IDENTIFICATION OF THE SITES OF ACTION OF INHIBITORS OF MAMMALIAN PHOSPHOLIPASE D2 (PLD2) AND THE ROLE OF INTERACTING PROTEIN PARTNERS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science.

By

RAMYA GANESAN

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY RAMYA GANESAN ENTITLED IDENTIFICATION OF SITES OF ACTION OF INHIBITORS OF MAMMALIAN PHOSPHOLIPASE D2 (PLD2) AND THE ROLE OF INTERACTING PROTEIN PARTNERS BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

______________________________
Julian Gomez-Cambronero, Ph.D.
Thesis Director

Committee on Final Examination

______________________________
Julian Gomez-Cambronero, Ph.D.
Professor

______________________________
Gerald M. Alter, Ph.D.
Professor

______________________________
Nancy J. Bigley, Ph.D.
Professor of Immunology

______________________________
Robert E.W. Fyffe, Ph.D
Vice President for Research and Dean of the Graduate School

______________________________
Barbara E. Hull, Ph.D.
Director of Microbiology and Immunology Program, College of Science and Mathematics
ABSTRACT

Ganesan, Ramya. M.S. Department of Microbiology and Immunology, Wright State University, 2013. Site of Action of Inhibitors of PLD2 and the Role of Interacting Partners of PLD2 during Inhibition.

Phospholipase D (PLD) is a key enzyme for the remodeling of phospholipids in the cell membrane. PLD has been implicated in many physiological functions such as chemotaxis and phagocytosis as well as pathological functions such as ischemia/reperfusion and cancer metastasis. Several small molecule inhibitors of PLD have been recently developed to overcome these pathological effects. We have concentrated on 5-Fluoro-2-Indolyl des-Chlorohalopemide (FIPI) which is an indole derivative of halopemide that inhibits the two mammalian isoforms PLD1 and PLD2. We also concentrate on N-[2-[1-(3-Fluorophenyl)-4-oxo-1,3,8-triazaspiro[4,5]dec-8-yl]ethyl]-2-naphthalenecarboxamide (NFOT) and N-[2-(4-oxo-1-phenyl-1,3,8-triazaspiro[4,5]dec-8-yl)ethyl]-2-naphthalenecarboxamide (NOPT) that are specific inhibitors of the PLD2 isoform. In spite of knowing their specificity derived from in vitro assays, the mechanism of action of these inhibitors has never been studied. Here for the first time, we show the site of action of FPI and NFOT on PLD as well as a mechanism of enzymatic action that explains in vivo actions. Our studies showed that FPI is a non-reversible inhibitor of PLD that binds to the HKD, whereas NFOT shows mixed type of inhibition, which suggests that inhibitor binding site might be anywhere in the regulatory PX or PH domains of PLD2 or the catalytic region HKD. We performed extensive mutational
analysis on PLD2 and our results clearly indicate that FIPI binds to S757 residue and S648 and a PIP2 binding site are critical for NFOT to inhibit PLD2, since mutating these key amino acid(s) resulted in partial resistance of the enzyme towards the inhibitor. Inhibitor dose dependent assays of the resistant PLD2 mutants F244N/L245A/L246A and S648A confirmed the same. Also, the mutants exhibited resistance against the inhibitors unlike wild-type, in performing chemotaxis further confirming physiological relevance. In addition, we uncovered the effect of PLD2 binding partners, Grb2 and Ras on the efficacy of inhibitors. Mechanistically it was found that Grb2 positively regulates PLD2, while Ras has a negative effect on PLD2 and that the small molecule inhibitor’s action on PLD2 is affected by the absence or presence of Grb2 or Ras. In conclusion, we consider the results obtained in this study regarding the key residues of PLD2 are crucial in order to develop more potent and efficient inhibitors that can be used in vivo for pathological processes such as chronic inflammation and cancer metastasis.
HYPOTHESIS AND SPECIFIC AIMS

Hypothesis:

It is hypothesized that one or more amino acids in either the regulatory PX and/or PH domains or within or in the near vicinity of the catalytic site is essential for the action of inhibitors on PLD2 and that mutating these key amino acid(s) will result in resistance of the enzyme towards the inhibitor. In addition, the inhibitor’s action on PLD2 is effected by the presence or absence of protein interacting partners of PLD2.

Specific aims: The hypothesis will be tested by three different specific aims.

Aim1: To determine the nature of the PLD inhibitors: reversible or irreversible

Aim2: To determine the site of action of PLD inhibitors on PLD by screening for mutants in the PX, PH and around the HKD motif and study biological relevance of these mutated sites

Aim3: To determine the role of interacting proteins in preserving PLD from PLD inhibitors
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LIST OF ABBREVIATIONS

PLD2= PhospholipaseD2

FIPI= 5-Fluoro-2-indolyl-deschloralopemide

NFOT= N-[2-[1-(3-Fluorophenyl)-4-oxo-1,3,8-triazaspiro[4.5]dec-8-yl]ethyl]-2-naphthalenecarboxamide

NOPT= N-[2-(4-oxo-1-phenyl-1,3,8-triazaspiro[4,5]dec-8-yl)ethyl]-2-naphthalenecarboxamide

DMEM= Dulbecco’s modified eagle medium

DMSO= Dimethyl sulfoxide

Grb2= Growth factor receptor-bound protein 2

Ras= Rat sarcoma
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INTRODUCTION

Phospholipase D (PLD) is a hydrolytic enzyme present in the cell membrane that catalyzes the conversion of phospholipid namely phosphocholine (PC) to produce free acid, phosphatidic acid (PA) (Fig 1). PA is involved in cellular signaling and membrane dynamics in all eukaryotes (Kooijman and Burger 2009). Phospholipase D belongs to the HKD superfamily, and as the name suggests PLD has “HxKxxDxxxx” motif in its catalytic site. This HKD motif accounts for the lipase activity of Phospholipase D. There are 6 isoforms of mammalian PLD, namely PLD1-6 (Fig 3). PLD1 and PLD2 belong to the classical HKD superfamily that possesses phosphoinositide binding (PX) and pleckstrin homology (PH) domains with 2 HKD motifs, while PLD3-6, cardiolipin synthase, etc. belong to the non-classical HKD superfamily that lack PX and PH domains and may have 1 or 2 HKD motifs. The PX and PH domains of PLD are the regulatory domains of PLD.

PLD is implicated in many physiological and pathological functions in cells. Apart from its lipase activity, PLD interacts with proteins such as Rac2, Grb2-SOS, WASp, S6K (Di Fulvio, Lehman et al. 2006; Lehman, Ledford et al. 2007; Kantonen, Hatton et al. 2011; Peng, Henkels et al. 2011) and Bcl-2 and performs functions such as chemotaxis, cell migration, DNA synthesis and anti-apoptosis (Banno 2002). Of all the 6 known mammalian PLD isoforms, PLD1 and PLD2 are well studied. PLD2 is constitutively active, unlike PLD1. Importantly dysregulation of PLD2 can result in
pathological conditions such as cancer metastasis, cell invasion and chronic inflammation. Hence it is important to have PLD2 inhibitors to protect cells from the pathological implications of PLD2. A number of PLD inhibitors have been developed so far. Some of the classical PLD inhibitors are ethanol and butanol. Due to some off target effects and non-specificity of these primary alcohols, other serine protease inhibitors were used to inhibit PLD.

