Published in final edited form as:

Nat Chem Biol. 2009 February ; 5(2): 108–117. doi:10.1038/nchembio.140.

Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness

Sarah A Scott^{1,5}, Paige E Selvy^{1,5}, Jason R Buck¹, Hyekyung P Cho¹, Tracy L Criswell², Ashley L Thomas¹, Michelle D Armstrong¹, Carlos L Arteaga^{2,3}, Craig W Lindsley^{1,4}, and H Alex Brown^{1,4}

¹Department of Pharmacology, Vanderbilt Institute of Chemical Biology, Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University School of Medicine, 2220 Pierce Avenue South, Nashville, Tennessee 37232-6600, USA.

²Department of Cancer Biology, Vanderbilt Institute of Chemical Biology, Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University School of Medicine, 2220 Pierce Avenue South, Nashville, Tennessee 37232-6600, USA.

³Department of Medicine, Vanderbilt Institute of Chemical Biology, Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University School of Medicine, 2220 Pierce Avenue South, Nashville, Tennessee 37232-6600, USA.

⁴Department of Chemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University School of Medicine, 2220 Pierce Avenue South, Nashville, Tennessee 37232-6600, USA.

Abstract

Phospholipase D (PLD) is an essential enzyme responsible for the production of the lipid second messenger phosphatidic acid. Phosphatidic acid participates in both G protein-coupled receptor and receptor tyrosine kinase signal transduction networks. The lack of potent and isoform-selective inhibitors has limited progress in defining the cellular roles of PLD. We used a diversity-oriented synthetic approach and developed a library of PLD inhibitors with considerable pharmacological characterization. Here we report the rigorous evaluation of that library, which contains highly potent inhibitors, including the first isoform-selective PLD inhibitors. Specific members of this series inhibit isoforms with > 100-fold selectivity both *in vitro* and in cells. A subset of inhibitors was shown to block invasiveness in metastatic breast cancer models. These findings demonstrate the power of diversity-oriented synthesis combined with biochemical assays and mass spectrometric lipid profiling of cellular responses to develop the first isoform-selective PLD inhibitors—a new class of antimetastatic agents.

Regulated production of lipid second messengers through the activation of G protein-coupled receptors (GPCRs) and tyrosine kinases modulates a wide range of critical cellular processes (Fig. 1a). Phosphatidic acid (**17**) is the product of phosphatidylcholine (**16**)

© 2009 Nature America, Inc. All rights reserved.

Correspondence should be addressed to H.A.B. (alex.brown@vanderbilt.edu).

⁵These authors contributed equally to this work.

Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

AUTHOR CONTRIBUTIONS

S.A.S. carried out the cell-based inhibitor screening. P.E.S. and M.D.A. carried out *in vitro* inhibitor assays and protein purification. J.R.B. and A.L.T. performed chemical synthesis and characterization. H.P.C. was responsible for siRNA, RT-PCR and western blots. T.L.C. performed transwell experiments. C.L.A., C.W.L. and H.A.B. were responsible for coordination, planning and data interpretation of different aspects of the project.

hydrolysis catalyzed by PLD, which is a phosphodiesterase ubiquitously expressed in eukaryotic cells. Phosphatidic acid is a precursor of diacylglycerol (DAG, **18**) and lysophosphatidic acid (LPA, **19**) and is strategically located at the intersection of several major cell signaling and metabolic pathways. Endogenous levels of phosphatidic acid are low in resting cells, and PLD activity is tightly regulated by mechanisms that control vesicular trafficking, secretion, migration, survival and proliferation of cells.

