

The First Potent Inhibitor of Mammalian Group X Secreted Phospholipase A₂: Elucidation of Sites for Enhanced Binding

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Abstract: Using the X-ray structure of human group X secreted phospholipase A₂ (hGX), we carried out structure-based design of indole-based inhibitors and prepared the compounds using a new synthetic route. The most potent compound inhibited hGX and the mouse orthologue with an IC₅₀ of 75 nM. This compound is the most potent hGX inhibitor reported to date and was also found to inhibit a subset of the other mouse and human sPLA₂s.

Secreted phospholipases A₂ (sPLA₂s) are a group of enzymes with conserved active sites and calcium-binding loops that catalyze the hydrolysis of the *sn*-2 ester of glycerophospholipids to release free fatty acids and 2-lysophospholipids.^{1–4} The sPLA₂s are low molecular weight (~14–20 kDa) enzymes with 5–8 disulfide bonds and require Ca²⁺ as a catalytic cofactor.^{1–4} To date, there are 10 distinct mammalian sPLA₂s (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA), with group IIC present as a pseudogene in humans.^{1,2} Biomedical interest in this class of enzymes stems from the observation that one or more plays a role in the liberation of arachidonic acid from cellular phospholipids for the biosynthesis of eicosanoids. Mammalian cells also contain a cytosolic phospholipase (cPLA₂-α).⁵ Recent evidence shows that in macrophages and other cells, sPLA₂s and cPLA₂-α work together to liberate arachidonic acid in agonist-stimulated mammalian cells.^{6–9} The molecular basis for the cross-talk between these two enzymes is not known. Potent and selective inhibitors of the sPLA₂ enzymes would help resolve some of the questions surrounding the mechanism of arachidonate release as well as probe other physiological functions of sPLA₂s.

For the past decade, the group IIA sPLA₂ has received the most attention among the sPLA₂s because it was the first nonpancreatic sPLA₂ to be discovered, and it is found in high levels in patients suffering from inflammatory diseases including rheumatoid arthritis¹⁰ and acute pancreatitis.¹¹ The group IIA sPLA₂ has been the focus of medicinal chemistry efforts at several pharmaceutical companies.^{12–16} Workers at Lilly Labs and Shionogi & Co. Ltd. have reported on substituted indoles, exemplified by compound **I**, as potent inhibitors of group IIA sPLA₂ (Figure 1).^{12–15} Among the various reported inhibitors of sPLA₂,¹⁷ these compounds appear to be the most potent and also appear to have the most drug-like properties. With the discovery of additional sPLA₂ enzymes, we have been interested in exploring these indole analogues as inhibitors of all of the members of the sPLA₂ enzyme class.¹⁸ We are particularly interested in the group X sPLA₂ because it appears to have the highest specific activity in promoting arachidonic acid release from mammalian cells.^{7,19,20} To date, very few inhibitors of the group X sPLA₂ have been reported, with the general sPLA₂

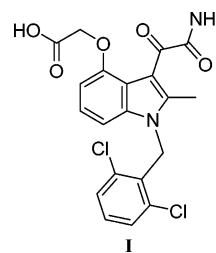


Figure 1. Representative structure of a substituted indole inhibitor of group IIA sPLA₂. Indole positions 1–7 are shown.

