIE 0.025 μΜ	IIF 5 μM	III 5 μM	V 0.33 μM	V 0.10 μM	Χ 0.33 μΜ	XIIA 5 µM
2,6-Cl ₂ -C ₆ H ₃ CH ₂	1	71	100	45	17	98	68	46		95	86	30	6
Phthalimido(CH ₂) ₄	2	25	2	5	9	86	17	37		50	34	15	7
C ₆ F ₅ CH ₂	3	63	33	6	20	72	0	14		32	3	9	9
4-Br-C ₆ H ₄ CH ₂	4	78	100	53	63	96	80	65		93	85	29	31
C ₆ H ₅ COCH ₂	5	14	61	27	0	36	0	22		10	8	0	14
3-(CH ₂ Br)-C ₆ H ₄ CH ₂	6	37	86	29	9	96	81	44		82	59	19	2
3-Cl-C ₆ H ₄ CH ₂	7	59	96	30	31	100	86	38		93	81	33	16
2-NapthaleneCH ₂	8	58	97	33	49	100	80	60		93	84	15	24
4-CN-C ₆ H ₄ CH ₂	9	36	29	4	34	100	42	52		69	44	13	13
2-CN-C ₆ H ₄ CH ₂	10	10	82	23	0	100	84	23		47	20	0	27
3-CN-C ₆ H ₄ CH ₂	11	9	86	29	0	95	47	22		71	37	1	11
(CH ₃) ₂ CHCH ₂	12	28	64	31	20	81	13	30		63	33	26	24
2-(OCH ₃)-5-(NO ₂)-C ₆ H ₃ CH ₂	13	37	7	0	16	49	0	16		4	0	2	0
$3-Br-C_6H_4CH_2$	14	59	95	27	70	100	88	44		93	84	37	13
$2-Br-C_6H_4CH_2$	15	50	100	22	26	100	96	45		78	49	26	18
$2-F-C_6H_4CH_2$	16	70	100	37	41	100	96	40		90	75	40	9
$4-F-C_6H_4CH_2$	17	67	80	34	52	97	69	41		88	73	24	9
$3-(NO_2)-C_6H_4CH_2$	18	24	57	32	5	97	46	29		82	46	30	1
4-(7-CH ₃ O)-coumarinylCH ₂	19	18	9	0	44	6	2	12		2	20	0	2
1-(8-CH ₂ Br)-naphthaleneCH ₂	20	24	24	2	37	91	18	52		23	5	18	8
(S)-(+)-CH ₃ CH ₂ CH(CH ₃)CH ₂	21	55	22	0	0	94	0	37	8	86	57	33	19
2-Cl-C ₆ H ₄ CH ₂	22	30	91	31	52	100	99	43		88	57	36	12
PhthalimidoCH ₂	23	9	0	9	38	53	0	17		6	2	0	3
$2-(CF_3)-C_6H_4CH_2$	24	29	93	30	23	100	93	31		79	48	19	14
2,4-F ₂ -C ₆ H ₃ CH ₂	25	78	84	7	89	98	82	41		93	70	27	13
2,5-F ₂ -C ₆ H ₃ CH ₂	26	62	76	1	34	98	52	22		80	44	13	10
2,6-F ₂ -C ₆ H ₃ CH ₂	27	79	91	12	34	98	82	44		92	68	16	11
3,4-F ₂ -C ₆ H ₃ CH ₂	28	32	84	22	48	96	54	31		94	60	12	7
2,3-F ₂ -C ₆ H ₃ CH ₂	29	73	96	15	29	99	85	37		98	68	47	16
3,5-F ₂ -C ₆ H ₃ CH ₂	30	55	84	20	60	90	16	22		55	4	24	6
$3-(CF_3)-C_6H_4CH_2$	31	55	97	30	0	95	81	8		92	76	18	10
4-(CF ₃)-C ₆ H ₄ CH ₂	32	60	83	15	23	100	62	41		79	49	3	19
$2-((2-CH_2Br)-C_6H_4)-C_6H_4CH_2$	33	83	97	25	43	87	23	51		95	66	17	33
$3-(OCF_3)-C_6H_4CH_2$	34	49	97	53	55	94	89	49		94	75	32	17
$3-I-C_6H_4CH_2$	35	59	98	51	59	96	89	58		92	75	20	19
2-(CF ₃)-4-F-C ₆ H ₄ CH ₂	36	36	93	45	7	95	77	26		64	43	0	13
2,3,6-F ₃ -C ₆ H ₂ CH ₂	37	56	84	9	8	94	63	19		76	20	4	15
2,5-(CF ₃) ₂ -C ₆ H ₃ CH ₂	38	15	7	1	45	54	0	23		2	0	0	14
4-(CH ₃)-C ₆ H ₄ CH ₂	39	46	88	9	30	100	85	38		85	62	5	14
CH ₃ CH ₂	40	0	39	13	27	17	0	3		1	1	0	10
$CH_3(CH_2)_2$	41	15	51	10	45	66	0	5		37	2	0	5
CH ₃ (CH ₂) ₃	42	72	69	18	33	84	0	26		52	31	4	0
$CH_3(CH_2)_4$	43	63	57	4	77	71	0	26		26	21	15	5
CH ₃ (CH ₂) ₅	44	66	79	9	78	84	3	43		23	35	15	8
$CH_3(CH_2)_6$	45	75	89	22	62	90	21	50		60	49	35	8
CH ₃ (CH ₂) ₇	46	80	100	59	100	92	31	60		64	53	42	15
C ₆ H ₅ CH ₂	47 48	68	82	24	29	97	84	42		73	65	22	0
2-(C ₆ H ₅)-C ₆ H ₄ CH ₂	Me-Indoxam	84	100	58	9	100	91	34		91	75	57	25

Analyses were performed in at least duplicate for hGIIA, hGV, and hGX sPLA₂ enzymes.