Halopemides, that were used as neuroleptic agents have been identified as PLD inhibitors. Recently, specific small molecule inhibitors of PLD (Table 1) have been developed including \( \text{N}-[2-[4-(2,3,5,6-tetrahydro-1H-benzo}[d]imidazol-1-yl]-1-piperidinyl]ethyl\)-5-fluoro-1H-indole-2-carboxamide (FIPI), \( \text{N}-[2-[(4-oxo-1-phenyl-1,3,5,6-tetrahydro-2-oxo-1H-benzo}[d]imidazol-1-yl]-1-piperidinyl]ethyl\)-5-fluoro-1H-indole-2-carboxamide (NOPT) and \( \text{N}-[2-[1-(3-Fluorophenyl)-4-oxo-1,3,8-triazaspiro[4.5]dec-8-yl]ethyl\]-2-naphthalene-carboxamide (NFOT), \( \text{N}-[2-[(4-oxo-1-phenyl-1,3,5,6-tetrahydro-2-oxo-1H-benzo}[d]imidazol-1-yl]-1-piperidinyl]-1-methylethyl\)-2-naphthalene-carboxamide (809), \( (1R,2R)-\text{N}-(\text{[S]}-1-\{4-[5-bromo-2-oxo-2,3-dihydro-1H-benzo(d)imidazol-1-yl]piperidin-1-yl\}propan-2-yl)-2-phenylcyclopropanecarboxamide (NBOD) and Apigenin (Scott, Selvy et al. 2009; Su, Chen et al. 2009; Lavieri, Scott et al. 2010). These inhibitors show greater inhibition \textit{in vitro}, while \textit{in vivo} they are not bio-assimilated and hence reduced bio-availability occurs despite using very micromolar concentrations of inhibitors.
Recently our lab showed that PLD2 is involved in tumor metastasis and invasion. Some of the specific small molecule inhibitors of PLD such as Apigenin, FIPI and NOPT inhibit tumor metastasis and cell invasion in mice models (Henkels, Boivin et al. 2013). To date there is no clear site of action of these specific small molecule inhibitors on PLD2 and the mechanism of action of these inhibitors on PLD is not known.

This study is the first determination of the site of action of three small molecule inhibitors on PLD. The importance of this is that knowledge about the mechanism and kinetics of the existing inhibitors will help in developing more potent PLD inhibitors to be used in pathological conditions.

This study shows that the site of action of the specific PLD2 inhibitor NFOT is serine 648, while the dual PLD inhibitor FIPI is serine 757, which is partly confirmed by enzyme competition assays showing mixed inhibition wherein, FIPI covalently modifies PLD2 and NFOT binds the regulatory domains of PLD2. Since, in vivo conditions PLD2 is surrounded by its binding protein partners, we also studied the influence of PLD2 binding partners, Grb2 and Ras on the effect of inhibitors. We found that these critically regulate PLD2 inhibition. Grb2 acts as a positive effector of lipase activity protecting sites for inhibitor binding, while Ras acts as a positive effector of lipase activity of PLD2 at low concentrations and does not affect inhibition as concentration increases. Grb2 increases the IC$_{50}$ of FIPI for PLD2 negatively regulates the inhibitor action.
LITERATURE REVIEW AND PRELIMINARY DATA

Enzymatic role of Phopholipase D

Phospholipase D (PLD), as the name indicates is a lipase that hydrolyzes phospholipids such as phosphatidylcholine (PC) to produce a phosphatidic acid (PA) and a soluble choline head group. In the presence of primary alcohol such as butanol, PLD hydrolyzes phosphatidyl choline producing phosphatidylbutanol by a biochemical process named transphosphatidylation. This catalytic reaction of PLD with butanol enables the measurement of in vitro PLD activity assay by inducing radiolabelled $[^3]H$-butanol which yields a product $[^3]H$- phosphatidylbutanol. This product is not metabolically changed any further and can be quantified in a scintillation counter after lipid extraction and chromatographic separation.

Phospholipase D: its structure and function

Phospholipase D is normally present in the cell membrane, but also localized in perinuclear and golgi membranes as well. There are mainly six isoforms of PLD namely PLD1-6. PLD protein PLD belongs to the HKD superfamily. The majority of members in this superfamily contain a short conserved sequence motif (H-x-K-x(4)-D), where x represents any amino acid residue), called the “HKD signature motif”. It consists of a histidine, a lysine and an aspartic acid in close proximity; normally a classical PLD molecule bears two HKD domains for full enzymatic activity.
Mammalian PLD1 and PLD2 belong to the classical HKD superfamily (Fig 2) that possesses phosphoinositide binding (PX) and pleckstrin homology (PH) domains with 2 HKD motifs, while PLD3-6, cardiolipin synthase and some endonuclease belong to the non-classical HKD superfamily that lack PX and PH domains and may have 1 or 2 HKD motifs. The PX and PH domains of PLD are the regulatory domains of PLD. The PX is a phosphoinositide binding domain involved in targeting of proteins to cell membranes. The PX domain was first identified in P40phox and p47phox of NADPH oxidase and later was also found in many PI3 kinases (Ponting 1996; Wishart, Taylor et al. 2001). Phox is an abbreviation for phagocytic oxidase.

The PH domain is approximately 120 amino acid sequence that occurs in many proteins that are involved in signaling or are constituents of cytoskeleton. This domain can bind PI lipids within the membrane (Wang and Shaw 1995), βγ subunits of heterotrimeric G proteins such as receptor tyrosine and serine/threonine kinases (Wang, Shaw et al. 1994) and protein kinase C (Yao, Kawakami et al. 1994).
Fig 1. PLD activity. Schematic representation of lipase activity showing hydrolysis and transphosphatidylation reactions catalyzed by PLD.
**Fig 2. HKD Superfamily.** The members of the HKD family are classified into classical and non-classical PLDs. The classical PLDs have PX and PH regulatory domains, while non-classical lack PX and PH domains.
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**Fig 3. Isoforms of PLD, their structure and functions**
**PLD: Mechanism of action**

Most PLD enzymes are monomeric proteins that possess two HKD-motif containing domains. These two HKD motifs from two such domains form a single active site. PLD6 in mitochondria has only one HKD motif per subunit but form an active dimer, which has a single active site at the dimer interface containing the two HKD motifs from both subunits. Despite differenced in catalytic functions and a very broad range of substrate specificities, the diverse group of PLD enzymes bind to and attack a phosphodiester moiety. Most of them are known to possess structurally similar active sites and thought to utilize a common two-step ping-pong catalytic mechanism, involving an enzyme-substrate intermediate, to cleave phosphodiester bonds.

Two-step mechanism (Fig 4):

1) The imidazole group of histidine in the HKD motif is a nucleophile that attacks the phosphate group of the substrate PC. A covalent bond is formed between the phosphate and the histidine generating a phosphatidyl-histidine intermediate. This is the rate limiting step.

2) In the second step, water or hydroxyl from an alcohol (eg. Butanol) nucleophilically attacks the phosphate in the intermediate releasing PA (or a phosphatidyl-alcohol) as the free enzyme.
Fig 4. Schematic of a two-step mechanism of action of PLD
**Mammalian PLD structure and sequence alignment**

PLD1 and 2 are the mammalian isoforms of PLD. PLD1 and PLD2 share ~50% homology. Two HKD motifs are necessary for their enzymatic activities. They possess the PX and PH regulatory domains (Fig 5).

The gene encoding PLD1 is on chromosome 3q.26 (human) and encodes a protein of ~120 KDa protein. PLD1 has many splice variants such as a, b, a₂, b₂. PLD1a is the longest of the splice variants and is 1074 amino acids long. Subcellularly PLD1 is localized in golgi, endoplasmic reticulum and endosomes. PLD1 requires small GTPases such as Arf, Rho and protein kinase C (PKC) and phosphatidyl inositol 4,5 bis phosphate (PIP₂) for its activation.

PLD2 gene is on chromosome 17p.13 (human). PLD1 is longer than PLD2 due to the presence of a “loop” in the protein and hence its molecular weight is 105 KDa. Splice variants of PLD2 are PLD2a, b and c. PLD2a is the longest with 933 amino acids. PLD2 localizes at cell membrane as well as cytosol. PLD2 requires only PIP2 and minimally PKC for its activity. PLD2 is constitutively active while PLD1 is not. Nevertheless, both isoforms are activated by cell agonists such as growth factors.
Fig 5. PLD1 and PLD2 protein. PLD protein structures showing the difference between the two mammalian isoforms PLD1 and PLD2. (Gomez-Cambronero 2011)
**Physiological functions of PLD**

PLD plays a major role in various physiological processes, including cell migration, phagocytosis (Kantonen, Hatton et al. 2011), chemotaxis (Lehman, Ledford et al. 2007), DNA synthesis (Di Fulvio, Lehman et al. 2006), membrane vesicle trafficking, actin cytoskeleton remodeling, membrane ruffling (Mahankali, Peng et al. 2011), cell growth and survival, anti-apoptosis (Oh, Lee et al. 2007) and also pathological conditions such as cancer cell metastasis, tumor progression (Henkels, Boivin et al. 2013) and inflammation (Peng, Henkels et al. 2011). PLD1 is essential for basal chemokinesis, while PLD2 is constitutively active.