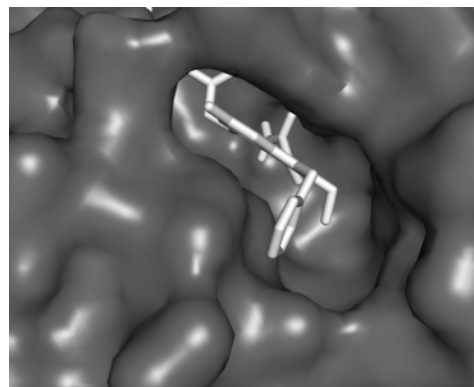


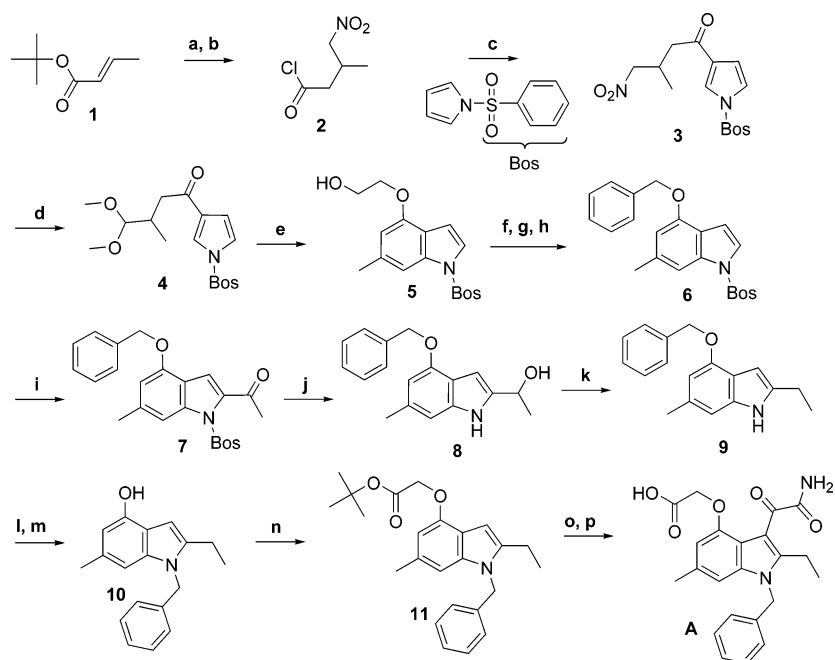
Figure 2. Compound A docked into the active site of hGX sPLA₂. As can be seen, the 2-ethyl substituent makes contact with the active site wall and the 6-methyl group sticks out of the active site.

inhibitor YM-26734 being the most potent at 0.20 μM against the group X enzyme.²¹ To this end, we recently determined the X-ray structure of the indole-based inhibitor Me-Indoxam bound to human group X sPLA₂ (hGX).¹⁸ We now report the development of a potent indole-based inhibitor of hGX that was designed based on this structural information.

In our first attempt to develop potent indole-based inhibitors of sPLA₂s other than the group IIA enzyme, we made a library of analogues in which the substituent attached to N1 of the indole ring was varied.¹⁸ The range of IC₅₀ values for this library was not very broad, and potent inhibitors of hGX were not obtained. Subsequent X-ray structural studies showed that this substituent largely points out of the active site of the sPLA₂, toward what would be the membrane plane when the enzyme is bound to the phospholipid bilayer.¹⁸ Thus, we turned to chemical modifications of other areas of the indole ring.

Inspection of the X-ray structure of Me-Indoxam bound to hGX sPLA₂¹⁸ reveals a large hydrophobic pocket near the 2-substituent that makes little interaction with the inhibitor. We hypothesized that a larger 2-alkyl substituent would bind to the hydrophobic pocket and increase the binding affinity of the inhibitor. Also, the 6-position of the indole points out of the enzyme active site and does not contribute to binding affinity. However, modification of the 6-position may be useful for modifying the physicochemical properties of the indole for subsequent use in whole animal studies to affect pharmacokinetics. Docking studies were performed on the hGX enzyme in which the indole inhibitor was modified to include a 2-ethyl and 6-methyl, and the N1 substituent replaced with a benzyl (Figure 2). As suspected, the larger 2-ethyl group contacts the inner wall of the enzyme much better than a 2-methyl group, and a 6-methyl group sticks out of the enzyme active site and should not affect binding. As the reported synthesis¹² of

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Scheme 1. Synthesis of sPLA₂ Inhibitor A^a

^a Reagents: (a) CH₃NO₂, KF, 18-Crown-6, ACN, reflux; (b) (i) H₂SO₄, CHCl₃, reflux; (ii) SOCl₂, reflux; (c) AlCl₃, ClCH₂CH₂Cl; (d) (i) NaOH, -20 °C, MeOH; (ii) H₂SO₄, -20 °C, MeOH; (e) p-TsOH, toluene, HOCH₂CH₂OH, reflux; (f) CCl₄, PPh₃; (g) 12 equiv of *n*-BuLi, THF; (h) NaH, BnBr, DMF; (i) *n*-BuLi, THF, -78 °C, Ac₂O; (j) LAH, THF, reflux; (k) NaBH₄, TFA, THF; (l) NaH, BnBr, DMF; (m) H₂, Pd/C, MeOH; (n) NaH, BrCH₂CO₂*t*-Bu, DMF; (o) (i) (ClCO)₂, CH₂Cl₂; (ii) NH₃; (p) TFA, CH₂Cl₂.

