Published in final edited form as:

Future Oncol. 2009 November ; 5(9): 1477. doi:10.2217/fon.09.110.

Targeting phospholipase D with small-molecule inhibitors as a potential therapeutic approach for cancer metastasis

Wenjuan Su,

Center for Developmental Genetics, Program in Molecular & Cellular Pharmacology and, Department of Pharmacology, Stony Brook University, NY, USA

Qin Chen, and

Center for Developmental Genetics, Program in Molecular & Cellular Pharmacology and, Department of Pharmacology, Stony Brook University, NY, USA

Michael A Frohman[†]

438 Center for Molecular Medicine, Center for Developmental Genetics and, Department of Pharmacology, Stony Brook University, Stony Brook NY 11794, USA

Abstract

Phospholipase D (PLD)1 and PLD2, the classic mammalian members of the PLD superfamily, have been linked over the past three decades to immune cell function and to cell biological processes required by cancer cells for metastasis. However, owing to the lack of effective small-molecule inhibitors, it has not been possible to validate these roles for the PLDs and to explore the possible utility of acute and chronic PLD inhibition *in vivo*. The first such inhibitors have recently been described and demonstrated to block neutrophil chemotaxis and invasion by breast cancer cells in culture, increasing the prospects for a new class of therapeutics for autoimmune disorders and several types of metastatic cancer.

Keywords

breast cancer; FIPI; halopemide; inhibitor; lipid signaling; neutrophil chemotaxis; phospholipase D

Classical phospholipase D (PLD) hydrolyzes the phosphodiester bond of the glycerolipid phosphatidylcholine (PC) to generate the signaling lipid/second messenger phosphatidic acid (PA) and choline [1].

Over the course of 50 years of study, PLD superfamily members have been found in organisms ranging from viruses and bacteria to yeast, plants and mammals. PLD activity and its product PA have been implicated in a wide range of cell biological processes, including signal transduction (activation of small G proteins [2,3] and kinases [3-7] and antiapoptotic signaling [8]), membrane vesicle trafficking (endocytosis [9,10], phagocytosis [11,12] and exocytosis/secretion [13-16]), actin [17,18] and myosin [19] cytoskeletal reorganization, and chemotaxis/

© 2009 Future Medicine Ltd

[†]Author for correspondence: 438 Center for Molecular Medicine, Center for Developmental Genetics and the Department of Pharmacology, Stony Brook University, Stony Brook, NY 11794, USA Tel.: +1 631 632 1476 Fax: +1 631 632 1692 michael@pharm.stonybrook.edu.

Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

cell migration for neutrophils and cancer cells [17,20-22]. These cell biological processes have been proposed to be relevant to human physiology and disease in several contexts, including diabetes [13,14], oncogenesis [2,3,8,21,23] and immune system function [2,11,17].

However, the lack of small-molecule inhibitors (and knockout mice) for PLD has prevented these proposed roles from being validated *in vivo* and therapeutic options explored in model systems. Fortunately, several recent reports have now characterized and described use of a new family of related PLD inhibitors. 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), an analog of halopemide (a therapeutic used in psychiatric disorders), is a potent PLD1 and PLD2 inhibitor [20,24] that inhibits neutrophil chemotaxis [17], and related isoform-selective analogs have also been developed and shown to block breast cancer cell migration and invasion in culture [21]. In this review, we discuss the PLD superfamily and focus on recent advances in understanding the cellular function of PLD and possible applications for PLD inhibitors in the clinical setting.

PLD superfamily

PLD family members

Members of the PLD superfamily are defined by the presence of one or more 'HKD' half-catalytic sites (formally known as HxK[x]₄D[x]₆GSxN) [1]. Classically, PLD is viewed as a lipid-modifying enzyme that hydrolyzes PC to generate PA or that uses glycerol or short-chain alcohols to generate phosphatidylalcohols. However, some family members possess quite divergent activities, including MitoPLD, which uses cardiolipin as a substrate [25], or cardiolipin synthase and phosphatidylserine synthase, which use the phosphatidyltransferase capacity to generate new lipids. The superfamily also includes endonucleases (Nuc), which use the phosphodiesterase activity to cleave the backbone of DNA, pox virus envelope proteins and their mammalian counterparts that are required for virion formation through an unknown biochemical mechanism, and the protein Tdp1, which resolves stalled topoisomerase-DNA complexes, involving covalent links between the protein and the DNA, again using the phosphodiesterase activity to sever them.

The classic mammalian PLD enzymes, PLD1 and PLD2, contain a number of recognized protein domains in addition to the regions that confer catalytic activity. In particular, both isoforms encode Pleckstrin homology, Phox homology and phosphoinositide (PtdIns[4,5]P₂)-binding domains or motifs that facilitate interaction of the enzymes with a wide variety of regulatory and target proteins (FIGURE 1). Furthermore, these regions regulate movement of the PLDs to membrane surfaces in different sub cellular compartments (e.g., the Golgi apparatus, secretory vesicles, and the plasma membrane).

Expression & subcellular localization of PLD

Mammalian PLD1 and PLD2 are expressed in a wide variety of cell and tissue types [26,27], although the expression levels vary dramatically. For example, high levels of PLD1 are found in secretory cells, such as human promyelocytic leukemia (HL-60) and pancreatic β -cells, whereas PC12K cells express only PLD2, and mouse thymoma (EL4) cells do not detectably express either PLD1 or PLD2. Most mammalian tissues express both isoforms, with the exception of peripheral leukocytes, in which no PLD2 expression is reportedly detected.

