### Title

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### Authors

Mouchlis, Varnavas D
Mu, Carol
Hammons, Renee
et al.



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# Lipidomics-based assays coupled with computational approaches can identify novel phospholipase $A_2$ inhibitors

**Varnavas D. Mouchlis**[*], **Carol Mu**, **Renee Hammons**, **Edward A. Dennis**[*]

Department of Chemistry and Biochemistry and Department of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, CA 92093-0601, USA

## Abstract

Phospholipase $A_2$ ($PLA_2$) enzymes play a major role in many diseases including the inflammatory cascade and specific potent small molecule inhibitors could be useful in studying their physiological role as well as for the development of drugs. In order to discover novel small molecule inhibitor platforms for members of the $PLA_2$ superfamily of enzymes, we have applied computational approaches to determine the binding mode of potent inhibitors specific for particular $PLA_2$s to the screening of chemical libraries. This has including the U.S. National Institutes of Health (NIH) National Cancer Institute (NCI) Diversity Set V and the ChemBridge commercial compound libraries. We have then subjected identified inhibitor structures to recently developed lipidomics based screening assays to determine the $X_I(50)$ and specificity of the identified compounds for specific $PLA_2$s. Herein we review this approach and report the identity of initial hits for both the Group IVA cytosolic $PLA_2$ and the Group VIA $Ca^{2+}$-independent $PLA_2$ that are worthy of further structural modification to develop novel platforms for inhibitor development.

### Keywords

Phospholipase $A_2$; virtual screening; enzymatic assays; dose-response inhibition; hit compounds

## 1. Introduction

Assaying the enzymatic activity of phospholipase $A_2$ ($PLA_2$) is a challenging task because the superfamily of $PLA_2$s include water-soluble enzymes acting on aggregated water-insoluble phospholipids (Dennis et al., 2011; Mouchlis et al., 2015). Thus, these enzymes must initially associate with the surface of the membrane to extract and bind their phospholipid substrate. Traditional $PLA_2$ assays have employed radio-labeled phospholipids that contain $^3H$- or $^{14}C$-labeled fatty acids at the *sn*-2 position of the phospholipid. Such

[*]Corresponding Authors: For Varnavas D. Mouchlis, vmouchlis@gmail.com; for Edward A. Dennis, edennis@ucsd.edu, phone: +1 858 534 3055.



CM and RLH carried out the computational selection and assays. VM and EAD designed and supervised the study and drafted the manuscripts.

phospholipids are challenging to synthesize, expensive, and very limited in terms of commercial availability (Yang et al., 1999).

The “surface dilution kinetics model” was developed and successfully employed by our laboratory to explain the action of $PLA_2$ enzymes on phospholipid/detergent mixed micelles (Carman et al., 1995; Roberts et al., 1977). The success of the surface dilution approach to explain kinetics of $PLA_2$ enzymes in mixed micelles, the stability of the micelle structure in the presence of various phospholipids and/or inhibitors, and the high efficiency in preparing mixed micelles makes mixed micelles an extremely suitable and attractive physical form of substrate to employ in a $PLA_2$ assay (Ribeiro and Dennis, 1975).

Lipidomics-based liquid chromatographic/mass spectrometric (LC/MS) approaches have proven to be very powerful in establishing novel high-throughput assays for $PLA_2$ enzymes (Mouchlis et al., 2018). These assays enabled us to perform detailed studies to define headgroup and *sn*-2 acyl-chain specificity on a wide variety of phospholipid substrates with great success (Mouchlis et al., 2018; Mouchlis and Dennis, 2019). We have used these assays for the three most well-studied human $PLA_2$ enzymes including cytosolic ($cPLA_2$), calcium-independent ($iPLA_2$), and secreted ($sPLA_2$) enzymes (Vasquez et al., 2018). In addition, dose-response inhibition studies on commercially available inhibitors allowed us to validate the application of these assays in identifying novel $PLA_2$ inhibitors (Mouchlis et al., 2019).