substituted 2-ethyl indoles was unsuccessful in our laboratory and the starting material for introduction of the 6-methyl substituent is not commercially available, a novel synthesis for 2-ethyl-6-methyl indoles was developed (Scheme 1). As there are few known literature reactions to functionalize the 6-position of an indole, the indole core had to be built up from pyrrole. Michael addition of nitromethane to *tert*-butyl crotonate followed by deprotection of this ester and subsequent treatment with thionyl chloride produced the acyl chloride **2**. This was then added to benzenesulfonyl protected pyrrole in the presence of aluminum trichloride to give ketone **3**. Treatment of **3** with NaOH in MeOH at low temperature followed by concentrated H₂SO₄ yielded dimethyl acetal **4**. Ring closure to form the 4-oxyethanol indole **5** was accomplished by addition of a catalytic amount of acid with refluxing in toluene/ethylene glycol solvent. Conversion to the chloride followed by addition of excess *n*-butyllithium and benzyl protection yielded indole **6**. Addition of *n*-butyllithium and acetic anhydride produced the desired 2-acetyl compound **7** due to the ortho-lithiating director used to protect the N1-position of the indole. Removal of the benzenesulfonyl protecting group and reduction of the ketone was accomplished in one step by refluxing in excess lithium aluminum hydride. Deoxygenation at the 2-position was accomplished using NaBH₄ and trifluoroacetic acid to produce the 2-ethyl indole **9**. N1-benylation and 4-hydroxy deprotection followed by addition of *tert*-butyl bromoacetate yielded the *tert*-butyl oxyethanoate **11**. Treatment of compound **11** with dilute oxalyl chloride followed by ammonia gas and deprotection of the *tert*-butyl ester with trifluoroacetic acid yielded the desired substituted 2-ethyl-6-methyl indole (compound **A**). Compounds **B–D** (Figure 3) were synthesized similarly (Supporting Information).

To test the indole analogues as sPLA₂ inhibitors, we used a fluorometric assay consisting of unilamellar vesicles of 1-hexadecanoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol.²² The sPLA₂-catalyzed liberation of 10-pyrenedecanoic acid

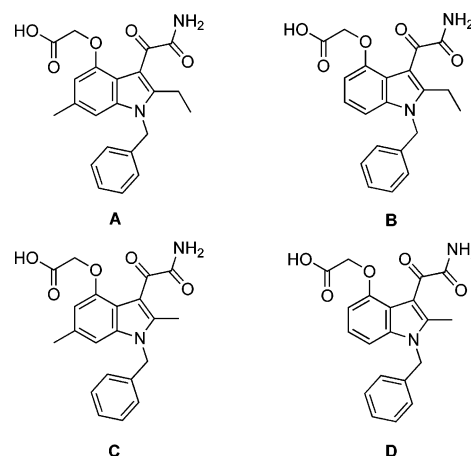


Figure 3. Structures of sPLA₂ inhibitors with varying alkyl groups at the 2- and 6-positions.

allows the fluorophore to dislodge from the vesicles and bind to albumin in the buffer phase where it now undergoes monomer fluorescent emission rather than excimer emission. The assay results (Table 1) show the 2-ethyl substituent to have a dramatic effect on binding to the hGX, with IC₅₀ values of 75 nM for compounds **A** and **B**. The 2-ethyl compounds (**A** and **B**) are 26-fold more potent than the analogous 2-methyl compounds (**C** and **D**) against hGX, which have IC₅₀ values of 2 μM. The 6-methyl substituent has no effect on hGX binding; compounds **A** and **B** have identical IC₅₀ values. The inhibitors were then screened against a panel of recombinant human and mouse sPLA₂s (hGIB, mGIB, hGIIA, mGIIA, hGIIE, mGIIE, hGV, mGV, hGX, and mGX). In all cases the 2-ethyl compounds are more potent than the 2-methyl derivatives, and the 6-methyl group is tolerated (Table 1). Compounds **A** and **B** should be useful in distinguishing the groups X and V sPLA₂s based on the ~10-fold increased potency for the former. This is significant because current evidence favors a role of these two