Studies of the subcellular localization of PLD1 and PLD2 have yielded varied results. PLD1 has been reported to have a perinuclear vesicular localization in many cell lines, consistent with a Golgi apparatus, endoplasmic reticulum, secretory vesicle and late endosome distribution [13]. However, some groups have not observed Golgi localization, and in some cell lines PLD1 clearly localizes to the plasma membrane [28]. Most investigators have reported that PLD2 localizes to the plasma membrane [9], but it has also been reported to have

a cytosol distribution and co-localize with β -actin, or to localize to the Golgi apparatus [16]. It is likely that the dynamicity of PLD1 and PLD2 resolves this issue. Upon stimulation, PLD1 translocates to the plasma membrane and then cycles to sorting/recycling and early endosomes [29]. Differences in the kinetics of cycling and primary steady-state localizations for PLD1 in the different cell lines could underlie the differences in the published reports. Translocation of PLD2 to membrane ruffles has been demonstrated in HeLa cells in response to serum and EGF, following which it cycles through sorting/recycling and early endosomes and potentially the Golgi, before returning to the plasma membrane.

Finally, PLD3 – a poorly understood isoform – localizes to the luminal side of the endoplasmic reticulum [30], and MitoPLD localizes to the outer surface of mitochondria [25].

Regulation of PLD activity

Phospholipase D is activated in response to G-protein coupled receptor (GPCR) and receptor tyrosine kinase stimulation subsequent to the mobilization and activation of cytosolic factors. PLD activity is tightly regulated by a large number of factors, including small GTPases, protein kinase C (PKC), phospho inositides, post-translational modification and inhibitory factors. The regulation is complex, and many of these factors act in concert to regulate PLD activity in either synergistic or antagonistic ways.

Members of the Rho and ARF subfamily play critical roles in PLD activation. Upon stimulation, such as by fMet-Leu-Phe (fMLP) or EGF, ARF translocates to membranes, resulting in co-localization with and activation of PLD. Rho family members interact directly with PLD and also regulate its activity. The phosphoinositide $\text{PtdIns}(4,5)\text{P}_2$ is a critical lipid co-factor for PLD activity. Curiously, the kinase, PI4P5K, which generates $\text{PtdIns}(4,5)\text{P}_2$, is itself stimulated by PA, the enzymatic product of PLD, as well as by ARF and Rho small GTPases, creating a positive feed-forward loop of signaling lipid generation. In many signaling events and cell biological processes, the small GTPases appear crucial for achieving the spatially regulated production of PA and $\text{PtdIns}(4,5)\text{P}_2$ through the temporal activation and localization of PLD and phosphatidylinositol-4-phosphate 5-kinase [31].

Protein kinase C (PKC) is another well-known stimulator of PLD that is activated subsequent to the stimulation of GPCRs and receptor tyrosine kinases. PLD and PKC also physically interact, resulting in strong activation of PLD1 activity *in vitro*. Inhibition of PKC decreases receptor-induced PLD activity, and PLD1 mutants unresponsive to PKC do not respond well to activation of GPCR. Taken together, one view of the complex and synergistic stimulation of PLD, mediated by the small GTPases, PKC and $\text{PtdIns}(4,5)\text{P}_2$, is that PLD functions as an integrator of all of these pathways, and is activated to a significant extent only when multiple and potentially all of the inputs converge spatially and temporally during signaling events.

PLD function

The PLD activators PIP2, ARF and Rho GTPases are well-defined regulators of actin reorganization and membrane transport. PLD acts as a downstream effector of ARF to mediate budding of Golgi vesicles, transport from the endoplasmic reticulum to the Golgi membrane, and release of nascent secretory vesicles from the trans-Golgi network [16]. PLD has also been demonstrated to be an important component of the exocytic pathway in adipocytes, neuroendocrine, mast cells, platelets and pancreatic β -cells [23]. Several sources of evidence have implicated a critical role of PLD1-generated PA in membrane fusion or exocytosis steps subsequent to the docking process [13-15,28]. PA may play a direct and/or an indirect role in membrane fusion. PA can act as a fusogenic lipid – when present on the inner leaflet of fusing membranes, PA promotes negative curvature, decreasing the energy required for fusion [13]. PA promotes fusion in an asymmetric manner, as has been shown using a cell-free system to

study soluble *N*-ethylmaleimide sensitive fusion protein attachment protein receptors (SNARE) complex-dependent membrane fusion [15]. In this setting, PA enhances fusion only when PA is present in the acceptor membrane, which is the equivalent to the plasma membrane. PA may also promote fusion indirectly by acting as a lipid anchor to recruit and activate phosphatidylinositol-4-phosphate 5-kinase to increase levels of PtdIns(4,5)P₂ or through recruitment of a calcium-activated protein [14] that facilitates late steps in the fusion process.

Analogously, a nonclassical mammalian PLD family member, MitoPLD, has been demonstrated to regulate mitochondrial fusion by hydrolyzing cardiolipin to produce PA on the outer membrane of mitochondria [25]. Unlike the exocytic fusion mediated by SNARE proteins, mitochondrial fusion is mediated by a separate protein machinery involving Mitofusins and Opa1, mutation of which causes the neuro-degenerative diseases Charcot–Marie–Tooth type II α and dominant optic atrophy. These findings suggest that PA, a fusogenic lipid, underlies different fusion processes, such as mitochondrial fusion and regulated exocytic fusion, even though they are mediated by distinct sets of protein machinery.

Finally, PLD functions in response to cell stress and in processes connected to reorganization of the actin cytoskeleton and cell migration, as discussed in the next sections. These functions, in particular, connect to current hypotheses concerning physiological functions for PLD that represent potential therapeutic targets.

PLD & cell movement

Increased PLD activity in acute and chronic inflammatory processes may be associated with protective responses of the tissue, and repair and remodeling. In addition to the established roles of PLD-generated PA in leukocyte function, such as phagocytosis and oxidative burst, a recurrent theme in the literature involves cell chemotaxis. This is a complex process that involves multiple cell biological pathways, including actin polymerization, cytoskeletal reorganization, morphological polarization, specific adhesiveness and cell-substratum detachment [32]. Although a variety of signaling cascades, including PLD, have been implicated in leukocyte motility, the precise contribution of each signaling pathway is not well understood.