The $PLA_2$ superfamily contains 16 groups of structurally and functionally diverse enzymes (Dennis et al., 2011). The six main types of $PLA_2$ enzymes include the secreted ($sPLA_2$), cytosolic ($cPLA_2$), calcium-independent ($iPLA_2$), platelet-activating factor acetylhydrolase (PAF-AH), also known as lipoprotein-associated $PLA_2$ (Lp-$PLA_2$), lysosomal $PLA_2$ ($LPLA_2$), and adipose-$PLA_2$ (AdPLA). Our recent studies have focused on three human recombinant enzymes, namely the Group IVA cytosolic ($cPLA_2$), Group VIA calcium-independent ($iPLA_2$), and Group V secreted ($sPLA_2$), which are all water-soluble, membrane-associated enzymes with distinct structures and biological functions (Mouchlis and Dennis, 2016, 2019). The structure of each enzyme contains a unique active site where the substrate binds and an interfacial surface that mediates association with cellular membranes (Mouchlis et al., 2015). Virtual screening using computational chemistry is a useful tool to identify new hit compounds for druggable targets (Mouchlis et al., 2011; Mouchlis et al., 2020). This manuscript discusses the application of a newly developed lipidomics-based high-throughput assay in combination with computational approaches to screen compound libraries for the identification of novel $PLA_2$ inhibitors.

## 2. Lipidomics-based $PLA_2$ assay

Advances in lipidomics enabled the identification and classification of numerus lipid metabolites using state-of-the-art LC/MS methods (Quehenberger et al., 2010). As part of our continuous efforts to advance the lipidomics field, we have developed a high-throughput assay that allowed as to define substrate specificity for $cPLA_2$, $iPLA_2$ and $sPLA_2$ (Mouchlis et al., 2018). A combination of hydrophilic interaction chromatography (HILIC), reversed-phase chromatography (C18), and multiple reaction monitoring (MRM), allowed

quantification of a variety of lysophospholipid and free fatty acid products (Figure 1) (Mouchlis et al., 2019). Lysophospholipids and free fatty acids were detected using both positive and negative ion mode, respectively (Mouchlis et al., 2018). Dose-response inhibition experiments on pyrrophenone which is a specific pyrrolidine $cPLA_2$ inhibitor (Seno et al., 2001), OTFP which is a specific fluoroketone $iPLA_2$ inhibitor (Mouchlis et al., 2016b), and Ly315920 which is a specific indole $sPLA_2$ inhibitor (Snyder et al., 1999) were employed to validate the applicability of the assay in identifying $PLA_2$ inhibitors.

## 3. Virtual Screening

Molecular docking and molecular dynamics are powerful computational techniques for understanding the interactions of an inhibitor with the active site of an enzyme. Normally, molecular dynamics simulations allow full flexibility for both the inhibitor and the enzyme, but such flexibility is time-consuming in terms of computational time especially when a large number of inhibitors is to be studied. Therefore, while ligand flexibility is well accounted for in molecular docking techniques, in the current application, the enzyme is not allowed to move during the calculations. The relaxed complex scheme combines the advantages of docking calculations with dynamic structural information provided by molecular dynamics simulations (Amaro et al., 2008). In particular, the enzyme-inhibitor complex is subjected to molecular dynamics simulations followed by clustering analysis to various selected conformations of the enzyme for molecular docking (Figure 2).

The U.S. National Institutes of Health (NIH) National Cancer Institute (NCI) Diversity Set V and the ChemBridge commercial compound libraries were virtually screened against the five “selected” conformations of each enzyme using GLIDE (Friesner et al., 2004). From each of the two compound libraries, eighty compounds were selected for *in vitro* screening based on the following criteria: (i) the docking score, (ii) the ability of the compound to form hydrogen-bonding with the oxyanion hole (Gly/Gly) of each enzyme, and (iii) the frequency that a compound appeared as top-ranked among the five conformations of each enzyme. Based on the above criteria 80 compounds were selected from the NCI Diversity Set V and 160 compounds from the ChemBridge compound libraries for each enzyme. Each compound was dissolved in DMSO at a concentration of 5 mM and tested for its inhibitory activity against $cPLA_2$ and $iPLA_2$.