PA as produced from PLD is a major lipid second messenger that is involved in several intracellular signaling pathways. Few of the pathways where PA is key player are described below. For example, PA initiates a signal cascade that results in PA binding to ribosomal S6 kinase (S6K) and subsequent actin polymerization and chemotaxis, wherein PA acts as a chemoattractant for leukocytes (Lehman, Ledford et al. 2007). PA interacts with many other proteins such as mTOR, Sos (Zhao, Du et al. 2007), Rac and Ras (Peng, Henkels et al. 2011) subsequently causing actin polymerization followed by cell migration or chemotaxis in normal as well as cancer cells. PA also interacts with Sos through Grb2 activating the Erk/MAPK pathway culminating in DNA synthesis (Di Fulvio, Lehman et al. 2006). PA functions in cellular signaling pathways as a direct lipid
second messenger or as an indirect precursor, once converted to other bioactive lipids such as LPA and DAG.

PA is essential for activating mTORC in mTOR, the protein involved in cell cycle progression and cell growth. p70S6 Kinase which is a downstream target of mTOR is activated by phosphorylation results in phosphorylation of the S6 protein resulting in protein synthesis (Fang, Vilella-Bach et al. 2001). PA also activates S6 independent of mTOR. PA is also involved in cell survival and anti-apoptosis via interactions with Bcl-2 and Bcl-xl (Oh, Lee et al. 2007). PA generated by PLD2 plays an important role in cell survival during Fas-mediated apoptosis through the increased Bcl-2 and Bcl-xL protein levels which resulted from PLA2 and AA-COX2 pathway (Kang, Jang et al. 1998).

Pathological role of PLD

PLD is involved in inflammation and cancer invasion or metastasis. A study showed that cancer cell invasiveness is dependent on the presence of PLD2 at the time of metastatic initiation. Recent studies have shown that PLD2 activity is regulated by phosphorylation and dephosphorylation. Three kinases EGFR, Src and JAK3 are found to phosphorylate PLD2 at specific tyrosine residues 296, 511 and 415 respectively. Y296 phosphorylation showed an inhibitory effect while that of Y415 showed activation functions. Also phosphorylation of Y511 is either activational or inhibitory depending on the cell-type. A high level of cell invasiveness of cancer cells was shown by a combined high JAK3/PLD2 phosphorylation and activity, involving PLD2’s Y415 residue (Henkels,
Peng et al. 2010). In COS7s prominent phosphorylation by JAK3 on Y415 activation site and moderate phosphorylation by EGFR kinase and Src on Y296 and Y511, led to great PLD2 activity. This study shows that importance of PLD2 activity in cancer by highlighting the regulation of phosphorylation-dephosphorylation of PLD2. In the low invasive breast cancer MCF-7s it was shown that phosphorylation of the inhibitory Y296 in PLD2 by EGFR kinase stimulation is a reason for low PLD activity in MCF-7s than aggressive MDA-MB-231s and normal COS7s.

PLD binds to Grb2 via phosphorylated Y169 and Y179 residues and recruits Sos, while PLD-derived PA directly binds to Sos, both ultimately promote GTP loading of Ras and stimulates the MEK/ERK pathways (Zhao, Du et al. 2007). The stimulation of MEK/ERK pathway results in cell proliferation and transformation. PLD has been implicated in colorectal cancer, wherein high level of PLD2 expression was observed in patients with colorectal cancer (Oshimoto, Okamura et al. 2003).

Another study in Japan involved studying a polymorphism in PLD2 1814 C \( \rightarrow \) T causing a mutation, Ile577Thr was predominant in colorectal cancer patients, but this polymorphism did not affect PLD activity (Yamada, Hamajima et al. 2003). In yet another study, 97 colon carcinoma samples were tested for PLD2 levels and it was observed that there was high PLD2 level in the tumor samples. The PLD2 levels varied between tumors but were relative to tumor size (Saito, Ohata et al. 2007).
In a recent study, it has been shown that PLD2 is very critical in cancer invasiveness and metastasis. It was shown that by subjecting SCID mice that have been injected with human breast cancer cells alone or human breast cancer cells silenced for PLD2, were also given PLD inhibitors at a fixed dose/mg/Kg/day. It was observed that tumor growth was inhibited significantly in the mice that were given PLD inhibitors. Also the mice that were silenced for PLD2 inhibited metastasis to a great extent (Henkels, Boivin et al. 2013). Similar studies have also shown that PLD1 plays a major role in tumor invasion and metastasis.

**Protein interacting partners of PLD2**

PLD2 interacts with several protein partners such as Grb2, Ras, Rac, WASp, Sos and S6K.

The proteins of interest for this study are Grb2 and Ras, the details of which are described below.

*i) Grb2 and its interaction with PLD2*

The Growth factor receptor-bound protein 2 (Grb2) is an adaptor protein involved in signal transduction. Grb2 has one SH2 domain and two SH3 domains. Its SH3 domains mediate complex formation with proline-rich residues, while SH2 domain interacts with phosphorylated tyrosine residues in target proteins such as EGFR and PDGFR (Lowenstein, Daly et al. 1992). Grb2 is a crucial protein for cell cycle progression and for
actin based cell motility. It is also involved in other complex processes such as angiogenesis, epithelial morphogenesis and vasculogenesis (Giubellino, Burke et al. 2008). Grb2 is involved in many cellular processes essential for cell survival, growth and proliferation. It translocates to the plasma membrane when activated by EGF and binds to EGFR through the phosphotyrosine containing proteins on its SH2 domains (Yu, Chen et al. 2008). Activation of Ras-guanine nucleotide exchange factor, Sos by interaction of SH3 of Grb2 with C-terminal Proline-rich regions in Ras and Sos is a well characterized mechanism that results in the activation of MAPK cascade and cell proliferation (Chardin, Camonis et al. 1993; Egan, Giddings et al. 1993).

The SH2 domain of Grb2 also serves the recruitment of phosphotyrosine motif pYxN located on the PH domain of PLD2 (Schlessinger 1994). PH domain of PLD2 binds to the SH2/SH3 containing tyrosine kinases (Ahn, Oh et al. 2003; Choi, Hiragun et al. 2004) and an increase in PLD activity correlates with increase in the overall tyrosine phosphorylation of PLD2 (Choi, Hiragun et al. 2004). PLD binds to Grb2 and recruits Sos, while PLD-derived PA directly binds to Sos, both ultimately promote GTP loading of Ras and stimulates the MEK/ERK pathways (Zhao, Du et al. 2007). PX residues of PLD2 Y169 and Y179 are necessary for binding to Grb2 and recruiting Sos, wherein Y169 modulates enzyme activity while Y179 is essential for tyrosine phosphorylation of the protein (Di Fulvio, Frondorf et al. 2007).
Also, Y511 is shown to be involved in tyrosine interactions between PLD2 and Grb2. When Y179 and Y511 are phosphorylated, they mediate enzyme phosphorylation and thus activate PLD2-Grb2 interaction mediating lipase activity, while on the other hand when they are unphosphorylated, the PLD2 activity decreases and signals for cell proliferation and de novo DNA synthesis (Henkels, Short et al. 2009). Since we know that Grb2 interacts with Y169/179 of PLD2 through its SH2 domains, we are interested to know if this Grb2 interaction with PLD2 affects the binding and action of small molecule PLD2 inhibitors.

ii) *PLD2 and Ras*

Ras is a small G protein that has GTPase activity and involved in intracellular signaling. Ras is a member of the Ras family of proteins that also include Rad, Rab, Rap, Ran, Rho, Rheb, Rit, and Arf. PLD2 is known to activate Ras via PA production. PA interacts with PH domain of Sos and thus stimulates Sos mechanisms to activate Ras while coupling Ras activation with extracellular EGFR signaling (Zhao, Du et al. 2007). Another study shows that PLD2 regulates Ras-GEF, Ras-GRF1 in response to intergrin, wherein PLD2 recruits RasGRF1 to the plasma membrane to activate Ras (Mor, Campi et al. 2007).

On the other hand, PLD activity is elevated by oncogenic stimulus via H-Ras and not V-Ras. H-Ras localizing in caveolin rich membrane fractions elevates PLD activity by stimulating the activity of Arf6 and RalA (Xu, Frankel et al. 2003). Earlier studies show that v-src activates PLD activity by G-proteins (Jiang, Alexandropoulos et al.)
These studies were followed by another study which states that v-src induced PLD activity is Ras mediated by providing three lines of evidence. First of which shows that in v-src transformed Balb/c 3T3 cells, PLD activity reduces by the use of neutralizing Ras monoclonal antibodies.