Table 2. Inhibition of mouse sPLA₂s by indoles bearing a 4-(2-oxy-ethanoic acid) side chain



R Group	Compd	% Inhibition against mouse sPLA ₂									
		IB 0.5 μM	ΠΑ 0.025 μΜ	ΠC 0.025 μM	IID 5 μM	ΠΕ 0.025 μΜ	IIF 5 μM	V 0.333 μΜ	Χ 1.0 μΜ		
2,6-Cl ₂ -C ₆ H ₃ CH ₂	1	96	85	70	27	56	2	95	70		
Phthalimido(CH ₂) ₄	2	66	17	42	17	21	4	55	35		
C ₆ F ₅ CH ₂	3	69	8	55	14	29	8	31	38		
4-Br-C ₆ H ₄ CH ₂	4	89	88	78	85	61	30	97	61		
C ₄ H ₅ COCH ₂	5	17	0	15	15	19	6	7	7		
$3-(CH_2Br)-C_2H_4CH_2$	6	52	47	64	16	62	5	87	51		
3-Cl-C/H4CH2	7	84	75	83	59	31	5	92	68		
2-NapthaleneCH ₂	8	86	83	82	92	27	30	87	64		
4-CN-C-H-CH	9	54	29	42	25	6	0	75	49		
$2 - CN - C_2 + L_2 + CH_2$	10	31	34	52	11	45	0	66	42		
3-CN-C H CH	10	27	17	11	12	12	4	63	33		
(CH) CHCH	11	50	10	28	64	12	15	73	24		
$(CH_3)_2$ CHCH ₂ 2 (OCH) 5 (NO) C H CH	12	57	10	14	12	0	15	5			
$2 - (OCH_3) - 3 - (INO_2) - C_6 H_3 CH_2$	15	57	4	14	12	24	8 7	5	4		
$3-BF-C_6H_4CH_2$	14	/8	75	80	20	20	12	96	73		
2-Br-C ₆ H ₄ CH ₂	15	81	74	93	55	45	12	86	/3		
2-F-C ₆ H ₄ CH ₂	16	81	/8	99	50	51	8	94	//		
4-F-C ₆ H ₄ CH ₂	1/	/6	/8	82	39	32	3	88	4/		
$3-(NO_2)-C_6H_4CH_2$	18	31	36	33	21	20	1	74	35		
$4-(7-CH_3O)$ -coumarinylCH ₂	19	20	0	12	18	0	13	8	7		
$1-(8-CH_2Br)-naphthaleneCH_2$	20	73	8	27	12	0	3	29	21		
$(S)-(+)-CH_3CH_2CH(CH_3)CH_2$	21	60	16	59	58	16	5	87	55		
$2-Cl-C_6H_4CH_2$	22	81	75	95	44	62	8	91	69		
PhthalimidoCH ₂	23	9	11	18	19	10	2	4	13		
$2-(CF_3)-C_6H_4CH_2$	24	62	46	78	3	60	4	84	69		
$2,4-F_2-C_6H_3CH_2$	25	78	66	86	40	56	8	95	54		
$2,5$ - F_2 - $C_6H_3CH_2$	26	74	49	88	32	34	0	77	58		
$2,6-F_2-C_6H_3CH_2$	27	77	72	83	30	45	0	89	80		
$3,4-F_2-C_6H_3CH_2$	28	73	45	61	25	26	0	76	41		
$2,3-F_2-C_6H_3CH_2$	29	72	73	92	29	57	8	93	70		
3,5-F ₂ -C ₆ H ₃ CH ₂	30	63	33	67	30	13	4	56	47		
$3-(CF_3)-C_6H_4CH_2$	31	63	54	67	49	47	10	92	63		
$4-(CF_3)-C_6H_4CH_2$	32	74	56	48	45	35	5	73	33		
$2-((2-CH_2Br)-C_6H_4)-C_6H_4CH_2$	33	84	43	89	11	31	40	90	71		
$3-(OCF_3)-C_6H_4CH_2$	34	68	66	73	62	37	16	87	51		
3-I-C ₆ H ₄ CH ₂	35	71	67	83	56	35	32	91	64		
$2-(CF_3)-4-F-C_6H_4CH_2$	36	71	47	68	17	37	13	66	23		
$2.3.6 - F_3 - C_6 H_2 C H_2$	37	68	57	73	16	27	5	71	62		
$2.5 - (CF_2)_2 - C_2 + C_2 + C_2$	38	37	0	16	13	7	24	14	10		
4-(CH ₂)-C ₄ H ₄ CH ₂	39	67	74	83	46	43	9	85	59		
CH ₂ CH ₂	40	18	0	6	48	4	11	12	2		
CH ₂ (CH ₂) ₂	41	58	5	31	74	8	10	44	8		
CH ₂ (CH ₂) ₂	41	79	35	55	71	10	4	70	40		
CH ₂ (CH ₂)	42	79	37	55	40	0	11	58	30		
$CH_{2}(CH_{2})_{4}$	- 1 5 44	80	50	60	71	0	13	65	30		
$CH_{(CH_{2})5}$	-++	00 97	59 71	80	/ I \$0	17	13	74	39 40		
$CH_{2}(CH_{2})_{6}$	4J AC	0/	/1	0U 92	07	1 / 6	23	/4 82	40		
$C \Pi_3(C \Pi_2)_7$	40	80 70	82 72	80 85	95 24	6	30	82 85	54		
$C_6H_5CH_2$	4/	12	12	85	34	69	11	85	69		
	48	0.0	C 1	62	20	•	~ 4	<u>.</u>			
$2 - (C_6H_5) - C_6H_4CH_2$	Me-Indoxam	90	81	92	39	28	34	94	67		