PLD isoenzymes mediate the parallel reactions of phospholipid hydrolysis and transphosphatidylation, and the PLD superfamily includes a broad array of bacterial, plant and mammalian enzymes¹. Some bacterial and all mammalian PLD enzymes share a conserved histidine, lysine, aspartate (HKD) amino acid domain that is thought to form the catalytic site². Two mammalian isoforms, PLD1 and PLD2, have been identified, with multiple splice variants of each. These isoforms share conserved phox homology (PX) and pleckstrin homology (PH) regulatory domains at the N terminus, and both isoforms have a requirement for phosphatidylinositol-4,5-bisphosphate (PIP2) for physiological activation^{3–5}. Despite structural similarities between the two isoforms, studies suggest distinct modes of activation and functional roles for PLD1 and PLD2. PLD1 has low basal activity that is highly regulated by protein kinase C (PKC), Arf and Rho GTPases⁶, whereas PLD2 has high basal activity and mediates a number of unique protein interactions⁷ (Fig. 1a).

Aberrant phosphatidic acid signaling is observed in a number of disease states⁸. Elevated PLD activity and overexpression results in cellular transformation and has been implicated in multiple human cancers including breast^{9,10}, renal¹¹, gastric¹² and colorectal¹³. Stable cells overexpressing PLD1 and PLD2 demonstrate anchorage-independent growth, upregulation of matrix metalloprotease secretion and tumorigenesis in nude mice^{14,15}.

Owing to the absence of well-characterized small-molecule inhibitors, previous studies of PLD function have relied heavily on primary alcohols such as *n*-butanol (**20**), which block phosphatidic acid production. In the PLD reaction mechanism, primary alcohols serve as competitive nucleophiles to water, resulting in a transphosphatidylation reaction that produces a phosphatidylalcohol, such as phosphatidylbutanol (PtdBuOH, **21**), instead of phosphatidic acid. Because PtdBuOH is metabolized slowly, this reaction has been frequently used as a stable readout of total cellular PLD activity¹⁶. Primary alcohols do not inhibit the enzyme, and it is known that PtdBuOH serves some of the same structural roles as phosphatidic acid¹⁷. Because alcohols and molecular genetic reagents are good tools, but not viable therapeutic options for blocking PLD activity, we set out to develop new small-molecule inhibitors of the two PLD isoenzymes.

Detailed characterization of potent and isoform-selective small-molecule inhibitors of PLD are limited, although some indirect inhibitors have been described. Such molecules include diethylstilbestrol (half-maximal inhibitory concentration (IC₅₀) > 50 μ M, **1**, Fig. 1b)¹⁸, resveratrol (IC₅₀ > 60 μ M, **2**, Fig. 1b)¹⁹ and honokiol²⁰ (**3**, Fig. 1b). SCH420789 (**4**, Fig. 1b) was one of the first reported PLD inhibitors^{21,22}. However, in-depth characterization of the anti-PLD activity of this compound has not been described. The mevalonate-derived product presqualene diphosphate ((Z)-PSDP, **5**, Fig. 1b) was shown to inhibit both non-HKD-containing bacterial (*Streptomyces chromofuscus*) PLD (IC₅₀ = 100 nM) and human PLD1b (IC₅₀ > 1 μ M) *in vitro*²³, but the mechanism of inhibition was not elucidated. A previous report from this laboratory implicated certain selective estrogen receptor modulators (SERMs) in the regulation of PLD activity. Raloxifene (**6**, Fig. 1b) inhibits PLD activity (IC₅₀ = 4 μ M) both *in vitro* and in breast cancer cell lines, whereas tamoxifen (**7**, Fig. 1b) stimulates PLD activity²⁴. In addition to SERMs, a recent report on a high-throughput screen suggested that the psychotropic agent haloperidol (**8**, Fig. 1b) inhibits PLD2 (ref. 25).

This report showed that halopemide and related congeners were potent inhibitors of PLD2. This report attracted our attention, as selective and potent PLD2 inhibitors would be invaluable tools to probe PLD functions. Though the initial report suggested PLD2 selectivity, the manuscript did not describe effects on PLD1 or demonstrate that the compounds act directly. We found that the PLD inhibitors in this report (*vide infra*) are a combination of dual PLD1 and PLD2 inhibitors and slightly PLD1-preferring inhibitors; moreover, none of the compounds displayed any preference for inhibition of PLD2 (ref. 25). Thus, our laboratories launched a campaign to develop potent and selective PLD1, PLD2 and dual PLD1 and PLD2 inhibitors using the halopemide scaffold as a starting point.