Phospholipase D/PA has recently been implicated in cell spreading. Cells in suspension exhibit high levels of PLD2 activity, elevating production of PA, which recruits myosin phosphatase and keeps it in an inactive state, thus promoting myosin hyperphosphorylation and ensuing symmetrical retraction of the cells into spherical shapes [19]. Upon cell attachment, downregulation of PLD2 activity decreases PA production, resulting in release and activation of myosin phosphatase, myosin dephosphorylation, actomyosin disassembly, and finally cell spreading. In other contexts, PLD activation is required for integrin-mediated cell spreading by acting as a membrane anchor for the small GTPase Rac1, which is a crucial regulator of actin cytoskeletal rearrangement, cell spreading and migration [33].

PLD1 knockdown by small-interfering RNA has been reported to abolish basal chemokinesis [34]. PLD2 knockdown also leads to cell migration arrest. Both isoforms are associated with cell polarity and directionality in the context of changes in adhesion and F-actin polymerization. Our data using a small-molecule inhibitor of PLD, FIPI, confirmed a role for PLD in fMLP-induced chemotaxis [20]. In neutrophils, Rac1 plays a critical role during chemokine gradient detection and polarized actin assembly via phosphatidylinositol 3-kinase (PI3K) pathways. Very recently, PLD-generated PA has been demonstrated to recruit the guanine nucleotide exchange factor of Rac1, DOCK2, to the leading edge of polarizing cells, resulting in increased local actin polymerization [17]. In the presence of the PLD inhibitor FIPI, neutrophils fail to form leading edges properly and exhibit defects in chemo taxis. This work elegantly demonstrates that PLD-generated PA coordinates with PI3,4,5P₃ to sequentially regulate the

recruitment and localization dynamics of DOCK2, which in turn localizes Rac activation during neutrophil chemotaxis. Additionally, studies on neutrophils responding to fMLP suggest that PLD activity may regulate inside-out activation of the $\beta 2$ integrin, CD11b/CD18, which plays an important role in leukocyte adhesion and migration [22,35].

These roles have an obvious implication in the setting of immune function, but are also relevant to cancer cell metastasis, as discussed in the following section.

PLD & cancer

During the past 15 years, many studies have reported that PLD activity is increased in response to mitogenic signals and activated oncoproteins, such as EGF, PDGF, FGF, insulin, v-Src, v-Ras and c-Fps [8]. Elevated PLD activity and expression have also been found in many types of human cancer, including breast, colon, gastric and kidney (Table 1) [8,23]. Expression levels of PLD2 correlate significantly with tumor size and survival of patients in colorectal carcinoma [36], and PLD2 point mutations are found in breast cancer [37]. These studies provide compelling evidence that increased PLD activity is functionally linked with oncogenic signals and tumorigenesis.

PLD as an alternative survival signal

Elevated PLD activity has been shown to contribute to cell transformation and survival [8]. c-Src does not lead to transformation by itself. However, in combination with elevated PLD1 or PLD2, it can transform rat fibroblasts [38] and enhance cellular proliferation [39]. Similarly, PLD1 or PLD2 can transform rat fibroblasts overexpressing the EGF receptor [38,40]. PLD1 and PLD2 can also induce anchorage-independent growth and promote cell-cycle progression of mouse fibroblasts [41]. More recent work has revealed an unexpected role of PLD2 in Ras activation in response to EGF stimulation, in which the PLD2-generated PA acts upstream of Ras by recruiting its immediate activator, Sos, to translocate to the plasma membrane, which is critical in the Ras activation pathway that leads to cell transformation [3]. Such types of evidence indicate that PLD activity can contribute to proliferation and transformation.

In addition to promoting cell proliferation, elevated PLD expression prevents cell-cycle arrest and apoptosis. Enhanced Ras or Raf signaling induces cellular senescence or apoptosis in the absence of serum [42,43]. However, if the cells express elevated levels of PLD1 or PLD2, then they continue to proliferate and successfully tolerate the high-intensity Raf signal [44]. Similarly, PLD1 and PLD2 provide survival signals that prevent apoptosis in c-Src overexpressing fibroblasts that would otherwise undergo apoptosis in response to growth factor withdrawal [45], and inhibiting PLD activity leads to apoptosis in serum-deprived v-Src-transformed cells [46]. Finally, PLD activity provides resistance to H_2O_2 - and glutamate-induced apoptosis [47].

Mammalian target of rapamycin (mTOR), a serine/threonine kinase, regulates a variety of cellular activities in response to environmental stress [48]. Cancer cells must overcome these stress responses to survive and proliferate; thus, mTOR is a crucial source of survival signals in cancer cells [49]. PA binds to the mTOR complex, resulting in activation of the mTOR substrate S6 kinase and phosphorylation of another mTOR substrate, eukaryotic initiation factor 4E binding protein-1 in HEK293 cells [50,51]. Exogenous expression of PLD2 to increase PA levels also increases S6 kinase phosphorylation in human breast adenocarcinoma (MCF-7) cells [52], and elevated levels of PLD1 increase S6 kinase phosphorylation in rat fibroblasts and block DNA damage-induced increases in p53 [53], whereas suppression of PLD1 expression blocks S6 kinase phosphorylation in melanoma cells. PLD2 has also been shown to form a functional complex with mTOR and its binding partner Raptor via a TOR signaling (TOS) motif in PLD2, and this interaction is essential for mitogen stimulation of

mTOR [5]. More recently, PLD1, but not PLD2, was found to be required for Rheb activation, which is the small G protein that acts upstream of mTOR signaling [54]. In addition, new evidence has revealed a PLD-generated PA requirement for the stabilization of both mTORC1 or mTORC2 complexes by maintaining the association of mTOR with Raptor or Rictor, respectively [55]. Accordingly, in addition to the ability of PA to activate mTOR, several studies have demonstrated a requirement of PLD for the activation of mTOR.