Table 3. Inhibition of $sPLA_{28}$ by indoles bearing a 5-(4-oxy-butanoic acid) side chain



R Group	Compd	% Inhibition			
		hGIIA 1 µM	hGX 1 μM		
2,6-Cl ₂ -C ₆ H ₃ CH ₂	49	85	11		
C ₆ F ₅ CH ₂	50	26	4		
4-Br-C ₆ H ₄ CH ₂	51	83	20		
C ₆ H ₅ COCH ₂	52	37	43		
3-Cl-C ₆ H ₄ CH ₂	53	87	36		
2-NapthaleneCH ₂	54	93	4		
4-CN-C ₆ H ₄ CH ₂	55	33	20		
$2-CN-C_6H_4CH_2$	56	0	13		
3-CN-C ₆ H ₄ CH ₂	57	10	10		
(CH ₃) ₂ CHCH ₂	58	29	0		
2-(OCH ₃)-5-(NO ₂)-C ₆ H ₃ CH ₂	59	63	20		
3-Br-C ₆ H ₄ CH ₂	60	83	11		
2-Br-C ₆ H ₄ CH ₂	61	75	43		
$4-F-C_6H_4CH_2$	62	49	26		
$3-(NO_2)-C_6H_4CH_2$	63	5	10		
4-(7-CH ₃ O)-coumarinylCH ₂	64	0	18		
2-Cl-C ₆ H ₄ CH ₂	65	77	0		
PhthalimidoCH ₂	66	0	18		
$2-(CF_3)-C_6H_4CH_2$	67	8	6		
$2,4-F_2-C_6H_3CH_2$	68	90	78		
$2,5-F_2-C_6H_3CH_2$	69	27	30		
$2,6-F_2-C_6H_3CH_2$	70	13	26		
$3,4-F_2-C_6H_3CH_2$	71	16	44		
$2,3-F_2-C_6H_3CH_2$	72	55	13		
$3,5-F_2-C_6H_3CH_2$	73	79	68		
$3-(CF_3)-C_6H_4CH_2$	74	46	59		
$4-(CF_3)-C_6H_4CH_2$	75	23	30		
$2 - ((2 - CH_2Br) - C_6H_4) - C_6H_4CH_2$	76	0	32		
$3-(OCF_3)-C_6H_4CH_2$	77	77	32		
$2-(CF_3)-4-F-C_6H_4CH_2$	78	3	15		
$4-(CH_3)-C_6H_4CH_2$	79	89	27		
$CH_3(CH_2)_3$	80	29	28		
$CH_3(CH_2)_4$	81	7	25		
$CH_3(CH_2)_5$	82	8	19		
$CH_3(CH_2)_6$	83	31	36		

Analyses were performed in at least duplicate for hGIIA and hGX sPLA $_2$ enzymes.

3. Discussion

3.1. Inhibition patterns among sPLA₂ enzyme species

The large amount of inhibition data obtained with the 4-(2-oxy-ethanoic acid) analogues and the human sPLA₂s (potency of 48 compounds on eight group I, II, V, and X enzymes) is conveniently summarized by the gray scale array shown in Figure 5A. Figure 5B shows the array for the same compounds as inhibitors of the eight mouse sPLA₂s. Figure 5C shows a comparison of the results obtained with human and mouse sPLA₂s (mGIIC is removed since there is no hGIIC and mGXIIA is removed since inhibition data is not available, see above). The general pattern for potency of

inhibition among the various enzyme species is readily apparent from inspection of these arrays and is summarized as follows. For the human sPLA₂s, several members of the 48-compound, Me-Indoxam analogue library are potent inhibitors (IC₅₀ $< 0.05 \,\mu$ M) of hGIIA, hGIIE and hGV. These compounds show intermediate potency against hGIB (several compounds with an $IC_{50} = 0.05 - 0.5 \mu M$) and hGX (several compounds with an IC₅₀ = 0.5–5 μ M), and are poor inhibitors of hGIID, hGIIF, and hGXIIA (IC₅₀ > 5 μ M). The same set of Me-Indoxam analogues are potent inhibitors of mGIIA, mGIIC, mGIIE, and mGV (several compounds with an $IC_{50} < 0.05 \mu M$), they show intermediate potency toward mGIB (several compounds with an $IC_{50} = 0.05$ -0.5 μ M) and mGX (several compounds with an $IC_{50} = 0.5-5 \mu M$), and are poor inhibitors of mGIID and mGIIF (IC₅₀ > 5 μ M). It can be seen from Figure 5C that for each human/mouse orthologue pair, the inhibition potencies for the Me-Indoxam analogues acting on the human sPLA₂ matches closely to that measured for its mouse orthologue (hGIB versus mGIB, hGIIA versus mGIIA, hGIID versus mGIID, hGIIE versus mGIIE, hGIIF versus mGIIF, hGV versus mGV, and hGX versus mGX). This is expected given that for each orthologue pair, the active site residues are highly conserved (based on the hGIIA and hGX X-ray structures and the active site models for the remaining groups I, II, V, and X enzymes).