## 4. In vitro screening and inhibitor binding interactions

Group specific assays were employed to determine the activity of human recombinant Group IVA cytosolic ($cPLA_2$) and Group VIA calcium-independent ($iPLA_2$) phospholipases $A_2$ in a mixed micelle 96 well-plate assay, as previously described (Mouchlis et al., 2019; Mouchlis et al., 2018). The strategy is schematically summarized in Figure 3. The substrate for each enzyme consisted of 100 $\mu$M phospholipid, 400 $\mu$M C12E8 surfactant, and 2.5 $\mu$M 17:0 LPC internal standard. For $cPLA_2$, the total phospholipid concentration (100 $\mu$M) consisted of 97 $\mu$M phospholipid substrate and 3 $\mu$M PI(4,5)P2 which enhances the activity of the enzyme. A specific buffer was prepared to achieve optimum activity for each enzyme. The buffer for $cPLA_2$ contained 100 mM HEPES pH 7.5, 90 $\mu$M $CaCl_2$, and 2 mM DTT (Mouchlis et al., 2016a). For $iPLA_2$, the buffer consisted of 100 mM HEPES pH 7.5, 2 mM

ATP, and 4 mM DTT Mouchlis et al., 2016b). The enzymatic reaction was performed in a 96 well-plate using a Benchmark Scientific H5000-H MultiTherm heating shaker for 30 min at 40 °C. Each reaction was quenched with 120 $\mu$L of methanol/acetonitrile (80/20, v/v), and the samples were analyzed using the HPLC-MS system. A blank in which enzyme was omitted, was also included for each experiment to determine the product of non-enzymatic hydrolysis and to detect any changes in the intensity of 17:0 LPC. Dose-response inhibition curves were generated using GraphPad Prism 5.0 and the non-linear regression by plotting percentage of inhibition vs log (mole fraction) to calculate the reported $X_I(50)$ values and their associated error.

Table 1 summarizes the structure, percent inhibition at a 5 mM concentration, and the $X_I(50)$ values of six novel hit compounds. Compounds 1, 3, and 4 showed better inhibitory activity toward $iPLA_2$ and they exhibited lower inhibitor activity towards $cPLA_2$. Compound 2 showed good activity toward $cPLA_2$ while its activity toward $iPLA_2$ was lower. Compound 5 showed low activity toward both enzymes. Compound 6 exhibited good activity toward both enzymes. The binding mode of compound 2 in the active site of $cPLA_2$ revealed hydrogen-bonding with the oxyanion hole of Gly197/Gly198 and pi-pi stacking of the aromatic rings with Phe199, Trp232, Phe295, and Phe683. The amide group participates in hydrogen-bonding with Asn555 (Figure 4A). The binding mode of compound 3 in the active site of $iPLA_2$ revealed hydrogen bonding with the oxyanion hole of Gly486/Gly487 and a halogen bond of the chlorine atom with the backbone amide of Met544. The aromatic rings participate in pi-pi stacking with Tyr643, Phe644, Phe722, and Tyr541 (Figure 4B).

## 5. Conclusion

In this manuscript, we have summarized our new lipidomics-based high-throughput assay approach for $PLA_2$ enzymes and explored the use of this assay to identify novel chemical platforms from which potent and selective inhibitors can be developed for members of this enzyme superfamily. The relaxed complex scheme was employed to virtually screen two compound libraries, the NCI Diversity Set V and the ChemBridge collection. Based on the docking score, the ability of the compound to form hydrogen-bonding with the oxyanion hole (Gly/Gly) of each enzyme, and the frequency that a compound appeared as top-ranked among the five conformations of each enzyme, 160 compounds from each library were selected for in vitro screening against $cPLA_2$ and $iPLA_2$. New hit compounds were identified which are now candidates for further hit-to-lead optimization.