Table 1. Inhibition Data against Mammalian sPLA₂s for Compounds A–D^a

sPLA ₂	compound IC ₅₀ (μM)			
	A	B	C	D
hGIB	0.80 ± 0.10	0.75 ± 0.15	2.00 ± 0.20	2.50 ± 0.25
mGIB	0.20 ± 0.05	0.14 ± 0.075	2.00 ± 0.10	2.20 ± 0.15
hGIIA	0.125 ± 0.03	0.125 ± 0.02	0.30 ± 0.05	0.275 ± 0.05
mGIIA	0.05 ± 0.01	0.07 ± 0.02	0.125 ± 0.02	0.125 ± 0.02
hGIIE	0.05 ± 0.01	0.05 ± 0.02	0.125 ± 0.03	0.075 ± 0.01
mGIIE	0.075 ± 0.02	0.075 ± 0.02	0.40 ± 0.05	0.40 ± 0.04
hGV	0.50 ± 0.1	0.50 ± 0.05	0.80 ± 0.05	0.80 ± 0.05
mGV	0.75 ± 0.15	0.75 ± 0.10	0.85 ± 0.05	1.00 ± 0.075
hGX	0.075 ± 0.01	0.075 ± 0.01	2.20 ± 0.10	2.00 ± 0.15
mGX	0.075 ± 0.01	0.075 ± 0.01	2.50 ± 0.15	2.50 ± 0.20

^a IC₅₀s are based on duplicate or triplicate analyses.

sPLA₂s in arachidonate liberation in mammalian cells. Although these compounds are also potent inhibitors of the group IIA sPLA₂s, the original lead compound Me-Indoxam is 50-fold more potent on hGIIA and mGIIA versus hGX and mGX.¹⁸ Thus, by carrying out studies with a combination of inhibitors, it should be possible to probe for the role of specific sPLA₂s in cellular processes.

In conclusion, the first potent inhibitor against hGX and mGX sPLA₂s has been discovered. A new chemical route to these indole-based sPLA₂ inhibitors has been developed.

Supporting Information Available: Experimental details including the synthesis of all compounds and assay procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- Valentin, E.; Lambeau, G. Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins. *Biochim. Biophys. Acta* **2000**, *1488*, 59–70.
- Six, D. A.; Dennis, E. A. The expanding superfamily of phospholipase A(2) enzymes: classification and characterization. *Biochim. Biophys. Acta* **2000**, *1488*, 1–19.
- Berg, O. G.; Gelb, M. H.; Tsai, M. D.; Jain, M. K. Interfacial enzymology: the secreted phospholipase A(2)-paradigm. *Chem. Rev.* **2001**, *101*, 2613–2654.
- Murakami, M.; Kudo, I. Phospholipase A2. *J. Biochem. (Tokyo)* **2002**, *131*, 285–292.
- Leslie, C. C. Properties and regulation of cytosolic phospholipase A2. *J. Biol. Chem.* **1997**, *272*, 16709–16712.
- Satake, Y.; Diaz, B. L.; Balestrieri, B.; Lam, B. K.; Kanaoka, Y.; Grusby, M. J.; Arm, J. P. Role of group V phospholipase A2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. *J. Biol. Chem.* **2004**, *279*, 16488–16494.
- Mounier, C. M.; Ghomashchi, F.; Lindsay, M. R.; James, S.; Singer, A. G.; Parton, R. G.; Gelb, M. H. Arachidonic acid release from mammalian cells transfected with human groups IIA and X secreted phospholipase A(2) occurs predominantly during the secretory process and with the involvement of cytosolic phospholipase A(2)-alpha. *J. Biol. Chem.* **2004**, *279*, 25024–25038.
- Balsinde, J.; Balboa, M. A.; Yedgar, S.; Dennis, E. A. Group V phospholipase A(2)-mediated oleic acid mobilization in lipopolysaccharide-stimulated P388D(1) macrophages. *J. Biol. Chem.* **2000**, *275*, 4783–4786.