3.2. Interactions between enzyme residues and the indole core of Me-Indoxam and its analogues

The trends in inhibition potency among the different sPLA₂ species noted above tend to hold as the N₁-substituent is varied. In addition, the contacts between the glyoxamide and carboxymethyl side chains of Me-Indoxam and the enzyme are conserved among all of the sPLA₂s. This suggests that it is the residues that interact with the remaining portion of Me-Indoxam, the indole core, that are responsible for the differences in inhibition potency among the sPLA₂ enzyme species. For the hGX-Me-Indoxam complex, these residues are Ile-2, Leu-5, Gly-28, and Leu-29 (Fig. 3). The residues at these positions for all of the human and mouse groups I, II, V, and X sPLA₂s are listed in Table 4 (supplemental information). For all of the enzymes, glycine is conserved at position-28. This leaves residues 2, 5, and 29 for further consideration. When residue 5 is a Phe, the edge of this aromatic group would form a favorable interaction with the aromatic face of the indole ring of Me-Indoxam. Such an interaction between two aromatic rings that are perpendicular to each other is thought to be highly favorable.55 This could explain why Me-Indoxam and many of its analogues are generally potent inhibitors of hGIB, mGIB, hGIIA, mGIIA, mGIIC, hGIIE, and mGIIE, all of which have a Phe-5 (Table 4, supplemental information). Within this group of enzymes, less potent inhibition is seen with hGIB and mGIB. This may be due to the fact that the group IB enzymes have a Val at position-2, which is not quite long enough to contact the indole core of the inhibitor. In contrast, the group IIA, IIC, and IIE sPLA₂s contain longer aliphatic side chains at position-2 (Leu, Ile, and Phe) which are predicted to make a direct contact with the indole core of Me-Indoxam, based on the contact between Ile-2 and inhibitor observed in the hGX-Me-Indoxam X-ray structure. Thus, the combination of Phe-5 and Leu, Ile, or Phe at position-2 seems to provide the most favorable interaction with the indole core of Me-Indoxam. The enzymes hGIID, mGIID, hGIIF, mGIIF, hGX, and mGX all have Leu at position-5 instead of Phe, and this may be the reason that Me-Indoxam and its analogues are generally poor inhibitors of these enzymes. The group V sPLA₂s represent an exception to these trends. They have Leu-2, which favors Me-Indoxam binding, but they contain Leu-5 instead of Phe-5, which should disfavor binding and yet many of the Me-Indoxam analogues are potent hGV and mGV inhibitors.



Figure 5. Array of potencies of inhibition of human and mouse sPLA₂s by Me-Indoxam and its analogues. Panel A is for the human enzymes indicated along the horizontal top edge of the array, panel B is for the mouse enzymes, and panel C is human and mouse enzymes combined. The vertical edge of the array gives the compound numbers for the Me-Indoxam analogues with the 4-(2-oxy-ethanoic acid) side chain (see Table 1). Inhibition potency was classified into 4 gray scale categories as follows: black is $IC_{50} < 0.05 \ \mu$ M, dark gray is $IC_{50} = 0.05-0.5 \ \mu$ M, light gray is $IC_{50} = 0.5-5 \ \mu$ M, and white is $IC_{50} > 5 \ \mu$ M.

However, the group V sPLA₂s are unique in containing a Trp at position-29, whereas all of the other sPLA₂s contain an aliphatic residue at this position (Ile, Leu, Val). The homology models of the group V sPLA₂ active sites suggest that the edge of the indole ring of Trp-29 could form a favorable interaction with the face of the indole ring of Me-Indoxam.

3.3. Interaction between enzyme residues and the N_1 -substituent of Me-Indoxam and its analogues

The X-ray structure of Me-Indoxam bound to hGX shows that the 2-phenyl group on the N₁-benzyl chain does not form significant interactions with the enzyme (discussed above). This is predicted to be the case for all group I, II, V, and X sPLA₂s, based on modeling. This presumably explains why the potency of Me-Indoxam and 47, which lacks the 2-phenyl group, are similar for all sPLA₂s (maximum difference in potency of \sim 3-fold).

Inspection of the hGX-Me-Indoxam X-ray structure suggests that a 2-, 3- or 4-substituent on the N_1 -benzyl group would not influence sPLA₂ inhibition potency since these substituents protrude away from the enzyme surface. Indeed compounds 4, 6, 7, 9-11, 14-18, 22, 24, 31–35, 39, and 48, which contain a single substitution at either the 2-, 3- or 4-position of the N₁-benzyl chain, display similar inhibition potency as compound 47, which contains an unsubstituted N₁-benzyl chain. This trend is true for all sPLA₂s as predicted from the active site modeling. The same applies for disubstituted N_1 -benzyl chains that include 2-, 3-, and 4-substituents (compounds 25, 28, 29, and 36). It is clear from the X-ray structure of the hGX-Me-Indoxam complex as well as the models for the other sPLA₂ active sites that compounds 13 and 38 are expected to be generally poor inhibitors, and this was found to be the case. These compounds have an N₁-benzyl group with a 2,5disubstitution pattern. This leads to a steric clash with the enzyme. If the 2-substituent points away from the enzyme surface, the 5-substituent would clash with Ala-6 of hGX (as well as corresponding residues of the other sPLA₂s). Flipping the aromatic ring would relieve the steric clash with the 5-substituent and Ala-6, but would position the 2-substituent in steric clash with the indole ring of Me-Indoxam. Poor inhibition was not observed for compound 26, which contains the smaller fluorine atoms at the 2- and 5-position. Compounds 1 and 27, which contain a 2,6-dichloro and 2,6-difluoro pattern of substitution on the benzyl group display similar potency as compound 47, which contains the unsubstituted benzyl chain. Presumably the fluorine and chlorine substituents cause less steric clash because they are smaller than those present on the poorly active compounds with 2,5-disubstituted benzyl chains (13 and 38 which contain larger substituents OMe, NO₂, and CF₃).