Secondly immobilized Ras protein tired cytosolic ability to stimulate PLD activity. Lastly expression of Ras negative mutant in v-src transformed cells reduced PLD activity levels to that of nontransformed cells (Jiang, Lu et al. 1995). Recent findings have shown that PLD2 acts as a GEF on Ras, wherein it has been shown that Ras is a better substrate for PLD2-GEF following faster kinetics than any other GTPase such as Rac2 or Rac1. The study showed that in the absence of PLD2-GEF cell growth and proliferation was affected and that PLD2 by itself is involved in Ras activation and not by its product PA (Henkels, Mahankali et al. 2013).

**PLD inhibitors**

Inhibitors of Phospholipase D, are molecules that inhibit the enzymatic activity of PLD by interacting at specific sites on PLD preventing it from converting PC to PA thus the downstream processes. Inhibitors of PLD are classified into PLD1, PLD2 and dual PLD inhibitors.
PLD1 and PLD2 inhibitors specifically inhibit PLD1 and PLD2 respectively, while the dual inhibitor is less specific, but efficiently inhibits both PLD1 and PLD2 isoforms.

PLD is known to be implicated in pathological conditions, and hence it is necessary to inhibit the pathological effects of PLD such as in inflammation, cancer metastasis and invasion. Early uses of PLD inhibitors included primary alcohols such as ethanol and butanol. But it was later realized that these alcohols did not inhibit PLD activity but deviated the product from phosphatidic acid to phosphatidyl-ethanol or –butanol, which is being used in in vitro measurement of lipase activity with the help of radiolabelled [3H]-butanol. Also the use of primary alcohols led to many off target effects wherein ethanol was found to inhibit neutrophil functions of PLD (Sato, Hongu et al. 2013).

Aluminum fluoride is known to activate PLD in intact cells, but was found later that it inhibited PLD in cell extracts/lysates and permeabilized cells. This difference was attributed to the fact that in extracts, Aluminum fluoride acts on inhibition-causing target in intact cells, which might be the enzyme itself (Li and Fleming 1999). During the purification of PLD from granulocytes, PLD activity was found to be inhibited by a commonly-used protease inhibitor cocktail. The cocktail consisted of six inhibitors, the serine protease inhibitor 4-(2- aminoethyl)-benezensulfonyl fluoride (AEBSF) was the sole inhibitor of PLD (Andrews, Bond et al. 2000). This correlation of a serine protease
inhibitor in inhibiting a lipase like PLD attributes to the fact that PLD has histidine and aspartic acid in its catalytic active site similar to a serine protease. Due to some drawbacks, such as high inhibitor concentration unsuitable for in vivo applications, more specific and efficient small molecule inhibitors of PLD have been developed recently.

The base for most of the current small molecule inhibitors of PLD2 are “halopemides” (Monovich, Mugrage et al. 2007). Halopemides were found to inhibit dopamine receptors and evaluated to be neuroleptic agents (Seeman, Grigoriadis et al. 1986). FIPI (5-fluoro-2 indolyl des-chlorohalopemide is a derivatives of halopemide and tested for its ability to inhibit PLD. It was observed that the 2-indolyl added to the halopemide made it a potent inhibitor of PLD (IC\textsubscript{50} \approx 0.020\mu M). Of the two compounds that had 2-indolyl, the one with a p-fluorophenyl in the side chain was even more potent during pharmaco-kinetics studies with a half-life greater than 5 h. And the potency versus PLD2 was improved to 75-fold that was unmatched in a set of fused bicyclic heterochromatic compounds (Monovich, Mugrage et al. 2007). Another study with PLD1 shows that FIPI is a potent PLD1 inhibitor. PLD1 has lower basal activity and hence stimulating it with PMA followed by inhibition with FIPI gave an IC\textsubscript{50}\approx1nM (Su, Yeku et al. 2009).

Since PLD1 has been well studied by many other scientists in the field such as Michael A. Frohman and Ryu, showing their importance in cancer and inflammation, we have been interested in PLD2. As in a recent study we have shown the importance of
PLD2 in cancer metastasis and invasion, it is necessary to inhibit PLD2 in such pathological conditions. Hence another inhibitor of interest is PLD2 specific N-[2-[1-(3-Fluorophenyl)-4-oxo-1,3,8-triazaspiro[4.5]dec-8-yl]ethyl]-2-naphthalenecarboxamide (NFOT). It has been shown in a recent study that NFOT is an isoform selective inhibitor of PLD specific to PLD2 (Lavieri, Scott et al. 2010).

The study also indicated that developing PLD2 specific inhibitors is difficult and that NFOT is 75 folds selective to PLD2 with an IC$_{50}$ of 20 nM. Another PLD2 specific inhibitors was developed an year earlier to NFOT called N-[2-(4-oxo-1-phenyl-1,3,8-triazaspiro[4,5]dec-8-yl)ethyl]-2-naphthalenecarboxamide (NOPT) (Lewis, Scott et al. 2009). This was more specific to PLD2 but has an IC$_{50}$ greater than NFOT and is less potent compared to NFOT. NFOT is also a [4.5]dec-8-yl]ethyl]-2-naphthalenecarboxamide derivative but has a fluorophenyl instead of oxo-phenyl making it more potent compared to NOPT.
MATERIALS AND METHODS

Materials

The COS-7, African black monkey kidney fibroblasts were purchased from ATCC. COS-7 culture medium Dulbecco’s modified eagle medium (DMEM), Fetal Bovine Serum and TransfectaGRO were obtained from Corning Cellgro® (Manassas, VA). Lipofectamine, Plus and EGF were purchased from Invitrogen (Carlsbad, CA). $^3$H$-$butanol was obtained from Perkin Elmer (Waltham, MA). All the phospholipids required for lipase enzyme activity assay including PC8 and PIP$_2$ were purchased from Avanti Polar Lipids. Matrigel inserts were purchased from BD (Franklin Lakes, NJ). Hematoxylin stain was obtained from Ricca (Arlington, TX). Phospholipase D (PLD) inhibitors were obtained from Cayman Chemicals, Avanti polar lipids and Tocris biosciences.

Methods

Cell culturing and Plasmid transfections

COS-7 cells were cultured and sub-cultured in DMEM with 10% FBS, 50 U/ml Penicillin, 50 U/ml Streptomycin and 50µg/ml Gentamycin at 37°C temperature and 5% carbon-dioxide in a CO$_2$ incubator. Cells were split at 80% confluency. The cells were transfected at 60% confluence per well with plasmids ranging from 1-6 µg of DNA, using 6µl Lipofectamine and 6 µl Plus diluted in 600 µl TransfectaGRO. Sterile glass culture
tubes housed each liposome (DNA+Lipid complex) prior to transferring the solution into 6-well plates of cells containing 1 ml of TransfectaGRO. The transfection mix was allowed for 3 hours and then the media is aspirated and changed to fresh complete DMEM. Transfection is allowed to go for 48 hours.

**Phospholipase activity assay**

Lipase activity measurement began with addition of the following reagents to 50 µl of lysates: 3.5 mM PC8 phospholipid, 0.9 mM PIP$_2$ phospholipid and 0.5 µCi of n-[$^3$H]butanol (Perkin-Elmer, MA) making the final reaction volume 120 µl. Samples were incubated for 20 minutes at 30°C with continuous shaking. The reactions were then stopped by adding 200 µl ice-cold stopping solution, 75 µl of Chloroform and 25 µl of water. After stopping the reactions, the eppendorfs were capped tightly, vortexed twice and spun at 14000g for 1 minute. Three layers were obtained after spinning. The top layer was discarded and the bottom-most layer was pipetted carefully and transferred into a fresh vial. This was dried under N$_2$ gas and resuspended in chloroform: methanol (9:1). This was spotted on Thin-layer chromatography plates along with phospholipid controls p-ethanol and p-butanol. The plate was kept in a chamber containing solvent (Ethyl acetate: Iso-octane: acetic acid: water) and allowed to separate by thin-layer chromatography. Then the samples were read in scintillation counter for lipase activity by measuring the radioactivity of the samples.