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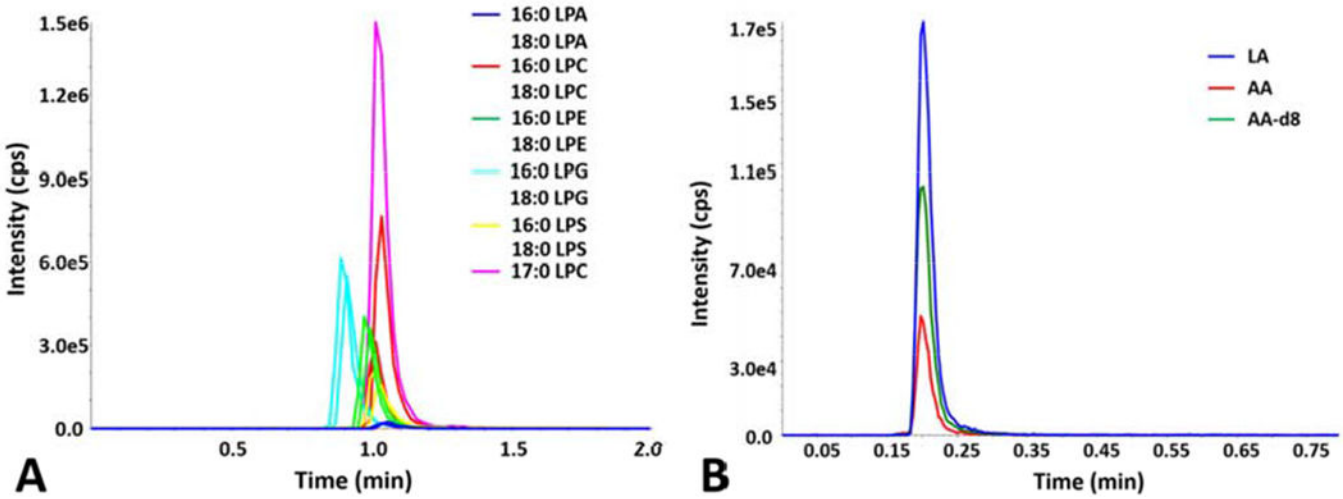



**Figure 1.**

Identification of lysophospholipid and free fatty acid products: (A) for lysophospholipids using a HILIC column, and (B) for free fatty acids using a C18 reversed-phase column (from reference Mouchlis et al., 2019).