Indole analogues containing non-benzyl substituents at the N₁-position (2, 5, 8, 12, 19, 20, 21, 23, 40, 41, 42, 43, 44, 45, and 46) generally display poor inhibition potency against human and mouse $sPLA_2s$. The hGX-Me-Indoxam X-ray structure shows the benzyl group of

Me-Indoxam pointing into the hydrophobic cavity of the enzyme and interacting favorably with Ile-2 and Ala-6. However, the non-benzyl substituents either sterically clash with Ala-6 (8 and 19) or lose their favorable interaction with Ile-2 and Ala-6 (2, 5, and 12) of hGX as seen from modeling of these substituents with the hGX-Me-Indoxam X-ray structure and models of the human and mouse groups I, II, V, and X sPLA₂s. Indole analogue 23 does not contact Ala-6, but instead its phthalimido group sterically clashes with Leu-29 and Gly-30 of hGX because the methylene group between the N_1 -position and the phthalimido group is too short to allow the phthalimido group to protrude out of the active site. Analogue 21 is similar to analogue 12 (branched alkyl group at the N₁-position) except that the side chain is lengthened by one carbon. This reestablishes the favorable interaction with Ala-6 of hGX, as the geometry of the chain rotates to mimic the benzyl substituent of Me-Indoxam. Indole analogues 40-46 display increasing inhibition potency against human and mouse group sPLA₂s. This is due to the filling of the hydrophobic cavity by the increasing size of the saturated alkyl chains. The favorable interactions with Ile-2 and Ala-6 of hGX (and the corresponding residues of the other sPLA₂s modeled) are realized as the chain length is increased to eight carbons. Presumably, after eight carbons this trend would no longer hold, as the remaining methylene groups would protrude out of the cavity and not interact with any protein residues.

3.4. Discovery of compounds useful for cellular studies of sPLA₂ function

With the series of Me-Indoxam analogues prepared in the current study, we have not been able to generate compounds that are highly specific for a single human or mouse sPLA₂. However, some compounds that will be useful in cell culture studies have been found. For example, the original lead compound Me-Indoxam and compound 5 are \sim 5-fold more potent on hGIIA than on hGV, and compound 21 was discovered to have a reverse preference (10-fold more potent on hGV compared to hGIIA). Compound 21 was re-tested on all of the human sPLA₂s to determine the precise IC_{50} values (based on enzyme activity measured in the presence of five inhibitor concentrations): hGIB, $IC_{50} = 0.77 \ \mu M$; hGIIA, $IC_{50} = 1.0 \ \mu M$; hGIID, no inhibition at 5 μ M; hGIIE, IC₅₀=0.04 μ M; hGIIF, no inhibition at $5 \,\mu\text{M}; \text{hGV}, \text{IC}_{50} = 0.1 \,\mu\text{M}; \text{hGX}, \text{IC}_{50} = 5 \,\mu\text{M}; \text{hGXIIA},$ <20% inhibition at 5 µM. Thus, Me-Indoxam or compound 5, when used in combination with compound 21, should be useful for distinguishing cellular events caused by hGIIA versus hGV.

4. Conclusion

In an attempt to find potent and selective inhibitors of mammalian secretory phospholipases A_2 , a library of 83 indole analogues was synthesized and tested against the full set of mouse and human sPLA₂s. Homology models of the human and mouse sPLA₂s were made based on the X-ray structures of the hGIIA and hGX and

inhibitors were designed with structure-guided techniques. The X-ray structure was determined of Me-Indoxam, an indole analogue that is a competitive tight binding inhibitor of several sPLA₂s, and modeling of this structure with the sPLA₂ homology models suggests a good deal of variation in the residues surrounding the N₁-position of the indole. Based on this structure and a series of previously published indoles bound to the hGIIA sPLA₂, a library was generated by varying the N₁-substituent of the indole and the position and length of the calcium coordinating carboxylate arm. All compounds generated were tested in a fluorometric assay employing vesicles with a pyrenedecanoic acid at the sn-2 position of the phospholipid. The structure-activity relationship suggests that a 4-(2-oxy-ethanoic acid) moiety and an N₁-position carrying a substituted benzyl group are the most highly potent against the sPLA₂ enzymes. However, 2,5- and 3,6-disubstituted benzyl groups at the N₁-position cause steric clash and significantly diminish the inhibition potency. Although no single compound was highly selective for a single sPLA₂ enzyme, a combination of compounds with a 5 to 10-fold inverse selectivity preference for the hGV versus hGIIA was found and should prove useful in cell culture studies.