Rapamycin is an mTOR inhibitor; however, rapamycin-based therapeutic strategies are unsuccessful in some cancer patients [8]. Interestingly, PA contributes to mTOR activation in a manner that is competitive to rapamycin, and increased PLD activity provides an alternative survival signal and confers rapamycin resistance in breast cancer cell lines. Inhibition of PLD activity using small interfering RNA triggers apoptosis in these tumor cell lines, whereas the PI3K inhibitor L294002 does not block the survival signal conferred by elevated PLD activity [52], indicating that PLD/mTOR functions via a pathway parallel to PI3K/Akt survival signaling.

PLD & metastasis

The most crucial step in progression to malignancy is gaining the ability to metastasize to distant sites where multiple tumors grow, ultimately resulting in lethality. The biological cascade of metastasis can be simplified as an orderly sequence of basic steps:

- Loss of cellular adhesion
- Increased motility and invasiveness
- Entry and survival in the circulation
- Exit into new tissue
- Eventual colonization in a distant site [56].

As discussed above, PLD activity has been shown to be important in cell motility/migration, which is essential for many physiological processes, such as embryogenesis and wound healing; however, inappropriate cell motility can accelerate the process of diseases such as spreading of cancers.

Phospholipase D activity has been shown to provoke reorganization of the actin cytoskeleton, which plays a central role in cell motility. PLD2 stimulates cell protrusion in v-Src-transformed cells and is required for EGF-induced membrane ruffling, consistent with a role of PLD in cell migration. PLD activity has also been implicated in tumor invasion [57,58]. Interestingly, MDA-MB-231 human breast cancer cells with high levels of PLD activity migrate and invade Matrigel™ in culture, unlike MCF-7 breast cancer cells with relatively low PLD activity. Recent data have shown that there is a rapid and dramatic increase in PLD activity in response to the stress of serum withdrawal [59]. Concomitant with increased PLD activity, serum withdrawal increases cell migration and invasion of Matrigel in MDA-MB-231 cells. The ability of MDA-MB-231 cells to both migrate and invade Matrigel is dependent on PLD. In EL4 lymphoma cells, PLD2 activity enhances Akt activation and cell invasion, whereas catalytically inactive PLD2 exerts inhibitory effects on adhesion, migration and invasion [60].

Invasive cancer cells also secrete proteases to promote metastasis, and PLD activity has been correlated with elevated protease secretion. PLD1 mediates matrix metalloproteinase (MMP)-9 secretion in HCT116 colon cancer cells in response to phorbol 12-myristate 13-acetate stimulation [61], whereas PLD1 depletion inhibits phorbol 12-myristate 13-acetate-induced MMP-9 secretion. Increased expression of PLD and its enzymatic activity have also been reported to stimulate the secretion and expression of MMP-2 and to induce glioma cell

invasiveness [62]. Thus, linkage of PLD with cytoskeleton organization and protease secretion suggests that PLD contributes to cell movement and invasiveness and may play a key role in metastasis of cancer cells.

Small-molecule inhibitors of PLD

PLD inhibitors

A number of compounds or proteins have been reported to inhibit PLD activity in *in vitro* assay systems. Fodrin, the nonerythroid form of spectrin, inhibits PLD activity by decreasing the amount of available PtdIns(4,5)P₂, which is a required cofactor for PLD activity [63]. Fodrin, accordingly, is not a specific PLD inhibitor and, in fact, also inhibits cytosolic phospholipase A2 and PLC activity owing to the decrease in PtdIns(4,5)P₂, which is the substrate for PLC and an enhancer of cytosolic phospholipase A2 activity. Similarly, synaptojanin, a PtdIns(4,5)P₂ 5-phosphatase, blocks PLD1 activity by lowering the level of PtdIns(4,5)P₂ [64]. In contrast, clathrin assembly protein 3 (AP3), a synapse-specific protein isolated from rat brain, inhibits PLD activity through the direct interaction between AP3 and PLD, although the mechanism of inhibition is unknown. Amphiphysins, key regulators of clathrin-mediated endocytosis, are another type of PLD inhibitor purified from rat brain [65]. Ceramide, a nonprotein product of sphingomyelinase, was shown to inhibit PLD activity in a cell-free system [66], and in fibroblasts and neutrophils by different mechanisms, including blocking its translocation to membranes, inhibiting its activation by RhoA [67] and suppressing its mRNA expression [68]. However, ceramide also inhibits the membrane translocation of the G proteins and PKCs that are required for PLD activation; therefore, its major effect could be indirect. Natural products were also found to inhibit PLD activity, such as honokiol, which blocks Ras-dependent PLD activity in human cancer cells through an indirect method [69].

These inhibitors, which are proteins and/or compounds that function indirectly, have not been considered useful for research and clinical settings. Thus, it is primary alcohols that have remained the most widely used PLD inhibitor in the research setting. PLD, as a transphosphatidylase, can use short-chain primary alcohols such as ethanol or 1-butanol to generate phosphatidylalcohol products. Phosphatidylalcohols are not normally found in biological membranes and are relatively inert and stable. Hence, stimulating phosphatidylbutanol formation by exposing cells to low levels of 1-butanol has become a convenient means to record and assay PLD activity. Moreover, addition of high levels of alcohol has been used as a means to divert PLD away from producing PA, and thus as a way to inhibit PLD-mediated pathways. However, the amounts of alcohol required to fully block PLD production of PA also affect other vital signaling pathways [20], such as those involved in many types of receptor tyrosine kinase signaling, adipocyte differentiation [13] and modulation of p42/44 mitogen-activated protein kinase signaling [70].

Halopemide variants as small-molecule PLD inhibitors & their clinical potential

In 2007, a small-molecule screen using an *in vitro* biochemical assay identified halopemide as a modest inhibitor of PLD2, and its analog FIPI as being even more potent [24]. We have reported that FIPI is a potent *in vivo* inhibitor of PLD1 and PLD2 that functions at sub-nM concentrations [20]. FIPI appears to function through direct inhibition of PLD enzymatic activity, and does not affect levels of PtdIns(4,5)P₂, PLD isoform localization, or other signaling pathways that we are aware of. FIPI inhibits PLD regulation of F-actin cytoskeleton reorganization, cell spreading and chemotaxis, indicating potential clinical applications for autoimmunity and cancer metastasis [17,20]. Interestingly, several biological processes blocked by 1-butanol are not inhibited by FIPI, suggesting that roles that have been proposed for PLD through the use of primary alcohol are in need of being re-evaluated.