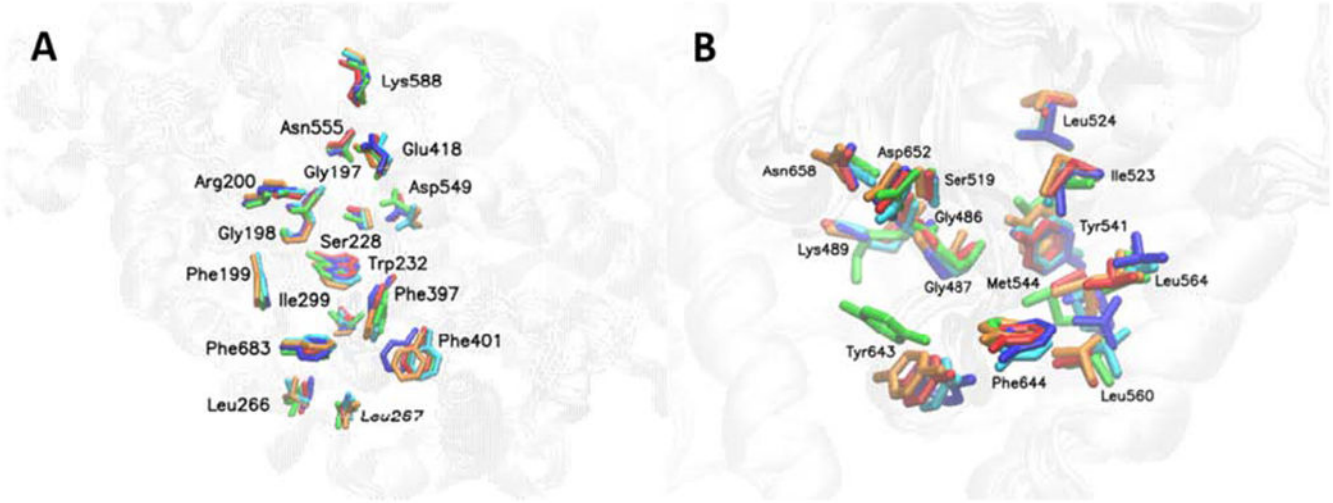



**Figure 2.**

Clustering analysis for (A) $cPLA_2$ and (B) $iPLA_2$. Five conformations of each enzyme are depicted in various colors.

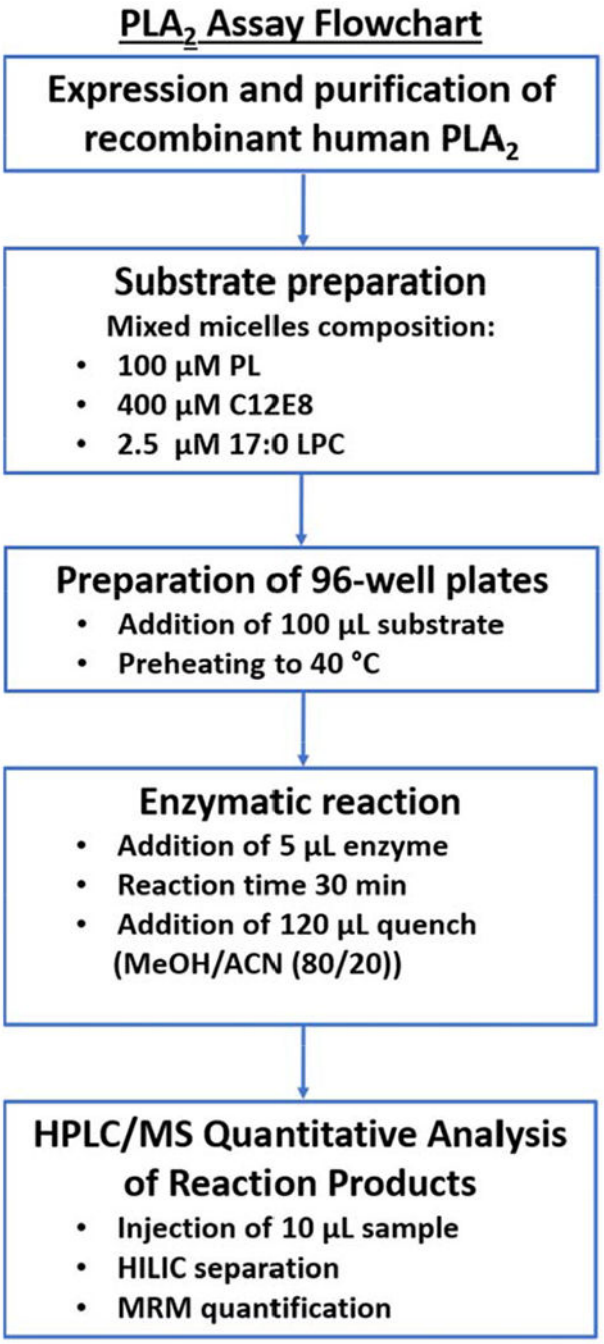



**Figure 3.**

Schematic representation of $PLA_2$ assay ($PLA_2$, phospholipase $A_2$; PL, phospholipid; C12E8, surfactant; LPC, lysophospholipid; MeOH, methanol; ACN, acetonitrile; HPLC/MS, high-performance liquid chromatography/mass spectrometry; HILIC, hydrophilic interaction chromatography; MRM, Multiple reaction monitoring).