**Enzyme Competition Assay**
1x 10⁶ Cells were cultured and transfected with 2µg PLD1 or PLD2, 48 h post-transfection, cells were starved for 2 h, stimulated with EGF (3nM) and made lysates. These lysates were treated with 100 nM PLD1 inhibitor (NBOD), PLD2 (NFOT) inhibitor or dual inhibitor (FIP1) for 25 min. After treatment with the inhibitor, the lysates were subject to lipase activity assay as mentioned above, but with increasing concentrations of PC8, which is the substrate of PLD. The phospholipase activity of the enzyme was measured by measuring the radioactivity in a scintillation counter.

**Western blotting**

The cells after stimulation were made into lysates. These lysates were made in desired volume of Special lysis buffer (50mM HEPES, pH 7.2, 100 µM Na₃VO₄, Triton X-100 and 5 mg/ml each of protease inhibitors (aprotinin and leupeptin)) and sonicated. BIO-RAD protein estimation assay was utilized to determine the protein concentration and to normalize for protein per reaction.

Desired concentration of protein was loaded into the wells of SDS-PAGE gels along with a molecular weight marker. These gels were then transferred for 1 h onto PVDF membrane, blocked for 45 min at room temperature with 1% BSA with TBS, 0.2% Tween 20 and probed with primary antibody overnight. The blots were then washed with TBS-T and probed with secondary antibody conjugated to horse radish peroxidase (HRP) and detected on x-ray films with the help of ECL western blotting detection reagents from GE Health Care (Fairfield, CT). The Kodak Gel Logic software used to perform
densitometry on western blots which consists of quantifying the bead of the protein of interest versus the band of the actin loading control.

**Cell migration**

Adherent cells were detached using 25% Trypsin/EDTA or a non-enzymatic cell dissociation buffer for COS-7 or RAW cells, respectively. A hemocytometer was used to count cells, and trypan blue exclusion was used to determine cell viability. 24-well plates and Transwell inserts were pre-wetted with DMEM containing 0.5% BSA. Cell concentration was adjusted to 5 x 10^4 cells per 400µl of DMEM containing 0.5% BSA per insert. 600µl of DMEM containing 5% BSA was added to the bottom of the well prior to placing the cells and insert in the well. Either 100 ng/µl EGF for COS7 cells was used as chemoattractants. The murine macrophage cells lines were allowed to chemotax for 3.5 hours and COS-7 cells chemotaxed for 1 hour at 37°C, 5% CO2. Afterward, inserts were removed and cells that had migrated to the bottom were fixed with 4% paraformaldehyde per each well. Cells that migrated to the bottom were then allowed to settle and adhere before being counted. Cells were then counted using an inverted microscope at 20x. The average numbers of 6 counts per well were taken and data is quantified.
RESULTS

Aim 1: To determine the kinetics of the PLD inhibitors: Is it Reversible or irreversible?

Rationale

All PLD inhibitors considered in the present study are compounds that inhibit the lipase activity of PLD. Earlier many non-specific inhibitors of PLD such as primary alcohols and AEBSF were found. But due to their non-specific interactions or very high concentration requirements they could not be used. Recently, many specific small molecule inhibitors of PLD have been developed. However it is not clearly known whether these inhibitors are competitive or non-competitive or mixed inhibitors.

If the inhibitor can be classified as competitive or non-competitive, it would be suggestive of the site of action, as competitive predominantly binds in the catalytic site and also in some allosteric site, but via non-covalent interactions that can be knocked out by higher substrate concentrations. While in case of non-competitive, substrate cannot be knocked out by increasing the substrate concentration.

In case of a mixed inhibition, we might say that in some regions inhibitor binding are by weak interactive forces that allow recovery of enzyme activity to a certain extent and saturate.

*Purification of Phospholipase D2 protein*
Experimental approach

Recombinant PLD2 was made by infecting Sf21 insect cells with pBacC1-mycPLD2a-WT passage 2 virus (Gomez-Cambronero and Henkels 2012). The cells were infected with 10 times of the virus to the number of cells in the culture. This infection was allowed to go for 5 days. 5 days post-infection, cells were harvested and made into lysates. The lysate was then purified by batch purification using Cobalt conjugated TALON His-Tag purification resin. The PLD2 virus used here has 6-HN repeats and is pulled down by the TALON resin. Finally the protein is eluted by a series of elution buffer washes, wherein the elution buffer has imidazole to release the Histidine-Cobalt bond.

Results

All the elutions, washes, flow through and crude samples were then run on a gel to test for purity and specificity of PLD2 protein. Coomassie brilliant blue staining (Fig. 6) was performed to look at the purity of the recombinant PLD2 protein. Also immunoblotting (Fig. 7) was done for myc-tag on PLD2. After having looked at the purified protein being PLD2, the lipase activities (Fig. 8) of the various samples were measured and we found that first 3 eluates have good lipase activity.

Optimization of PLD assay time

Experimental approach
The enzyme competition assays were performed with recombinant PLD2 and all other experiments were done with either whole cells or cell lysates. It is important to optimize the duration of lipase assay in order to obtain the highest activity under uninhibited conditions, while getting the largest inhibition when treated with inhibitor. The recombinant protein and the lysates were treated with inhibitors first and then subject to lipase activity assay for different time points of 0, 5, 10, 15 and 20 mins.

**Results**

It was found that 15 mins was an optimal time point to stop the PLD assay as the lipase activity (Fig. 9 and 10) was the highest, while the inhibition was largest when treated with inhibitors.

*IC₅₀ of specific PLD inhibitors on lipase activity*

It is important to know the IC₅₀ of inhibitors in order to verify the potency and specificity of the inhibitors to their targets. Moreover, the IC₅₀ varies from cell to cell. The IC₅₀ of all the PLD inhibitors was determined (Fig. 11-17) and it was observed that only the PLD2 specific inhibitors and the dual PLD inhibitor worked efficiently and hence studies were performed with dual PLD inhibitor, FIPI, and PLD2 specific NFOT.

The IC₅₀ of the specific PLD inhibitors NFOT and FIPI in COS7 cells was found to be ~11 nM and ~6 nM respectively. (Fig. 13 and 15)
All the PLD assay experiments were done by starving and stimulating the cells prior to inhibitor treatment. Increasing concentrations of PLD inhibitors ranging from 0nM to 1000 nM were used.

**Enzyme-kinetics of PLD2 with inhibitors FIP1 or NFOT and substrate PC**

**Experimental approach**

All the enzyme competition assays were performed with recombinant PLD2. Before treating PLD2 with inhibitors, the IC$_{50}$ of PLD inhibitors was determined and it was observed that only the PLD2 specific inhibitors and the dual PLD inhibitor worked efficiently and hence studies were performed with dual PLD inhibitor, FIP1, and PLD2 specific NFOT and/or NOPT.

To test if PLD2 inhibition was reversible or irreversible increasing concentrations of the purified PLD2 enzyme was treated with a fixed concentration of the substrate PC (3.5 mM) and inhibitor 300 nM FIP1 or NFOT and enzyme competition assay was performed.

Another assay to study the mechanism was enzyme kinetics assay or the lipase assay of recombinant purified PLD2 protein with increasing substrate concentrations and treatment with vehicle or 300 nM inhibitor FIP1 or NFOT. Recombinant PLD2 was treated with inhibitor first, after which they were subject to treatment with increasing
concentrations of the substrate PC8 ranging from 0mM to 55mM and a fixed concentration of the effector PIP$_2$ (0.09mM).

**Results**

It was found that with FIPI the inhibition is irreversible as the PLD activity did not increase with increasing concentrations of the enzyme even after a E-I threshold was surpassed, whereas with NFOT, the inhibition was reversible as the PLD activity (Fig. 18) was regained after increasing the enzyme concentration.

For the second assay it was observed that in control group (Vehicle (DMSO) treated), PLD activity increased with increasing concentrations of the substrate. However, in 300 nM FIPI and NFOT treated cells, PLD activity (Fig. 19 and 20) dropped initially, but increased at higher concentrations of the substrate PC approaching the control levels.

Michaelis-menton kinetics and line-weaver burk plot were used to plot the data suggesting different $K_m$ and $V_{max}$ when treated with FIPI or NFOT.

*Enzyme-kinetics of PLD2 with inhibitors FIPI or NFOT and effector molecule PIP$_2$*

**Experimental approach**

Recombinant PLD2 was treated with inhibitor first, after which they were subject to treatment with increasing concentrations of the effector PIP$_2$ ranging from 0nM to 3µM and a fixed concentration of the substrate PC8 (3.5mM) at the beginning of lipase assay.
Lipase assay of recombinant purified PLD2 protein with increasing concentrations of its positive effector PIP2, in the presence of vehicle or 300 nM FIPI or 300nM NFOT was also performed to observe changes in $K_m$ and $V_{max}$.