- Kuwata, H.; Nonaka, T.; Murakami, M.; Kudo, I. Search of factors that intermediate cytokine-induced group IIA phospholipase A2 expression through the cytosolic phospholipase A2- and 12/15-lipoxygenase-dependent pathway. *J. Biol. Chem.* **2005**, *280*, 25830–25839.
- Hara, S.; Kudo, I.; Chang, H. W.; Matsuta, K.; Miyamoto, T.; Inoue, K. Purification and characterization of extracellular phospholipase A2 from human synovial fluid in rheumatoid arthritis. *J. Biochem. (Tokyo)* **1989**, *105*, 395–399.
- Gronroos, J. M.; Nevalainen, T. J. Increased concentrations of synovial-type phospholipase A2 in serum and pulmonary and renal complications in acute pancreatitis. *Digestion* **1992**, *52*, 232–236.
- Dillard, R. D.; Bach, N. J.; Draheim, S. E.; Berry, D. R.; Carlson, D. G.; Chirgadze, N. Y.; Clawson, D. K.; Hartley, L. W.; Johnson, L. M.; Jones, N. D.; McKinney, E. R.; Mihelich, E. D.; Olkowski, J. L.; Schevitz, R. W.; Smith, A. C.; Snyder, D. W.; Sommers, C. D.; Wery, J. P. Indole inhibitors of human nonpancreatic secretory phospholipase A2. 1. Indole-3-acetamides. *J. Med. Chem.* **1996**, *39*, 5119–5136.
- Dillard, R. D.; Bach, N. J.; Draheim, S. E.; Berry, D. R.; Carlson, D. G.; Chirgadze, N. Y.; Clawson, D. K.; Hartley, L. W.; Johnson, L. M.; Jones, N. D.; McKinney, E. R.; Mihelich, E. D.; Olkowski, J. L.; Schevitz, R. W.; Smith, A. C.; Snyder, D. W.; Sommers, C. D.; Wery, J. P. Indole inhibitors of human nonpancreatic secretory phospholipase A2. 2. Indole-3-acetamides with additional functionality. *J. Med. Chem.* **1996**, *39*, 5137–5158.
- Draheim, S. E.; Bach, N. J.; Dillard, R. D.; Berry, D. R.; Carlson, D. G.; Chirgadze, N. Y.; Clawson, D. K.; Hartley, L. W.; Johnson, L. M.; Jones, N. D.; McKinney, E. R.; Mihelich, E. D.; Olkowski, J. L.; Schevitz, R. W.; Smith, A. C.; Snyder, D. W.; Sommers, C. D.; Wery, J. P. Indole inhibitors of human nonpancreatic secretory phospholipase A2. 3. Indole-3-glyoxamides. *J. Med. Chem.* **1996**, *39*, 5159–5175.
- Schevitz, R. W.; Bach, N. J.; Carlson, D. G.; Chirgadze, N. Y.; Clawson, D. K.; Dillard, R. D.; Draheim, S. E.; Hartley, L. W.; Jones, N. D.; Mihelich, E. D.; Olkowski, J. L.; Snyder, D. W.; Sommers, C.; Wery, J. P. Structure-based design of the first potent and selective inhibitor of human nonpancreatic secretory phospholipase A2. *Nat. Struct. Biol.* **1995**, *2*, 458–465.
- Beaton, H. G.; Bennion, C.; Connolly, S.; Cook, A. R.; Gensmantel, N. P.; Hallam, C.; Hardy, K.; Hitchin, B.; Jackson, C. G.; Robinson, D. H. Discovery of new nonphospholipid inhibitors of the secretory phospholipases A2. *J. Med. Chem.* **1994**, *37*, 557–559.
- Reid, R. C. Inhibitors of secretory phospholipase A2 group IIA. *Curr. Med. Chem.* **2005**, *12*, 3011–3026.
- Smart, B. P.; Pan, Y. H.; Weeks, A. K.; Bollinger, J. G.; Bahnson, B. J.; Gelb, M. H. Inhibition of the complete set of mammalian secreted phospholipases A(2) by indole analogues: a structure-guided study. *Bioorg. Med. Chem.* **2004**, *12*, 1737–1749.
- Bezzine, S.; Koduri, R. S.; Valentin, E.; Murakami, M.; Kudo, I.; Ghomashchi, F.; Sadilek, M.; Lambeau, G.; Gelb, M. H. Exogenously added human group X secreted phospholipase A(2) but not the group IB, IIA, and V enzymes efficiently release arachidonic acid from adherent mammalian cells. *J. Biol. Chem.* **2000**, *275*, 3179–3191.
- Hanasaki, K.; Ono, T.; Saiga, A.; Morioka, Y.; Ikeda, M.; Kawamoto, K.; Higashino, K.; Nakano, K.; Yamada, K.; Ishizaki, J.; Arita, H. Purified group X secretory phospholipase A(2) induced prominent release of arachidonic acid from human myeloid leukemia cells. *J. Biol. Chem.* **1999**, *274*, 34203–34211.
- Hamaguchi, K.; Kuwata, H.; Yoshihara, K.; Masuda, S.; Shimbara, S.; Oh-ishi, S.; Murakami, M.; Kudo, I. Induction of distinct sets of secretory phospholipase A(2) in rodents during inflammation. *Biochim. Biophys. Acta* **2003**, *1635*, 37–47.
- Dudler, T.; Chen, W. Q.; Wang, S.; Schneider, T.; Annand, R. R.; Dempcy, R. O.; Cramer, R.; Gmachl, M.; Suter, M.; Gelb, M. H. High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A2. *Biochim. Biophys. Acta* **1992**, *1165*, 201–210.

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