Other halopemide analogs have been reported to exhibit preferential PLD1 or PLD2 inhibition, albeit with an IC₅₀ much higher than FIPI [21]. Use of these isoform-preferential analogs revealed that the most effective inhibition of migration of breast cancer cell lines is achieved when both PLD1 and PLD2 are inhibited; thus, the pan-inhibitor FIPI, which is the most potent and best-characterized compound to date, may be preferable for use as a cancer therapeutic, rather than these particular isoform-selective compounds. Nonetheless, utilization of FIPI as a leading compound to design and synthesize potent isoform-specific PLD inhibitors may continue to generate new and useful research tools and also have clinical implications, in particular for PLD-mediated processes in which only one isoform plays a role. Improved isoform-selective halopemide analogs with low nM potency and high selectivity (preferring PLD1 1700-fold, or PLD2 40-fold) have already been described [71,72], and further research is required to determine their utility for use in inhibition of PLD in cells and animals.

Phospholipase D has been linked to a variety of diseases as discussed in the previous section, suggesting the potential of PLD and its associated regulators as drug targets. The lack of effective small-molecule pharmaceutical inhibitors has hindered the examination of physiological roles for PLD in animals and the clinical application of PLD inhibition in disease. Halopemide is an approved psychiatric therapeutic, and the pharmacokinetics of FIPI indicates that, *in vivo*, it has a suitable half-life and bioavailability for animal studies [24]. Thus, FIPI and its derivatives are good candidates as *in vivo* agents to examine the function of PLD in animal models and determine whether inhibiting PLD could be useful in the therapeutic setting.

Conclusion

Phospholipase D is a highly conserved enzyme that generates a lipid signal important for directing several cell biological processes critical for normal functioning of the immune system and for the progression of tumors towards successful metastasis. Recent development of small-molecule inhibitors that target PLD effectively now permit examination of the hypothesis that acute ablation of PLD activity will be therapeutically beneficial in the setting of autoimmune disorders and oncogenesis.

Future perspective

Over the next few years, we will learn if *in vivo* administration of the new small-molecule inhibitors has an impact on the spread of cancer in mouse models, or on different paradigms of autoimmunity. Encouraging results will lead to the generation of better inhibitors, and we hope that clinical trials will eventually take place. In parallel, PLD knockout animals have been generated and are being studied in several laboratories. The findings from these mice should provide complementary information that assists in the design of future *in vivo* inhibitor studies.

Executive summary

Phospholipase D superfamily

- There are two classic phospholipase D (PLD) family members, PLD1 and PLD2.

Expression & subcellular localization of PLD

- PLD1 and PLD2 are expressed widely in cells and tissues.

Regulation of PLD activity

- PLD1 and PLD2 are activated by many types of G protein-coupled receptors and receptor tyrosine kinases, and generate the signaling lipid phosphatidic acid (PA).

PLD & cell movement

- PLD and PA direct critical aspects of cytoskeletal reorganization that are important for chemotaxis and cancer cell migration and invasion.

PLD & cancer

- PLD activity provides pro-survival, antiapoptotic signals to cancer cells.

PLD & metastasis

- PLD is activated in chemotaxis and in migrating cells, and is upregulated in many types of cancers.

Small-molecule inhibitors of PLD

- New small molecule inhibitors offer the potential of therapeutics in autoimmunity and cancer.

Acknowledgments

Supported by NIH awards GM071520 and GM084251 and the Carol Baldwin Breast Cancer Foundation.

Bibliography

Papers of special note have been highlighted as:

▪ of interest

▪▪ of considerable interest

1. McDermott M, Wakelam MJ, Morris AJ. Phospholipase D. *Biochem. Cell Biol* 2004;82(1):225–253. [PubMed: 15052340]
2. Mor A, Campi G, Du G, et al. The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane ras via phospholipase D2. *Nat. Cell Biol* 2007;9:712–719.
3. Zhao C, Du G, Skowronek K, Frohman MA, Bar-Sagi D. Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to ras activation by sos. *Nat. Cell Biol* 2007;9:707–712. ▪ Landmark paper linking phospholipase D2 (PLD2) to direct activation of Ras, a key signaling player in many types of cancer.
4. Delon C, Manifava M, Wood E, et al. Sphingosine kinase 1 is an intracellular effector of phosphatidic acid. *J. Biol. Chem* 2004;279(43):44763–44774. [PubMed: 15310762]
5. Ha SH, Kim DH, Kim IS, et al. PLD2 forms a functional complex with MTOR/Raptor to transduce mitogenic signals. *Cell. Signal* 2006;18(12):2283–2291. [PubMed: 16837165]
6. Jarquin-Pardo M, Fitzpatrick A, Galiano FJ, First EA, Davis JN. Phosphatidic acid regulates the affinity of the murine phosphatidylinositol 4-phosphate 5-kinase-I β for phosphatidylinositol-4-phosphate. *J. Cell. Biochem* 2007;100(1):112–128. [PubMed: 16888807]
7. Kim JH, Kim JH, Ohba M, Suh PG, Ryu SH. Novel functions of the phospholipase d2-phox homology domain in protein kinase c- ξ activation. *Mol. Cell. Biol* 2005;25(8):3194–3208. [PubMed: 15798205]
8. Foster DA. Phosphatidic acid signaling to MTOR: signals for the survival of human cancer cells. *Biochim. Biophys. Acta* 2009;91(9):949–955. [PubMed: 19264150] ▪ Excellent summary of reports linking PLD activity of pro-survival signals transmitted through the mTOR complex.
9. Du G, Huang P, Liang BT, Frohman MA. Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. *Mol. Biol. Cell* 2004;15(3):1024–1030. [PubMed: 14718562]
10. Shen Y, Xu L, Foster DA. Role for phospholipase D in receptor-mediated endocytosis. *Mol. Cell. Biol* 2001;21(2):595–602. [PubMed: 11134345]
11. Corrotte M, Chasserot-Golaz S, Huang P, et al. Dynamics and function of phospholipase D and phosphatidic acid during phagocytosis. *Traffic* 2006;7(3):365–377. [PubMed: 16497229]