Here we describe a new screening approach that rigorously evaluates the effects of small molecules in both *in vitro* enzymatic assays and cell-based assays in order to directly compare the PLD inhibitory activities of existing compounds as well as a library of new compounds generated in our laboratory. A number of existing cell lines were screened, and a new cell line was developed to obtain cell-based systems that provide PLD1- and PLD2-selective responses, respectively. The 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one halopemide scaffold was used as a starting point to initiate a diversity-oriented synthesis campaign to develop potent and, importantly, isoform-selective PLD inhibitors. In this report we demonstrate specific structural modifications to this scaffold, produce new compounds and describe the first series of potent and isoform-selective PLD inhibitors using both enzymatic- and cell-based PLD assays.

PLD aberrant activity is associated with several human cancers. Potent and selective small-molecule inhibitors are necessary to further understand the role of PLD in these signaling pathways and disease states. Here, we report that the inhibition of PLD activity in breast cancer cell lines with PLD1, PLD2 or dual PLD1 and PLD2 inhibitors affords a marked decrease in invasiveness. In combination with RNA interference and other molecular approaches, we have developed the first set of well-characterized inhibitors that can be used to interrogate the roles of PLD in cellular functions.

RESULTS

Design and synthesis of small-molecule PLD inhibitors

For initial characterization, a variety of novel small molecules, based on the halopemide report, were synthesized and evaluated for inhibition of PLD1 and PLD2 in both enzymatic- and cell-based assays (*vide infra*, Table 1). IC₅₀s obtained for compound **8** demonstrated potency against PLD2 comparable to that previously reported²⁵, but no isoform preference for PLD2 over PLD1 was noted (PLD1 IC₅₀ = 220 nM, PLD2 IC₅₀ = 310 nM), although a slight PLD1 preference was observed in the cell-based assays (PLD1 IC₅₀ = 21 nM, PLD2 IC₅₀ = 300 nM). Of the chemotypes previously reported as PLD inhibitors, the 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one series (as exemplified by **8**) was the most noteworthy in terms of chemical tractability and modular design; therefore, **8** was chosen as the lead compound and an optimization program was initiated. This program used a diversity-oriented approach to explore structure-activity relationships (SAR), improve potency for dual PLD1 and PLD2 inhibition and identify structural changes that provide selective inhibition of individual PLD isoforms. We dissected the lead compound into three sections (Fig. 2a), synthesized analog libraries and purified the new compounds by mass-directed preparative HPLC to analytical purity (> 98%). An initial 263-compound library was prepared based on a 3 × 3 × 30 matrix using three scaffolds, three linker moieties and a diverse set of 30 amide capping reagents. The libraries were synthesized as shown (Fig. 2b) by using solution phase parallel synthesis (polymer-supported reagents/scavengers and microwave-assisted organic synthesis). Specifically, scaffold **9** underwent a reductive amination sequence with one of three Boc-protected α -aminoaldehyde linkers (**10**) and MP-

B(OAc)₃H to provide **11**. Deprotection with 4 N HCl/dioxane liberated the free amine **12**, which was used without further purification in an acylation reaction with an acid chloride and polymer-supported tertiary amine under microwave irradiation to deliver analogs **13**, which were then purified (specific examples and variations on chemical synthesis can be found in Supplementary Methods online). The entire 263-compound library was screened at a single concentration in both enzyme- and cell-based assays to identify PLD inhibitors. To our knowledge, this is one of the most rigorous screening and characterization procedures for PLD inhibitors reported so far.