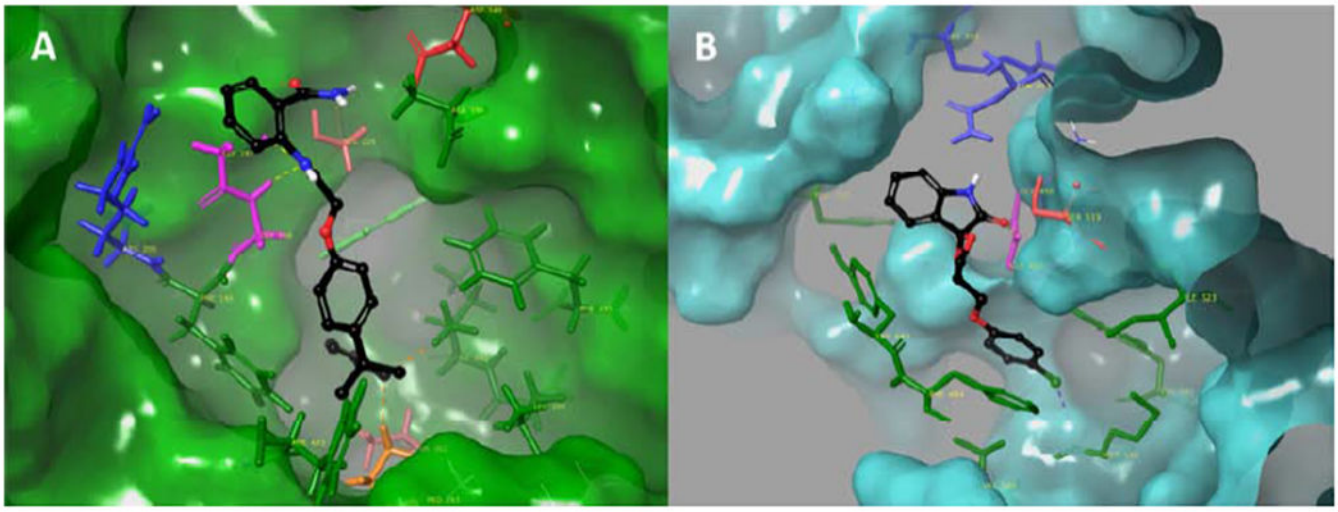
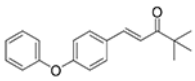
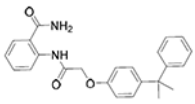
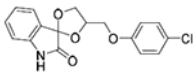
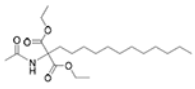
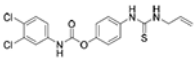
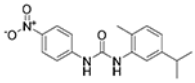



**Figure 4.**

Binding mode of $PLA_2$ inhibitors. (A) compound 2 in the active site of $cPLA_2$ and (B) compound 3 in the active site of $iPLA_2$.

**Table 1.**

$X_I(50)$ values of $cPLA_2$ and $iPLA_2$ inhibitors.

| | | | $cPLA_2$ | | $iPLA_2$ | |
|---|---|---|---|---|---|---|
| **No** | **Code** | **Structure** | **% Inh.** | **$X_I(50)$** | **% Inh.** | **$X_I(50)$** |
| 1 | 643029 (Div. Set V) |  | 94 | 1.5 ± 1 | 99 | 0.05 ± 0.01 |
| 2 | 6353053 (ChemBridge) |  | 98 | 0.05 ± 0.05 | 100 | 0.1 ± 0.1 |
| 3 | 6937786 (ChemBridge) |  | 98 | 0.3 ± 0.1 | 100 | 0.020 ± 0.008 |
| 4 | 20192 (Div. Set V) |  | 97 | 0.2 ± 0.2 | 100 | 0.040 ± 0.005 |
| 5 | 204262 (Div. Set V) |  | 94 | 0.5 ± 0.2 | 84 | 0.10 ± 0.08 |
| 6 | 46492 (Div. Set V) |  | 96 | 0.03 ± 0.02 | 100 | 0.020 ± 0.005 |