**Results**

It was found that PIP$_2$ has two binding sites. It suggests that PIP2 exerts both a positive effect and a negative effect on PLD2. At low (<1 mM) concentration of PIP2, PLD is activated, but as the substrate increase, the activity returns to basal level. It also suggests that the specific PLD2 inhibitor NFOT binds to the site occupied by PIP2, so NFOT blocks PIP2 binding and negates its positive effect on PLD2 activity. While on the other hand, FIPI is found to be irreversibly inhibiting PLD2 by covalent modifications not allowing PIP$_2$ to bind to PLD2 (Fig. 21).
**Aim 2: To determine the site of action of PLD inhibitors.**

**Rationale**

PLD has many physiological as well as pathological roles. Some of the physiological roles of PLD are chemotaxis, cell migration, DNA synthesis and phagocytosis. Unregulated activity of PLD2 results in pathological role of PLD in chronic inflammation, cancer metastasis. Therefore there is a need to find a specific small molecule inhibitor for PLD. Elucidating the mechanism of action of existing small molecule inhibitors of PLD is one approach to design more potent inhibitors. This specific aim is designed to screen various PLD mutants whether they exhibit resistance for PLD inhibitors. The site of action of these small molecule inhibitors needs to be known to develop more potent and specific PLD inhibitors. It is also know that these inhibitors are hydrophobic in nature and hence mutating highly hydrophobic residues to less hydrophobic suggesting if that mutant is resistant to be inhibition by the FPI and/or NFOT, then that is the possible site of action of inhibitor.

**Screening for mutants with intact lipase activity**

**Experimental approach**

Several mutants of PLD (Fig. 22 and 23) in the PX, PH and around HKD regions were designed in order to screen the resistance against inhibitors. Some of the mutants in PX region are: F107Y, L110A, L126A, F129Y, H158Q, Y165F, L166A, R172C, L173A,
R172C/L173A, Y169F, Y179F, Y169F/Y179F. Mutations in the PX regions were made in order to make hydrophobic residues such as leucine, arginine and tyrosine to either less hydrophobic or hydrophilic alanine.

Mutants designed in PH and around the HKD region are: R210A/R212A, F244N/L245A/L246A, P263A, K292A, Y296F, Y415F, S648A, D649A, R651A, S757A and S771A. Even the PH mutants were designed by changing the hydrophobicity of the residues from highly hydrophobic to less hydrophobic or hydrophilic to hydrophilic alanine. HKD mutants were designed by docking the substrate and the inhibitor on PLD2 by the use of bioinformatics.

All the above listed mutants were screened for intact lipase activity when compared to PLD2-WT. Only those mutants with lipase activity more than 60% when compared to WT were chosen for further experiments with inhibitors. It is important to know that the mutation is intact as making mutations might affect the secondary and tertiary structure of the protein causing faulty enzymatic activity or loss in enzyme activity.

**Results**

Among the 12 PX mutants F107Y, L126A, F129Y, H158Q, Y165F, R172C, L173A, R172C/L173A, Y169F, Y179F, Y169F/Y179F were found to have intact lipase activity (Fig. 24).
Among the PH mutants (Fig. 25) only F244N/L245A/L246A, P263A and K292A had intact lipase activity. Of the five HKD mutants (Fig. 26) S648A, D649A, R651A, S757A and S771A, all except S771A (found to be lipase inactive) had intact PLD activity. Since PLD2 is our molecule of interest, the inhibitors used in this study are NFOT (PLD2 specific) and FIPI (dual PLD).

**Effect of DMSO on lipase activity**

*Experimental approach*

Most of the PLD inhibitors are hydrophobic and soluble only in organic solvents like DMSO. DMSO by itself has a role in cell differentiation: 1.25% DMSO is used of cell differentiation and might alter the enzyme activities in a cell. Therefore it is essential to determine the effect of DMSO on lipase activity. In order to normalize the percentage of DMSO that can be subjected to the cells and cell lysates, the cells were treated with increasing concentrations of DMSO ranging from 0 % to 1 %.

*Results*

Figure 27 suggests that in cells treated with DMSO up to 1%, the lipase activity is not affected. We therefore were safe at using 0.16% final concentration of DMSO per reaction.
Effect of inhibitors of PLD activity of PLD2 mutants

Experimental approach

All the mutants of PLD were treated with 300 nM FIPI and 300 nM NFOT, since at least 3-10 times the IC₅₀ is recommended for use. Cells were transfected with the screened (for intact lipase activity) mutant plasmids and 48 h post-transfection, lysates were made in special lysis buffer with protease inhibitors. These lysates were either treated with inhibitors or with vehicle (DMSO) for 25 min and these samples were then subjected to PLD activity assay (Fig. 28-30).

The PX, PH and HKD mutants that were resistant or partially resistant to inhibition by NFOT and/or FIPI were subject to increasing doses of the inhibitors FIPI and NFOT (Fig. 31-34).

Results

It was observed that the mutants PLD2-F244N/L245A/L246A and PLD2-S648A are resistant to inhibition over a range of inhibitor concentrations of NFOT (Fig. 35).

We speculate that the residues F244/L245/L246 in the PH region and S648 in the HKD motif has to be intact for the action of NFOT. Using the coordinates of F244/L245/L246 and S648 specifically, more potent and sensitive inhibitors can be designed.
**Functional relevance of PLD2 mutations and PLD2 inhibition**

**Experimental approach**

In order to see if the mutations of PLD2 or PLD2 inhibition had any effect on the functions of PLD such as chemotaxis, we selected the most promising mutants and subjected them to chemotaxis (Fig. 36). The cells that were over-expressed with mock or PLD2-WT or one of the 7 mutants of PLD2 such as F129Y, H158Q, Y165F, R172C, L173A, R210A/R212A, F244N/L245A/L246A and S648A were treated with vehicle or 300 nM NFOT or FIPI and subject to chemotaxis (COS7s).

**Results**

It was found that the chemotactic ability of these mutants was intact but all the mutants except S648A were inhibited when treated with the inhibitors FIPI and/or NFOT (Fig. 37). These findings suggest that the mutants have functional relevance to PLD2-WT.
Aim 3: To determine the role of interacting proteins on the action of PLD inhibitors.

Rationale

As mentioned earlier, PLD2 has several protein-binding partners in the cell at any given time (Knapek, Frondorf et al. 2010). Upon inhibitor treatment in vivo, protein partners of PLD might interfere with inhibitor action. Therefore in this aim, we sought to determine the inhibitors action in the presence or absence of binding partners. In the present study we choose two binding partners of PLD2, Grb2 and Ras. These proteins were chosen because of their important role in both physiological processes like cell migration as well as in cancer metastasis.

In a recent study it was shown that in the presence of Grb2 PLD2 activity increases in vivo stimulating the Erk/MEK pathway of cell proliferation (Henkels, Short et al. 2009). Another study showed that Grb2 is in a heterotrimer of PLD2-Grb2-WASp stimulates leukocytes phagocytosis wherein PLD2 interacts with Grb2 and grb2 interacts with WASp through its SH3 domains resulting the activation of Arp2/3 which is involved in actin nucleation and forms filopodia and a phagocytic cup (Kantonen, Hatton et al. 2011).

If these binding partners indeed show positive or negative effect on the action of inhibitors, other binding partners will be studied further. Also it will provide important information regarding inhibitor doses employed in situations of inflammation and cancer.
Under such pathological conditions the protein partner’s effect on inhibitor action might have to be taken into consideration while employing inhibitor doses.

**Effect of binding partners of PLD2 upon treatment with PLD inhibitors**

**Experimental approach**

COS7s were silenced with 200 nM siRas and overexpressed with PLD2-WT for 48-72 h with a boost of 100 nM siRas a day after overexpression of PLD2-WT. Cells were harvested and lysates were prepared, which were subjected to increasing concentrations of the inhibitors FIPI and NFOT for each experimental group (PLD2-WT only or siRas + PLD2-WT).

**Results**

In a lipase assay following treatment with increasing doses of inhibitor, it was found that silencing Ras (Fig. 38 and 40) showed a positive effect on PLD2 lipase activity that increased marginally. But treatment with inhibitor did not protect this activity showing decreased PLD2 activity with increasing inhibitor concentrations.