5. Experimental

5.1. Crystallization and X-ray data collection

The purified recombinant hGX sPLA₂ was prepared as described elsewhere.⁵² The hGX-Me-Indoxam complex was co-crystallized at ambient temperature in 2 μ L hanging drops of a mixture of 1 μ L of a pre-equilibrated solution containing 15 mg/mL protein, 0.56 mM Me-Indoxam, 10 mM CaCl₂, 10% (v/v) MPD, 0.25% (v/v) DMSO, and 1 μ L of a crystallization reservoir solution of 10 mM CaCl₂, 12% (w/v) PEG 3500, 10% (v/v) MPD, 2% (v/v) ethylene glycol and 0.2 M Hepes buffer, pH 7.4. Crystals of rectangular shape appeared within a week and were allowed to grow for two weeks before data collection.

X-ray diffraction data were collected on a Rigaku RU-H3R rotating anode generator with a RAXIS IV image plate area detector. Crystals were pre-treated with a cryo-protecting and stabilizing solution made of the crystallization condition reported above with the addition of 10% (v/v) glycerol and flash frozen in the -180 °C nitrogen cryo-stream. X-ray diffraction data were collected from a single crystal that diffracted to a resolution of 1.97 Å. The programs DENZO and SCALEPAK were used for data processing.⁵⁶ The data collection statistics are shown in Table 5 (supplemental information). The protein crystal belongs to space group C222₁ with 2 copies of the hGX-Me-Indoxam complex in the asymmetric unit.

5.2. X-ray structure solution and refinement

The structure was solved by molecular replacement using the program AMORE⁵⁷ using a ligand free struc-

ture of hGX⁵² (pdb code 1LE6) as the search model. Standard refinement techniques in the program CNS-1.1⁵⁸ such as rigid body, positional and simulated annealing refinement were employed during cycles of refinement. Manual model improvement was done using the graphic programs CHAIN⁵⁹ and O.⁶⁰ The Me-Indoxam ligand was built into one of the two subunits of the protein when the R_{free} and $R_{working}$ were at 0.339 and 0.279, respectively at resolution 2.2 Å. Following a round of refinement, the electron density difference maps (2Fo-Fc) showed clear electron density for the active site ligand. Next, one round of positional and simulated annealing refinement was performed using Xray diffraction data that was extended to a resolution of 2.0 Å. Electron density difference maps directed the building of a second Me-Indoxam molecule into the second subunit's active site and was refined. Finally, a total of 164 solvent molecules were built into difference electron density (Fo–Fc), and iterative rounds of model adjustment and refinement converged. The final model has a value of R_{free} and R_{working} of 0.269 and 0.221, respectively with good geometry.

5.3. Synthesis of Me-Indoxam analogues

5.3.1. General methods. Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification and all non-aqueous reactions were carried out under an argon atmosphere with oven-dried glassware. THF was distilled under argon from sodium/benzophenone and CH2Cl2 was distilled over CaH₂ immediately prior to use. Reaction progress was monitored by thin-layer chromatography on Merck 60F₂₅₄ silica plates. Unless otherwise specified, extracts were dried over MgSO₄ and solvents were removed with a rotary evaporator at aspirator pressure. All compounds were purified by column chromatography using Merck 60 230-400 mesh silica gel. NMR spectra were recorded on Bruker AC-200 (200 MHz) or AC-300 (300 MHz) spectrometers. Unless otherwise specified, all spectra were obtained in CDCl₃ and chemical shifts are reported in parts per million relative to TMS. Low-resolution mass spectral data was obtained for all compounds using a Bruker Daltonics Esquire electrospray-ion trap mass spectrometer. High-resolution mass spectral data was obtained on a Bruker Daltonics APEX III 47e Fourier transform (Ion Cyclotron Resonance) Mass Spectrometer for two key compounds of the library, compounds 5 and 21. (Compound 5: HR-ESI-MS m/z 369.1426 calculated for C₁₈H₂₂N₂O₅Na [M+Na]⁺, found 369.1427. Compound 21: HR-ESI-MS m/z 417.1063 calculated for C₂₁H₁₈N₂O₆Na [M+Na]⁺, found 417.1068.)

({3-[Amino(oxo)acetyl]-1-(biphenyl-2-ylmethyl)-2methyl-1*H*-indol-4-yl}oxy) acetic acid (Me-Indoxam). Prepared according to the literature procedure³³ except that the starting material used was the commercially available 4-hydroxy-2-methylindole (A) from TCI America. ¹H NMR (300 MHz, DMSO- d_6 , δ): 2.35 (s, 3H), 4.66 (s, 2H), 6.36 (s, 2H), 6.40 (d, 1H), 6.51 (d, 1H), 6.88 (d, 1H), 7.02 (t, 1H), 7.19–7.58 (m, 8H), 7.73 (s, 1H), 12.90 (br s, 1H).

tert-Butyl [(2-methyl-1H-indol-4-yl)oxylacetate (B). The commercially available 4-hydroxy-2-methylindole (A) (3.00 g, 20.38 mmol) and *t*-butylbromoacetate (3.98 g, 100 g)20.38 mmol) were dissolved in 100 mL acetone (freshly distilled from $CaSO_4$) in a 250 mL round bottom flask. To this solution at room temperature was added anhydrous potassium carbonate (5.66 g, 40.80 mmol) and the stirred mixture brought to reflux for 12 h. The solid was removed by filtration and washed with two 20 mL portions of acetone. The solvent was removed by rotary evaporation to leave an off-white solid. This solid was dissolved in 50 mL EtOAc and washed with 50 mL of a brine solution in a separatory funnel. The aqueous phase was extracted twice with 20 mL portions of EtOAc and the organic layers combined, dried over MgSO₄ and evaporated to leave an off-white solid. The product was purified by flash column chromatography on silica gel using 20% EtOAc/hexanes as eluant to give 4.131 g of a white powder (77% yield). This compound decomposes quickly in solution when not under argon. The product of decomposition is a bright green color. Therefore, it is imperative to work quickly, including the purification step. ¹H NMR (200 MHz, CDCl₃, δ): 1.51 (s, 9H), 2.42 (s, 3H), 4.65 (s, 2H), 6.40-6.47 (m, 2H), 6.85 (d, 1H), 7.07 (t, 1H), 7.91 (br s, 1H).