Newly synthesized analogs inhibit PLD *in vitro*

Detailed characterization was performed on 30 compounds (Table 1 and Fig. 2c) selected from the initial library based on PLD inhibition, isoform selectivity and structural diversity. Concentration response curves (CRCs) were generated for these compounds using the exogenous substrate enzymatic assay^{3,26}. IC₅₀ values for human PLD isoforms PLD1 and PLD2 normalized to purified myr-Arf-1-stimulated activity are compared to those of the N-terminally truncated enzyme PLD1.d311 (Table 1). This construct lacks the first 311 amino acids of the N terminus but maintains the two catalytic HKD domains and has high basal activity that is further stimulated by myr-Arf-1 (ref. 27). Concentrations of recombinant PLD enzyme used in this assay were chosen based on similar myr-Arf-stimulated activities within the linear range of the assay. Equimolar PLD concentrations were also used as a basis for normalization for a few of the inhibitors, and no substantial changes in IC₅₀ values were observed.

Raloxifene (**6**), tamoxifen (**7**) and 4-OH tamoxifen behaved in manners consistent with previously reported findings²⁴. Halopemide (**8**) and a series of 1-(piperidin-4-yl)-1*H*-benzo[d]imidazol-2(3*H*)-one analogs, including **14** and **15** (referred to previously²⁵ as 4k and 4m, respectively), were previously identified as PLD2 inhibitors²⁵. IC₅₀s obtained for **8** demonstrated potency against PLD2 similar to that previously reported, but the compound was found to have no preference for PLD2 over PLD1. In fact, neither of the previously published analogs, **14** or **15**, showed any isoform selectivity for inhibition of recombinant PLD1 or PLD2. It is important to note that none of the previously published raloxifene or 1-(piperidin-4-yl)-1*H*-benzo[d]imidazol-2(3*H*)-one compounds and none of the newly generated compounds are as potent against the N-terminally truncated PLD1 as against full-length PLD1 (Table 1). Differences in potency may suggest that compounds have distinct sites of interaction. *In vitro* CRCs were also performed on two classes of bacterial enzymes (data not shown). *Streptomyces* sp. PMF PLD was included as a bacterial HKD-containing PLD, and *S. chromofuscus* PLD was included because even though it lacks conserved HKD domains, this enzyme can sustain both phosphatidylcholine hydrolysis and trans-phosphatidylolation, similar to the mammalian and PMF enzymes under specific conditions²⁸. Inhibition of either bacterial PLD was only observed at very high concentrations (> 2 μM), and a number of compounds, including **56**, resulted in no inhibition of activity. The failure to inhibit the PMF enzyme suggests that the compounds are unlikely to act at the HKD catalytic site and are more likely to serve as allosteric modulators at sites specific to the mammalian isoenzymes.

In vitro identification of isoform-preferring compounds

Compounds were classified based on the *in vitro* IC₅₀ values for their ability to differentially inhibit full-length PLD1 and PLD2. Of the 30 extensively characterized compounds, 13 showed no preferential inhibition between PLD1 and PLD2. Compounds **56** and **64** demonstrated similar potency for both enzymes (IC₅₀s of 81 nM and 79 nM for PLD1, and 240 nM and 170 nM for PLD2, respectively), and are representative dual PLD1 and PLD2

inhibitors (Fig. 3a). The lack of isoform selectivity of these compounds is similar to that of previously published compounds, such as **8**, **14** and **15**.

In a second class of compounds, potent inhibition of PLD1 over PLD2 was observed. Unlike previously published PLD inhibitors, these are the first 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one analogs to have PLD1 selectivity (Fig. 3b). Compound **58** demonstrated a tenfold preference for inhibition of PLD1 over PLD2 (IC₅₀s of 660 nM and 6800 nM, respectively). Compound **69** more potently inhibits PLD1 over PLD2, with a 20-fold difference in IC₅₀ values (46 nM and 933 nM).