**Role of interacting proteins Grb2 and Ras on PLD activity**

**Experimental approach**
To confirm the effect of Grb2 in PLD2 inhibition, COS7s were then overexpressed with PLD2 only or both PLD2 and Grb2. Normally an increase of 4-5 folds in activity is observed in lysates from cells that have been transfected 1-2 days with pcDNA3.1-mycPLD2a-WT. After 48 h of transfection, cells were harvested and lysates were prepared. These lysates were subjected to PLD activity assay and Western blot analysis.

Results

Overexpression of Grb2 showed an increase in PLD activity compared to mock transfected cells. Upon treatment with 300nM of inhibitors FIPI or NFOT, lipase activity of mock cells decreased rapidly, while the Grb2 overexpressed cells (Fig. 41) when treated with 300nM NFOT showed resistance, but when treated with 300nM FIPI PLD2 activity was inhibited (Fig. 39). Summarily, these inhibition experiments with binding partners conclude that while employing inhibitors in vivo, several factors including binding partners of the target protein has to be taken into consideration. A schematic summarizing this shows the interaction of Grb2 and PLD2 suggesting PLD2 activity is positively regulated by Grb2, and this interaction protects PLD2 from inhibition by NFOT (Fig. 42).

Overexpression of Grb2 was confirmed by western blot analysis. The assay showed an increase in PLD activity upon co-transfection with Grb2 in COS7s.
DISCUSSION

Our findings suggest that Phenylalanine244 through leucine 246 and serine 648 within the allosteric site and the catalytic site are essential for the action of inhibitors on PLD2 and that mutating these key amino acid(s) results in partial resistance of the enzyme towards the inhibitor. In addition, the inhibitor’s action on PLD2 is effected by the presence or absence of protein interacting partners of PLD2 such as Grb2.

PLD binds to Grb2 and recruits Sos, while PLD-derived PA directly binds to Sos, both ultimately promote GTP loading of Ras and stimulates the MEK/ERK pathways (Zhao, Du et al. 2007) that are key regulators of cell migration and DNA synthesis. In addition PLD2 itself acts as GEF for Ras (Henkels, Mahankali et al. 2013). Activation of Ras-guanine nucleotide exchange factor, Sos by interaction of SH3 of Grb2 with C-terminal Proline-rich regions in Ras and Sos is a well characterized mechanism that results in the activation of MAPK cascade and cell proliferation (Chardin, Camonis et al. 1993; Egan, Giddings et al. 1993). Our finding suggests that Ras negatively regulates PLD2, wherein in the absence of Ras, PLD2 activity is increased significantly. In addition, inhibitor experiments with Ras and PLD2 suggested that the presence of Ras did not affect inhibition of PLD2 by FIP1 or NFOT.

As we know that Grb2 is central to many intracellular signaling pathways and that Grb2 interacts with phosphotyrosine residues on PLD2 via SH2 domains triggering chemotaxis and cell migration, it is essential to see the effect of Grb2 in PLD2 inhibition
by PLD2 inhibitors. We found that Grb2 positively regulates PLD2 at lower concentrations of inhibitors by protecting it from inhibition, but at higher concentrations the activity is inhibited. This suggests that Grb2 interaction with PLD2 allows the binding of inhibitors thereby enhances efficiency of the inhibitors.

This is the first study where we attempted to elucidate the kinetics of PLD2 inhibitors. Lineweaver-burk (LB) plot (Fig. 17) shows that FIPI and NFOT inhibit PLD by increasing $K_m$ and decreasing $V_{max}$ values, which therefore is by mixed inhibition. Mixed inhibition suggests a wide repertoire of binding sites on PLD2 including both catalytic and regulatory regions. However, mixed inhibition of PLD2 by FIPI and NFOT does not suggest anything specific about their binding site on PLD2. To explore the site of action, we designed wide range of mutants within the PX, PH and HKD regions of PLD2.

PIP2 exerts both a positive effect and a negative effect on PLD2. At low (<1 mM) concentration of PIP2, PLD is activated, but as the substrate increase, the activity returns to basal level. If PIP is added to cell lysates of overexpressing PLD2 a strong negative can be documented. We also report that the specific PLD2 inhibitor, N-[2-[1-(3-Fluorophenyl)-4-oxo-1,3,-8-triazaspiro[4.5]dec-8-yl]ethyl]-2-naphthalenecarboxamide (NFOT) binds to PLD2 at two different sites, one being at the catalytic site (HKD motifs) at S648, and another to an allosteric site that is the natural site occupied by PIP2, so NFOT blocks PIP2 binding and negates its positive effect on PLD2 activity.
Our data suggests that the mutants F244N/L245A/L246A and S648A are resistant to inhibition by the inhibitor NFOT. Thus the binding site of the inhibitor NFOT are found to be F244/L245/L246 and S648 suggesting it binds to regulatory regions of PLD2 possibly affecting the binding of the effector PIP2 binding to PLD2 as well as the catalytic site.

Thus we developed a working model with this study showing the effect of inhibitors, type of inhibition on PLD2 and the role of interacting partner Grb2 in inhibition of PLD2 by FIPI or NFOT (Fig. 43). The model shows that NFOT binds to an allosteric site with high affinity and with comparatively low affinity to the catalytic site on PLD2. Upon PLD2 interactions with Grb2 via phosphorylated tyrosine residues, PLD2 activity is resistant to inhibition by NFOT, while FIPI results in an irreversible inhibition, presumably by covalently modifying the catalytic site. Ras has a negative effect on PLD2 activity.

From our findings on the action of the two PLD2 inhibitors, FIPI and NFOT, one could say the two have different modes of action. FIPI could possibly act by modifying the enzyme totally not allowing the protein partners such as Grb2 to interact with PLD2, whereas NFOT interacts at both the catalytic and allosteric sites suggesting two sites of action. The two sites for action of NFOT could possibly be accounted for by its structure wherein the fluoride possibly interacts at the catalytic site and the two benzyl rings interact at the allosteric site. Another finding from our study that would correlate with
FIPI action is the inhibition of PLD2 significantly higher than PLD1 by NBOD that also has fluoride, is available as a selective PLD1 inhibitor in the market.

Determining the site of action, mode and microenvironment of inhibitor action would help in developing more potent inhibitors of PLD2 which can be used in vivo in studying their effects on pathological conditions implicated by PLD2.
### Table 1. PLD inhibitors