¹⁴C-Labeled *tert*-butyl ({3-[amino(oxo)acetyl]-2-methyl-1*H*-indol-4-yl}oxy)acetate (C). To ¹⁴C-labeled oxalic acid solid (50 µCi, 10 mCi/mmol) from American Radiolabeled Chemicals Inc. in an amber vial under argon atmosphere was added freshly distilled, unlabeled oxalyl chloride (185 µL, 2.12 mmol) and approximately 3 mg of phosphorus pentachloride solid. The exact weight of the phosphorus pentachloride could not be obtained, as it is sensitive to moisture, which causes the weight to change rapidly. The mixture was refluxed for 10 min until the evolution of bubbles had ceased upon standing at room temperature. The entire contents of the vial were transferred to a 1 L round bottom flask containing 400 mL freshly distilled CH₂Cl₂. Compound (B) (0.490 g, 1.86 mmol) was added as a solution in 100 mL freshly distilled CH₂Cl₂ over 1 h under an argon atmosphere via addition funnel. The bright yellow solution was left to stir for 8 h at room temperature before ammonia gas was bubbled into the solution for 15 min, at which point the mixture became pale yellow and a fluffy white solid precipitated. The precipitate was removed by filtration and the solvent removed by rotary evaporation to leave a yellow solid. The crude solid was dissolved in 150 mL EtOAc and washed with water followed by brine. The organic layer was dried over MgSO4 and purified by column chromatography on silica gel using EtOAc as the eluant. The product was obtained in 87% yield as a bright yellow powder with a specific activity of 7160 cpm/ μmol. ¹H NMR (200 MHz, CD₃OD, δ): 1.46 (s, 9H), 2.58 (s, 3H), 4.61 (s, 2H), 6.50 (dd, 1H), 6.98–7.12 (m, 2H).

Library synthesis of indole analogues (D). A collection of various alkyl bromides from Aldrich Chemicals was purchased in small quantities (10–12 mg each) and each was delivered in a separate 4 mL amber vial. These compounds were dissolved in 1.00 mL of freshly distilled THF and capped. When not in use the vials were stored at -20 °C in jars containing CaSO₄ as desiccant. Fifty μ L of each of these solutions was added to individual 4 mL vials awaiting a reaction with compound (C). A 4 mL vial was charged with compound (C) (20.0 mg, 0.06 mmol) and flushed with argon. The solid was dissolved in 2.25 mL freshly distilled THF. To this solution was added NaH (3.5 mg, 0.15 mmol) without mineral oil and the reaction left to stir at room temperature for 10 min, at which point it became a bright yellow color. The entire contents of the vial were removed by gas-tight syringe and 25 μ L aliquots were added to each of the vials containing the alkyl bromide/THF solutions. These vials were then heated at 60 °C for 2 h followed by removal of solvent by entrainment with air. The vials containing the crude products were stored free of solvent at -20 °C until purified by HPLC. To each vial was added neat trifluoroacetic acid (100 µL, 1.30 mmol), vials were mixed on a vortex mixer for 15 s and left for 10 min at room temperature. The excess trifluoroacetic acid was removed by entrainment with air. The compounds were then purified using high performance liquid chromatography and eluting with a methanol/water/0.06% TFA system on a Vydac C-18 reverse phase semi-preparative 218TP1010 column using UV-Vis detection at 262 nm. The purified compounds were identified by electrospray mass spectrometry with characteristic fragmentation patterns. A speed vac (Savant Instruments) was used to remove the HPLC solvents from the fractions containing the desired products to leave solids. These solids were dissolved in 50 µL DMSO and transferred to Eppendorf tubes. An aliquot of this DMSO solution was used to quantify the compound using a liquid scintillation counter. Dilutions were made of each of the stock DMSO/inhibitor solutions in preparation for inhibition assays and stored at -20 °C.

tert-Butyl 4-[(2-methyl-1H-indol-5-yl)oxy]butanoate (F). A portion of 5-hydroxy-2-methylindole (E) (200.0 mg, 1.36 mmol) from Biosynth AG and tert-butyl 4-bromobutanoate (455 mg, 2.04 mmol) (prepared according to the literature procedure⁶¹) were dissolved in 20 mL acetone (freshly distilled from CaSO₄) in a 50 mL round bottom flask under argon. To this solution at room temperature was added anhydrous potassium carbonate (1.00 g, 6.8 mmol) and the stirred mixture brought to reflux for 3 days. The solid was removed by filtration and washed twice with 10 mL portions of acetone. The solvent was removed by rotary evaporation to leave a yellow oil. This oil was dissolved in 50 mL EtOAc and washed with 50 mL of brine in a separatory funnel. The aqueous phase was extracted with three 20 mL portions of EtOAc and the organic layers combined, dried over MgSO₄ and evaporated to leave a yellow oil. The product was purified by flash column chromatography on silica gel using 20% EtOAc/hexanes as eluant to give 359 mg (77% yield) of yellow oil. ¹H NMR (300 MHz, CDCl₃, δ): 1.49 (s, 9H), 2.04 (m, 2H), 2.42 (s, 3H), 2.46 (s, 2H), 4.03 (t, 2H), 6.12 (br s, 1H), 6.75 (dd, 1H), 6.99 (d, 1H), 7.17 (dd, 1H), 7.80 (br s, 1H).