Compounds that preferentially inhibited PLD2 were also identified (Fig. 3c). The only compound previously shown to selectively inhibit PLD2 without inhibiting PLD1 is 4-OH tamoxifen (Table 1). The PLD2-preferring compounds identified here are fewer in number than the PLD1-preferring class and contain greater variance in chemical structure from the initial indole 2-carboxylic acid scaffold. These compounds do not have the 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one scaffold but instead have a 1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one scaffold. Structure **82** showed at least a 10-fold preference for PLD2 inhibition with an IC₅₀ of 2.5 μM, whereas PLD1 inhibition was not as potent, with an IC₅₀ value of >20 μM (Fig. 3c). Unlike other isoform-preferring compounds, **82** did not inhibit PLD1 in a sigmoidal manner, which suggests that the mechanism of PLD1 inhibition may differ from that of PLD2. Compound **72** (lower panel Fig. 3c) was substantially PLD2-preferring, with an approximately 40-fold difference in IC₅₀ between PLD2 and PLD1 (140 nM and 5.1 μM, respectively).

Compounds inhibit PLD in a direct manner

Rigorous *in vitro* characterization was performed on compounds proposed to be PLD inhibitors to confirm that the mechanism of inhibition is direct and not due to interference with myr-Arf-1 GTPase stimulation. For this set of experiments (Fig. 4), PLD1.d311 was highly expressed in Sf21 insect cells and isolated by affinity purification to >98% homogeneity (Fig. 4c). PLD1.d311 has high basal activity that allows inhibition to be detected in the absence of myr-Arf-1 stimulation. CRCs were performed for two compounds on basal and myr-Arf-1-stimulated PLD1.d311. Differences in activity were approximately twofold between basal and myr-Arf-1-stimulated PLD1.d311. Compounds **56** and **64**, noted to be dual-isoform PLD inhibitors that have mid to high potency inhibition of PLD1.d311, were used *in vitro* to generate extensive concentration response curves with purified PLD1.d311. These compounds directly inhibited both basal and myr-Arf-1-stimulated activity of PLD1.d311 with no noticeable difference in inhibition between the two for either compound (Fig. 4b). These data demonstrate that the compounds act directly on PLD. In addition, because the PLD1.d311 construct is N-terminally truncated (Fig. 4a), the results suggest that the compounds directly interact with the catalytic domains of the enzyme, and do not require binding with the regulatory PX-PH domain to result in inhibition.

Cell assay to identify PLD isoform-specific inhibitors

The *in vitro* enzyme activity assay was essential for the identification and characterization of direct isoform-selective PLD inhibitors, but demonstration of activity in cells was a primary goal of the endeavor. After extensive screening of a number of cell lines (both basal and ligand-stimulated), we chose the following cell-based systems in which to interrogate potential inhibitors. PLD activity in the human non-small-cell lung cancer (NSCLC) cell line, Calu-1, is mediated predominantly by PLD1. RNA interference was used to confirm that the PLD1 isoform is activated by the known stimulator phorbol 12-myristate 13-acetate (PMA)²⁹ in Calu-1. These cells were transfected with small interfering RNA directed against PLD1, PLD2, PLD1+PLD2 or GAPDH as a control. Cells were then stimulated with

PMA in the presence of 1-butanol- d_{10} to trap the PLD protein product as phosphatidylbutanol- d_9 (PtdBuOH- d_9)²⁶. This method exploits PLD-mediated transphosphatidylation, allowing PLD activity to be determined by mass spectrometric analysis of the levels of individual PtdBuOH- d_9 species. In PLD1 siRNA transfections, overall PLD activity was significantly decreased by 67% ($P < 0.005$) compared with GAPDH control (Fig. 5a), whereas PLD2 siRNA transfection produced no change. The combination PLD1+PLD2 knockdown yielded a 65% decrease in PtdBuOH- d_9 production, similar to levels seen in the PLD1 knockdown samples. These data confirm that PMA stimulation of PLD activity in Calu-1 cells is a PLD1-mediated response with no detectable contribution from PLD2. Protein level knockdown was confirmed via western blot for PLD1 (Fig. 5b), and due to poor commercially available PLD2 antibodies, RT-PCR was used to show decreased PLD2 mRNA in these cells (Fig. 5c).