12. Iyer SS, Barton JA, Bourgoin S, Kusner DJ. Phospholipases D1 and D2 coordinately regulate macrophage phagocytosis. *J. Immunol* 2004;173(4):2615–2623. [PubMed: 15294978]
13. Huang P, Altshuller YM, Hou J Chunqiu, Pessin JE, Frohman MA. Insulin-stimulated plasma membrane fusion of glut4 glucose transporter-containing vesicles is regulated by phospholipase D1. *Mol. Biol. Cell* 2005;16:2614–2623. [PubMed: 15772157]
14. Waselle L, Gerona RR, Vitale N, Martin TF, Bader MF, Regazzi R. Role of phosphoinositide signaling in the control of insulin exocytosis. *Mol. Endocrinol* 2005;19(12):3097–3106. [PubMed: 16081518]
15. Vicogne J, Vollenweider D, Smith J, Huang P, Frohman M, Pessin J. Asymmetric phospholipid distribution drives *in vitro* reconstituted snare-dependent membrane fusion. *Proc. Natl Acad. Sci. USA* 2006;103(40):14761–14766. [PubMed: 17001002]
16. Yang J-S, Gad H, Lee S, et al. Copi vesicle fission: a role for phosphatidic acid and insight into golgi maintenance. *Nat. Cell Biol* 2008;10:1146–1153. [PubMed: 18776900]
17. Nishikimi A, Fukuhara H, Su W, et al. Sequential regulation of dock2 dynamics by two phospholipids during neutrophil chemotaxis. *Science* 2009;324:384–387. [PubMed: 19325080] ■ Landmark paper on the role of PLD in immune cell polarization and migration.
18. Rudge SA, Wakelam MJ. Inter-regulatory dynamics of phospholipase D and the actin cytoskeleton. *Biochim. Biophys. Acta* 2009;1791(9):856–861. [PubMed: 19422932] ■ Excellent review on the relationship of PLD signaling to cytoskeletal reorganization.
19. Du G, Frohman MA. A lipid-signaled myosin phosphatase surge disperses cortical contractile force early in cell spreading. *Mol. Biol. Cell* 2009;20:200–208. [PubMed: 18946083]
20. Su W, Yeku O, Olepu S, et al. FIPI, a phospholipase D pharmacological inhibitor that alters cell spreading and inhibits chemotaxis. *Mol. Pharmacol* 2009;75:437–446. [PubMed: 19064628] ■ First characterization of a small-molecule inhibitor for PLD on cell behaviors such as chemotaxis.
21. Scott SA, Selvy PE, Buck JR, et al. Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness. *Nat. Chem. Biol* 2009;5(2):108–117. [PubMed: 19136975] ■ Description of isoform-preferential analogs of the PLD small-molecule inhibitor and inhibition of breast cancer migration and invasion.
22. Powner DJ, Pettitt TR, Anderson R, Nash GB, Wakelam MJ. Stable adhesion and migration of human neutrophils requires phospholipase D-mediated activation of the integrin CD11b/CD18. *Mol. Immunol* 2007;44(12):3211–3221. [PubMed: 17346796]
23. Huang P, Frohman MA. The potential for phospholipase D as a new therapeutic target. *Expert Opin. Ther. Targets* 2007;11:707–716. [PubMed: 17465727]
24. Monovich L, Mugrage B, Quadros E, et al. Optimization of halopemide for phospholipase D2 inhibition. *Bioorg. Med. Chem. Lett* 2007;17(8):2310–2311. [PubMed: 17317170]
25. Choi SY, Huang P, Jenkins GM, Chan DC, Schiller J, Frohman MA. A common lipid links mfn-mediated mitochondrial fusion and snare-regulated exocytosis. *Nat. Cell Biol* 2006;8:1255–1262. [PubMed: 17028579]
26. Katayama K, Kodaki T, Nagamachi Y, Yamashita S. Cloning, differential regulation and tissue distribution of alternatively spliced isoforms of ADP-ribosylation-factor-dependent phospholipase D from rat liver. *Biochem. J* 1998;329(Pt 3):647–652. [PubMed: 9445394]
27. Colley WC, Altshuller YM, Sue-Ling CK, et al. Cloning and expression analysis of murine phospholipase D1. *Biochem. J* 1997;326(Pt 3):745–753. [PubMed: 9307024]
28. Zeniou-Meyer M, Zabari N, Ashery U, et al. Phospholipase D1 production of phosphatidic acid at the plasma membrane promotes exocytosis of large dense-core granules at a late stage. *J. Biol. Chem* 2007;282(30):21746–21757. [PubMed: 17540765]
29. Du G, Altshuller YM, Vitale N, et al. Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. *J. Cell Biol* 2003;162(2):305–315. [PubMed: 12876278]
30. Munck A, Bohm C, Seibel NM, Hosseini Z Hashemol, Hampe W. Hu-k4 is a ubiquitously expressed type 2 transmembrane protein associated with the endoplasmic reticulum. *FEBS J* 2005;272(7):1718–1726. [PubMed: 15794758]
31. Santarius M, Lee CH, Anderson RA. Supervised membrane swimming: Small G-protein lifeguards regulate PIPK signalling and monitor intracellular PtdIns(4,5)P2 pools. *Biochem. J* 2006;398(1):1–13. [PubMed: 16856876]