The table shows a list of PLD inhibitors and their structure.
Fig 6. Coomassie brilliant blue gel staining of PLD2 protein purification. This gel shows the purity of recombinant PLD2-WT made by batch purification.
Fig 7. **Western blot analysis of myc-PLD2.** This image shows the purified PLD2 protein has myc tag.
Fig 8. Lipase activity of PLD2 purification samples. This figure shows the intact lipase activity of the eluates of PLD2 obtained during batch purification.
Fig 9. Lipase activity of recombinant PLD2. This figure shows the determination of optimal time of lipase activity and inhibition of recombinant PLD2.
**Fig 10. Lipase activity of overexpressed PLD2.** This figure shows the determination of optimal time of lipase activity and inhibition of PLD2 overexpressed in cells.
Fig 11. RBPC IC$_{50}$. Shows the dose-response curve of PLD1 specific inhibitor RBPC in COS7s, wherein PLD2 is inhibited instead of PLD1
Figure 12. **NBOD IC$_{50}$**. Shows the dose response curve of a PLD2 specific inhibitor that inhibits PLD1 and PLD2 with IC$_{50}$ of 9 nM and 20 nM respectively in COS7s.
Fig 13. NFOT IC$_{50}$. Shows the dose response curve of a PLD2 specific inhibitor that inhibits PLD2 alone with an IC$_{50}$ of 10 nM in COS7s
Fig 14. NOPT IC$_{50}$. Shows the dose response curve of a PLD2 specific inhibitor that inhibits PLD2 with an IC$_{50}$ of 45 nM in COS7s
**Fig 15. FPI IC$_{50}$.** Shows the dose response curve of a dual PLD inhibitor that inhibits both PLD1 and PLD2 with an IC$_{50}$ of 10 nM and 8 nM respectively in COS7s
**Fig 16. NFOT IC$_{50}$ in whole cells.** Shows the dose response curve of a PLD2 specific inhibitor that inhibits only PLD2 with an IC$_{50}$ of 10 nM respectively in COS7s.
Fig 17. FICI $IC_{50}$ in whole cells. Shows the dose response curve of a dual PLD inhibitor that inhibits both PLD1 and PLD2 with an $IC_{50}$ of 7 nM and 8 nM respectively in COS7s.
**Fig 18. Enzyme Kinetics: Reversible or irreversible.** Shows the lipase activity assay of FIPI and NFOT respective to the control, suggesting FIPI is irreversible and NFOT is reversible.
**Fig 19. Enzyme competition assay: Michaelis-menton kinetics.** Shows the michaelis-menton kinetics plot of FICI and NFOT respective to the control, suggesting FICI and NFOT are non-competitive or mixed PLD inhibitors.
Fig 20. Enzyme competition assay: Lineweaver-burke plot. Shows the lineweaver-burke plot of FIPI and NFOT respective to the control, suggesting FIPI and NFOT are non-competitive or mixed PLD inhibitors as the inhibitor $K_m$ and $V_{max}$ are not equal to control suggesting both the catalytic binding and rate of action of the enzyme are affected by the inhibitors.
**Fig 21. Allosteric regulator (PIP₂) of PLD: Enzyme kinetics.** Shows a plot of PLD enzyme activity vs increasing dose of PIP₂ in the presence or absence of FIPI or NFOT, suggesting FIPI inhibits PLD2 at a different site than the allosteric regulator PIP₂ interaction site, while NFOT inhibition is partially rescued by increasing concentrations of PIP₂ suggesting binding site of PIP₂ could be possibly the site of interaction of NFOT as well.
Fig 22. Mutations in PLD2. This is a representative of the various mutations in PLD2 in the regulatory PX and PH domains, as well as in and around the catalytic HKD motifs. The mutations in the regulatory domains are made based on hydrophobicity, making more hydrophobic residues to less hydrophobic or hydrophilic and the mutations in catalytic domains are made by docking the substrate and inhibitors on PLD2. Also the residues in and around HKD were mutated into less hydrophobic alanine.
Fig 23. Optimization of mutants for transfection. All the selected mutations from PX, all the mutations made in PH and HKD were optimized for transfection shown in a western blot representative of the protein expression post-transfection.
Fig 24. Screening of intact lipase mutants in the PX region. All the mutations made in the PX domain were screened for intact lipase activity as making mutations might affect the protein structure and function. Mutants that had 60% or more activity compared to WT were considered intact.
**Fig 25. Screening of intact lipase mutants in the PH region.** All the mutations made in the PH domain were screened for intact lipase activity as making mutations might affect the protein structure and function. Mutants that had 60% or more activity compared to WT were considered intact.
Fig 26. Screening of intact lipase mutants in the HKD region. All the mutations made in and around the HKD motifs were screened for intact lipase activity as making mutations might affect the protein structure and function. Mutants that had 60% or more activity compared to WT were considered intact. Interestingly we found that S771 which is a HKD mutant is lipase inactive.
**Fig 27. Effect of DMSO on lipase activity.** Most of the potent and specific PLD inhibitors are hydrophobic and soluble in organic solvents like DMSO. This figure shows the effect of increasing concentrations of DMSO on lipase activity. It is shown that concentrations of DMSO up to 1% do not affect PLD activity. All the experiments with inhibitors are hence performed at safe levels of DMSO of 0.16%.
Fig 28. Effect of FIPI and NFOT on mutations in the PX region. All the mutants in the PX region that were screened for intact lipase activity were treated with 300 nM FIPI or NFOT to screen for a resistant mutant that could be the possible site of action. It was found that none of the mutations in the PX domain were resistant to inhibition by FIPI. But the mutants L126A, F129Y, Y165F, R172C and R172C/L173A were partially resistant to inhibition by NFOT.
**Fig 29. Effect of FIPI and NFOT on mutations in the PH region.** All the mutants in the PH were screened for intact lipase activity were treated with 300 nM FIPI or NFOT to screen for a resistant mutant that could be the possible site of action. R210A/R212A and F244N/L245A/L246A were found partially resistant to NFOT.
Fig 30. **Effect of FICI and NFOT on mutation in HKD region.** All the mutants in the HKD were screened for intact lipase activity were treated with 300 nM FICI or NFOT to screen for a resistant mutant that could be the possible site of action. S648A was resistant to NFOT and S757A was partially resistant to NFOT and FICI.
Fig 31. Effect of mutation in PX region on NFOT action. To confirm the results obtained earlier, the lipase activity of resistant PX mutants in the presence of a range of concentration of NFOT was measured. None of the PX mutants showed resistance to inhibition by NFOT.
Fig 32. Effect of mutation in PH region on NFOT action. To confirm the results obtained earlier, the lipase activity of R210A/R212A and F244N/L245A/L246A mutants in the PH region in the presence of a range of concentrations of NFOT was measured. It was found that R210A/R212A and F244N/L245A/L246A were partially resistant to inhibition by NFOT.
**Fig 33. Effect of mutation in HKD region on NFOT action.** To confirm the results obtained earlier, the lipase activity of S648A and S757A mutants in the HKD in the over a concentration range of NFOT was measured. It was found that S757A and S648A mutants were partially resistant to inhibition by NFOT.
Fig 34. Effect of mutation in HKD region on NFOT action in cell lysates. To confirm the results obtained earlier, the lipase activity of S648A in the HKD in the over a concentration range of NFOT was measured in cell lysates. It S648A was partially resistant to inhibition by NFOT.
Fig 35. Effect of mutation in PH region or HKD region on NFOT: quantification.

The graph showing partial resistance was quantified and subject to paired t-test for showing statistical significance. The inhibition of WT was highly significant (P<0.005), while that of the triple mutant and S648A was not significant.
Fig 36. Chemotaxis of intact PX, PH and HKD mutants. To test for the biological relevance of these mutations, the mutants were tested for their chemotactic ability. It was observed that many of the mutations showed similar chemotaxis respective to their activities.
Fig 37. Effect of inhibitors FIPI and NFOT selected PLD mutations: Functional relevance. Some of the studies showed R210A/R212A, F244N/L245A/L246A, S648A and S757A are resistant to inhibition by NFOT. And S757A was partially resistant to inhibition by FIPI as well. In order to study the biological relevance, we studied the chemotaxis of these mutations and found that these mutants had intact chemotaxis with no inhibitor treatment and they are resistant to inhibition by NFOT but not by FIPI except S757A.
Fig 38. Role of silencing Ras in PLD2 inhibition by NFOT. Silencing Ras increased PLD2 activity suggesting Ras could be negatively regulating PLD2 in the cells, but PLD2 activity was inhibited upon treatment with inhibitors.
Fig 39. Role of overexpressed Grb2 in PLD2 inhibition by FIPI and NFOT. As we already know that Grb2 positively regulates PLD activity, we wanted to look at the role of overexpressing Grb2 in inhibition of PLD activity by FIPI and NFOT. Overexpression of Grb2 showed an increase in PLD activity compared to mock transfected cells. Upon treatment with 300nM of inhibitors FIPI or NFOT, lipase activity of mock cells decreased rapidly, while the Grb2 overexpressed cells when treated with 300nM NFOT showed significant resistance (P<0.005), but when treated with 300nM FIPI PLD2 activity was inhibited.
**Fig 40. Western blot of silencing Ras.** This is a western blot showing Ras silencing in COS7 cells.
**Fig 41. Western blot of overexpressing PLD2 and Grb2.** This is a western blot showing co-expression of PLD2 and Grb2 or PLD2 alone in COS7 cells.
Fig 42. Model for Grb2 and Ras role in PLD2 inhibition. The model shows that NFOT binds to an allosteric site with high affinity and with comparatively low affinity to the catalytic site on PLD2 upon PLD2 interactions with Grb2. On the other hand, Ras has a negative effect on PLD2 activity.
**Fig 43. Summary model.** This is a model of the study showing FIPI is an irreversible (or non-competitive inhibitor) and NFOT is a reversible (mixed inhibitor). The possible sites of action of NFOT are F244/245/246 or nearby in the PH region and S648 or nearby in the HKD region and the possible site of action of FIPI is S757 or nearby in the HKD region. Ras negatively regulates PLD2 and does not significantly affect the inhibition of PLD2. Grb2 positively regulates PLD2 by binding to PLD2, and affects the binding site of action of NFOT on PLD2 thus showing partial resistance to inhibition by NFOT, while FIPI on the other hand could possibly be modifying the protein irreversibly and hence the lipase activity is significantly inhibited.
REFERENCES