HEK293 cells that stably overexpress green fluorescent protein (GFP)-tagged PLD2 were also subjected to siRNA protein knockdown of PLD1, PLD2 and PLD1+PLD2. The HEK293-gfpPLD2 cells have high basal PLD activity due to the overexpression of PLD2, which is known to be constitutively active³⁰. Cells transfected with siRNA against PLD2 showed a significant ($P < 0.05$) decrease in PtdBuOH- d_9 formation (33%), whereas siRNA against PLD1 had no effect (Fig. 5d). The combination PLD1+PLD2 siRNA-treated cells had decreased PtdBuOH- d_9 production (42%), comparable to PLD2 siRNA-transfected samples alone. Thus, unstimulated basal activity in the HEK293-gfpPLD2 is dominantly PLD2-mediated and does not include a significant PLD1 component. The PLD2 siRNA markedly decreased the GFP-tagged PLD2 protein present but did not eliminate all of the protein, as was shown by western blot (Fig. 5e). The partial knockdown of PLD2 would account for the incomplete inhibition of PLD activity in this experiment. These two cell lines provide systems to independently assess preferential isoform inhibition, which complements findings from the *in vitro* enzyme activity assay described above.

Potent and selective inhibitors of cellular PLD isoforms

Selected compounds characterized in the enzyme activity assay were similarly tested for PLD inhibition in the Calu-1 and HEK293-gfpPLD2 cellular systems. Cells were treated with vehicle or each of the 30 inhibitors in the presence of 1-butanol- d_{10} to trap the PtdBuOH- d_9 product. CRCs were generated for each inhibitor (highlights shown in Fig. 6), and cellular IC_{50} s were estimated with values ranging from 1 nM to $>20 \mu M$ (Table 1). Of the 30 compounds tested, 21 were shown to be potent ($IC_{50} < 1 \mu M$) in the Calu-1 cells, while 15 demonstrated a potency of $< 1 \mu M$ in the HEK293-gfpPLD2 cell line (Table 1). Many compounds, such as raloxifene (**6**), halopemide (**8**), **14**, **15**, **47**, **48**, **50**, **56** and **64**, were dual-specificity PLD1+PLD2 inhibitors (Fig. 6a and Table 1). Some showed selectivity with a > 100 -fold difference in IC_{50} values for PLD1 over PLD2, such as compounds **58** (IC_{50} s of 35 nM and 3,900 nM, respectively) and **69** (IC_{50} values of 11 nM and 1,800 nM, respectively) (Fig. 6b and Table 1). Others such as **72** and **82** were PLD2-preferring inhibitors, although with less cellular potency (Fig. 6c and Table 1).

The unstimulated HEK293-gfpPLD2 stable cell line had greater absolute PLD activity than the PMA-stimulated Calu-1 line, due in part to the former's overexpression of PLD2 enzyme. Therefore, it is challenging to precisely compare IC_{50} s for each of these compounds across the different cell types due to differences in PLD protein expression. However, the strength of this screening platform is the ability to compare inhibitor selectivity and potency patterns observed in the *in vitro* enzyme assay (Fig. 3) with those in the cellular assay (Fig. 6). Notably, good agreement was observed between results obtained from the two assays, and isoform preference and selectivity in these studies is defined on the

basis of substantial differences in potency observed in both the cell-based and enzymatic assays.