32. Cathcart MK. Signal-activated phospholipase regulation of leukocyte chemotaxis. *J. Lipid Res* 2009;50(Suppl):S231–S236. [PubMed: 19109234]
33. Chae YC, Kim JH, Kim KL, et al. Phospholipase D activity regulates integrin-mediated cell spreading and migration by inducing GTP-rac translocation to the plasma membrane. *Mol. Biol. Cell* 2008;19(7):3111–3123. [PubMed: 18480413]
34. Lehman N, Di Fulvio M, Mccray N, Campos I, Tabatabaian F, Gomez-Cambronero J. Phagocyte cell migration is mediated by phospholipases PLD1 and PLD2. *Blood* 2006;108(10):3564–3572. [PubMed: 16873675]
35. Carrigan SO, Pink DB, Stadnyk AW. Neutrophil transepithelial migration in response to the chemoattractant fmlp but not C5a is phospholipase D-dependent and related to the use of CD11b/CD18. *J. Leukoc. Biol* 2007;82(6):1575–1584. [PubMed: 17724165]
36. Saito M, Iwadata M, Higashimoto M, Ono K, Takebayashi Y, Takenoshita S. Expression of phospholipase D2 in human colorectal carcinoma. *Oncol. Rep* 2007;18(5):1329–1334. [PubMed: 17914593]
37. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. *Science* 2007;318(5853):108–1113. • First identification of driver mutations in PLD2 in the context of breast cancer.
38. Joseph T, Wooden R, Bryant A, Zhong M, Lu Z, Foster DA. Transformation of cells overexpressing a tyrosine kinase by phospholipase D1 and D2. *Biochem. Biophys. Res. Commun* 2001;289(5):1019–1024. [PubMed: 11741292]
39. Ahn BH, Kim SY, Kim EH, et al. Transmodulation between phospholipase D and c-src enhances cell proliferation. *Mol. Cell. Biol* 2003;23(9):3103–3115. [PubMed: 12697812]
40. Lu Z, Hornia A, Joseph T, et al. Phospholipase D and RalA cooperate with the epidermal growth factor receptor to transform 3y1 rat fibroblasts. *Mol. Cell. Biol* 2000;20(2):462–467. [PubMed: 10611224]
41. Min DS, Kwon TK, Park WS, et al. Neoplastic transformation and tumorigenesis associated with overexpression of phospholipase D isozymes in cultured murine fibroblasts. *Carcinogenesis* 2001;22(10):1641–1647. [PubMed: 11577003]
42. Samuels ML, McMahon M. Inhibition of platelet-derived growth factor- and epidermal growth factor-mediated mitogenesis and signaling in 3t3 cells expressing δ raf-1:Er, an estradiol-regulated form of raf-1. *Mol. Cell. Biol* 1994;14(12):7855–7866. [PubMed: 7969125]
43. Kerkhoff E, Rapp UR. Cell cycle targets of ras/raf signalling. *Oncogene* 1998;17(11 Reviews):1457–1462. [PubMed: 9779991]
44. Joseph T, Bryant A, Frankel P, et al. Phospholipase D overcomes cell cycle arrest induced by high-intensity raf signaling. *Oncogene* 2002;21(22):3651–3658. [PubMed: 12032867]
45. Zhong M, Shen Y, Zheng Y, Joseph T, Jackson D, Foster DA. Phospholipase D prevents apoptosis in v-src-transformed rat fibroblasts and mda-mb-231 breast cancer cells. *Biochem. Biophys. Res. Commun* 2003;302(3):615–619. [PubMed: 12615079]
46. Nozawa Y. Roles of phospholipase D in apoptosis and pro-survival. *Biochim. Biophys. Acta* 2002;1585(23):77–86. [PubMed: 12531540]
47. Kim KO, Lee KH, Kim YH, Park SK, Han JS. Anti-apoptotic role of phospholipase D isozymes in the glutamate-induced cell death. *Exp. Mol. Med* 2003;35(1):38–45. [PubMed: 12642902]
48. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mtor pathway. *Curr. Opin. Cell Biol* 2005;17(6):596–603. [PubMed: 16226444]
49. Tee AR, Blenis J. Mtor, translational control and human disease. *Semin. Cell Dev. Biol* 2005;16(1):29–37. [PubMed: 15659337]
50. Veverka V, Crabbe T, Bird I, et al. Structural characterization of the interaction of MTOR with phosphatidic acid and a novel class of inhibitor: compelling evidence for a central role of the frb domain in small molecule-mediated regulation of mtor. *Oncogene* 2008;27(5):585–595. [PubMed: 17684489]
51. Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J. Phosphatidic acid-mediated mitogenic activation of MTOR signaling. *Science* 2001;294(5548):1942–1945. [PubMed: 11729323]
52. Chen Y, Rodrik V, Foster DA. Alternative phospholipase d/MTOR survival signal in human breast cancer cells. *Oncogene* 2005;24(4):672–679. [PubMed: 15580312]