¹⁴C-Labeled *tert*-butyl 4-($\{3-[amino(oxo)acetyl]-2-methyl-1H-indol-5-yl\}oxy$)butanoate (G). To ¹⁴C-labeled oxalic acid solid (50 µCi, 3 mCi/mmol) from American Radio-

labeled Chemicals Inc. in 1 mL dry CH₂Cl₂ in an amber vial was added freshly distilled, unlabeled oxalyl chloride (100 μ L, 1.15 mmol) and approximately 3 mg of phosphorus pentachloride solid (see compound (C)). The mixture was refluxed for 30 min under an argon atmosphere and then the entire contents of the vial were transferred to a 1-liter round bottom flask containing 400 mL freshly distilled CH₂Cl₂. Compound (F) (219 mg, 0.756 mmol) was dissolved in 100 mL dry CH₂Cl₂ and added over 1 h under an argon atmosphere. The solution was left to stir for 12 h at room temperature before ammonia gas was bubbled into the solution for 15 min, at which point a white solid precipitated. The solid was removed by filtration and the solvent removed by rotary evaporation to leave an off white solid. The crude solid was dissolved in 100 mL EtOAc and washed with water followed by brine. The organic layer was dried over MgSO₄ and purified by column chromatography on silica gel using 75% EtOAc/hexanes as eluant. The product was obtained as an off-white powder with a specific activity of 11,723 cpm/µmol (85% yield). ¹H NMR (300 MHz, CD₃OD, δ): 1.43 (s, 9H), 2.03 (m, 2H), 2.42 (t, 2H), 2.64 (s, 3H), 4.00 (t, 2H), 6.81 (dd, 1H), 7.22 (dd, 1H), 7.61 (d, 1H).

Library synthesis of indole analogues (H). The alkyl bromides used in the generation of the first library were also used to generate this library of compounds. The alkyl bromides were stored as solutions in dry THF in a dessicated jar at -20 °C. Twenty-five µL aliquots of each of these solutions were added to individual 4 mL vials awaiting a reaction with compound (G). A 4 mL vial was charged with compound (G) (20.0 mg, 0.06 mmol) and flushed with argon. The solid was dissolved in 4 mL freshly distilled THF. To this solution was added NaH (3.5 mg, 0.15 mmol) without mineral oil and the reaction left to stir at room temperature for 10 min, at which point the solution turned yellow-green. The entire contents of the vial were removed by syringe and 50 µL aliquots were added to each of the vials containing alkyl bromide/THF solutions. These vials were then placed in an oven at 60 °C for 2 h. The resultant compounds were treated in the same manner as for the generation of the first library, purified by HPLC, and characterized by electrospray ionization mass spectrometry. The compounds were quantified using liquid scintillation counting and DMSO/inhibitor stock solutions were made as for the first set of library compounds.

Microtiter plate assay of sPLA₂s for inhibition analysis. To each well of a 96-well microtiter plate was added 100 µL of solution A in assay buffer (27 µM bovine serum albumin, 50 mM KCl, 1 mM CaCl₂, 50 mM Tris– HCl, pH 8.0) followed by the desired concentration of sPLA₂ inhibitor (\leq 3 µL in DMSO from serial diluted stock solutions) or 3 µL of DMSO only for control reactions. Solution B was prepared immediately prior to each set of assays, which consisted of only eight wells, to avoid loss of enzymatic activity due to sticking to the walls of the container. Solution B was delivered in 100 µL portions to all eight wells except the first well and had the same composition as Solution A plus 5–2000 ng of sPLA₂ (depending on specific enzymatic activity⁴²). In place of Solution B was added an additional 100 µL portion of Solution A as a minus enzyme control to the first of the eight wells in the assay. Quickly after the addition of Solution B, the assay was initiated by the addition of 100 µL of Solution C (4.2 µM 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (Molecular Probes) vesicles in assay buffer) with a repeating pipettor to all eight wells. The fluorescence (excitation = 342 nm, emission = 395 nm) was read with a microtiter plate spectrophotometer (Fluorocount, Packard Instruments). Control reactions without enzyme or inhibitor were run with each assay of eight wells and the percent inhibition calculated from the initial slopes of fluorescence versus time. The amount of enzyme used per well are as follows: hGIB, 0.5 ng; hGIIA, 0.5 ng; hGIID, 200 ng; hGIIE, 500 ng; hGIIF, 30 ng; hGIII, 5 ng; hGIII, 5 ng; hgV, 1 ng; hGX, 1 ng; hGXIIA, 1,500 ng; mGIB, 1.6 ng; mGIIA, 8 ng; mGIIC, 12 ng; mGIID, 2,000 ng; mGIIE, 5 ng; mGIIF, 100 ng; mGV, 6 ng; mGX, 3.5 ng. All of the recombinant mouse and human sPLA₂s were prepared as described.42

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