53. Hui L, Abbas T, Pielak RM, Joseph T, Bargonetti J, Foster DA. Phospholipase D elevates the level of mdm2 and suppresses DNA damage-induced increases in p53. *Mol. Cell. Biol* 2004;24(13):5677–5686. [PubMed: 15199126]
54. Sun Y, Fang Y, Yoon MS, et al. Phospholipase D1 is an effector of Rheb in the mtor pathway. *Proc. Natl Acad. Sci. USA* 2008;105(24):8286–8291. [PubMed: 18550814]
55. Toschi A, Lee E, Xu L, Garcia A, Gadir N, Foster DA. Regulation of MTORC1 and MTORC2 complex assembly by phosphatidic acid: competition with rapamycin. *Mol. Cell. Biol* 2009;29(6):1411–1420. [PubMed: 19114562]
56. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. *Nat. Rev* 2009;9(4):274–284.
57. Honda A, Nogami M, Yokozeki T, et al. Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein arf6 in membrane ruffle formation. *Cell* 1999;99(5):521–532. [PubMed: 10589680]
58. Shen Y, Zheng Y, Foster DA. Phospholipase D2 stimulates cell protrusion in v-src-transformed cells. *Biochem. Biophys. Res. Commun* 2002;293(1):201–206. [PubMed: 12054584]
59. Zheng Y, Rodrik V, Toschi A, et al. Phospholipase D couples survival and migration signals in stress response of human cancer cells. *J. Biol. Chem* 2006;281(23):15862–15868. [PubMed: 16595654]
60. Knoepp SM, Chahal MS, Xie Y, et al. Effects of active and inactive phospholipase D2 on signal transduction, adhesion, migration, invasion, and metastasis in el4 lymphoma cells. *Mol. Pharmacol* 2008;74(3):574–584. [PubMed: 18523140]
61. Kang DW, Park MH, Lee YJ, et al. Phorbol ester up-regulates phospholipase D1 but not phospholipase D2 expression through a PKC/RAS/ERK/NF κ B-dependent pathway and enhances matrix metalloproteinase-9 secretion in colon cancer cells. *J. Biol. Chem* 2008;283(7):4094–4104. [PubMed: 18084005]
62. Park MH, Ahn BH, Hong YK, Min do S. Overexpression of phospholipase D enhances matrix metalloproteinase-2 expression and glioma cell invasion via protein kinase C and protein kinase A/Nf- κ B/SP1-mediated signaling pathways. *Carcinogenesis* 2009;30(2):356–365. [PubMed: 19126647]
63. Lukowski S, Mira JP, Zachowski A, Geny B. Fodrin inhibits phospholipases A2, C, and D by decreasing polyphosphoinositide cell content. *Biochem. Biophys. Res. Commun* 1998;248(2):278–284. [PubMed: 9675127]
64. Chung JK, Sekiya F, Kang HS, et al. Synaptojanin inhibition of phospholipase D activity by hydrolysis of phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem* 1997;272(25):15980–15985. [PubMed: 9188500]
65. Lee C, Kim SR, Chung JK, Frohman MA, Kilimann MW, Rhee SG. Inhibition of phospholipase D by amphiphysins. *J. Biol. Chem* 2000;275(25):18751–18758. [PubMed: 10764771]
66. Venable ME, Bielawska A, Obeid LM. Ceramide inhibits phospholipase D in a cell-free system. *J. Biol. Chem* 1996;271(40):24800–24805. [PubMed: 8798752]
67. Abousalham A, Liossis C, O'Brien L, Brindley DN. Cell-permeable ceramides prevent the activation of phospholipase D by ADP-ribosylation factor and RhoA. *J. Biol. Chem* 1997;272(2):1069–1075. [PubMed: 8995404]
68. Yoshimura S, Sakai H, Ohguchi K, et al. Changes in the activity and mRNA levels of phospholipase D during ceramide-induced apoptosis in rat c6 glial cells. *J. Neurochem* 1997;69(2):713–720. [PubMed: 9231731]
69. Garcia A, Zheng Y, Zhao C, et al. Honokiol suppresses survival signals mediated by ras-dependent phospholipase D activity in human cancer cells. *Clin. Cancer Res* 2008;14(13):4267–4274. [PubMed: 18594009]
70. Pannequin J, Delaunay N, Darido C, et al. Phosphatidylethanol accumulation promotes intestinal hyperplasia by inducing zonab-mediated cell density increase in response to chronic ethanol exposure. *Mol. Cancer Res* 2007;5(11):1147–1157. [PubMed: 18025260]
71. Lewis JA, Scott SA, Lavieri R, et al. Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part I: Impact of alternative halogenated privileged structures for pld1 specificity. *Bioorg. Med. Chem. Lett* 2009;19(7):1916–1920. [PubMed: 19268584]

72. Lavieri R, Scott SA, Lewis JA, et al. Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part II. Identification of the 1,3,8-triazaspiro[4,5] decan-4-one privileged structure that engenders PLD2 selectivity. *Bioorg. Med. Chem. Lett* 2009;19(8):2240–2243. [PubMed: 19299128]
73. Uchida N, Okamura S, Nagamachi Y, Yamashita S. Increased phospholipase D activity in human breast cancer. *J. Cancer Res. Clin. Oncol* 1997;123(5):280–285. [PubMed: 9201251]
74. Noh DY, Ahn SJ, Lee RA, et al. Overexpression of phospholipase D1 in human breast cancer tissues. *Cancer Lett* 2000;161(2):207–214. [PubMed: 11090971]
75. Yamada Y, Hamajima N, Kato T, et al. Association of a polymorphism of the phospholipase D2 gene with the prevalence of colorectal cancer. *J. Mol. Med* 2003;81(2):126–131. [PubMed: 12601529]
76. Uchida N, Okamura S, Kuwano H. Phospholipase D activity in human gastric carcinoma. *Anticancer Res* 1999;19(1B):671–675. [PubMed: 10216475]
77. Zhao Y, Ehara H, Akao Y, et al. Increased activity and intranuclear expression of phospholipase d2 in human renal cancer. *Biochem. Biophys. Res. Commun* 2000;278(1):140–143. [PubMed: 11185526]

Figure 1. Motifs and domains found within mammalian PLDs including PLD1, PLD2 and mitoPLD HKD domains are essential for catalysis. The PX and PH domains are lipid-binding domains important for the regulation of PLD localization. The loop region, which is absent in PLD2, may play a negative regulatory role for PLD activity. The PIP₂-binding domain is important for PLD localization and activity. The sites of interaction of PLD1 with its regulators are also highlighted.

ARF: ADP-ribosylation factor; HKD: Histidine–lysine–aspartic acid; PH: Pleckstrin homology; PKC: Protein kinase C; PLD: Phospholipase D; PtdIns: Phosphoinositide; PX: Phox homology.

Table 1

Cancers with altered phospholipase D expression and/or activity.

Cancer	PLD expression/activity	Ref.
Breast	Elevated PLD activity	[73]
	Increased PLD1 expression	[74]
	Driver mutations in PLD2	[37]
Colorectal	Polymorphism of PLD2 associated with prevalence	[75]
	Expression levels of PLD correlate with tumor size and survival	[36]
Gastric	Increased expression of PLD2	[76]
Renal	Increased expression of PLD2	[77]
PLD: Phospholipase D.		