PLD inhibitors block invasion in breast cancer cell lines

PLD has been implicated in human cancer progression^{9–13}, as well as reorganization of the actin cytoskeleton and cell motility³¹. As such, PLD may be an unappreciated molecular target whose inhibition could block tumor cell invasion^{32–34}. Small-molecule PLD inhibitors, identified in both enzyme activity and cell-based assays, were further assessed for the ability to decrease invasive migration of cancer cells. This was done in three cancer model cell lines: the MDA-231 human breast cancer cell line, the mouse metastatic breast cancer line model 4T1 and PMT cells derived from mammary tumors in MMTV/polyomavirus middle T antigen transgenic mice³⁵. These three cell lines are well-characterized model systems of highly metastatic breast cancer.

Compound **56** was able to significantly ($P < 0.05$) decrease the ability of all three invasive cell lines to migrate in a transwell invasion assay when tested at 20 μM (Fig. 7a). Compound **56** is a dual PLD1+PLD2 inhibitor and therefore inhibited both PLD isoforms in this assay. Isoform-selective PLD1 and PLD2 inhibitors were also tested. PLD1-selective compounds **58** and **69** and the PLD2-preferring compounds **72** and **82** were used at concentrations of 0.2 and 20 μM in invasion assays for each of the three cell lines. At the high concentration (20 μM), all four inhibitors blocked both PLD1 and PLD2 activity, whereas at the low concentration (200 nM) each compound preferentially inhibited either PLD1 or PLD2 (shown in Fig. 6). In transwell assays, each PLD inhibitor significantly decreased cell migration when used at 20 μM in all cell lines tested (P values of < 0.05 , Fig. 7b). At the lower isoform-selective concentration, decreases in cell migration were observed in most instances regardless of the specific isoform inhibited. This evidence suggests that a single PLD isoform is not preferentially used in MDA-231 or PMT cell migration, but there is a distinct pattern of isoform influence observed in the 4T1 cells. In 4T1 cells, both PLD2-preferring compounds showed little ability to block migration at 200 nM. PLD1-preferring compounds **58** and **69** significantly decreased cell migration compared with the vehicle control ($P < 0.05$, Fig. 7b). These data suggest that PLD1 may have an important role in mediating cell migration in the 4T1 cell line.

To confirm that these cellular effects were being mediated through inhibition of PLD activity and not through some nonspecific interaction, we transfected the human breast cancer cell line MDA-231 with siRNA targeted against either PLD1, PLD2, PLD1+PLD2 or GAPDH as a negative control. When PLD2 was knocked down, there was a significant decrease in cell migration (Fig. 7c, $P < 0.001$). Cells that were transfected with PLD1+PLD2 siRNA had a more substantial decrease in cell migration than those transfected with PLD2 alone. Although some minor effects were noted, PLD1-alone knockdowns did not have a statistically significant effect on invasion in these studies. To confirm that siRNA transfection decreased targeted protein levels, PLD1 knockdown was confirmed by western blot showing that in both the PLD1 and PLD1+PLD2 conditions, PLD1 protein was nearly undetectable (Fig. 7d). Owing to the lack of suitable antibodies that specifically identify native PLD2 protein by western blot, we confirmed knockdown of PLD2 using RT-PCR to verify that mRNA levels were significantly ($P < 0.001$) decreased in all conditions that received siRNA transfections against PLD2 (Fig. 7e). Taken together, the results of the small-molecule inhibitor and siRNA studies strongly suggest that PLD is essential to invasive migration.

DISCUSSION

Developing a complete understanding of PLD physiology has been elusive, in part due to the absence of high-quality reagents that could be used to define its function in cells. The field has been restricted by the absence of potent and isoform-selective inhibitors, and primary alcohols have been used almost exclusively as the only means of preventing phosphatidic acid production. Transphosphatidylolation products such as PtdBuOH maintain some of the structural functions of phosphatidic acid³⁶, and such primary alcohols are not optimal tools to study PLD activity or phosphatidic acid function in cellular processes. Here we report the characterization of potent dual PLD1 and PLD2 inhibitors, and the discovery of isoform-selective PLD1 and PLD2 small-molecule inhibitors. To our knowledge, this is the first description and rigorous biochemical characterization of isoform-selective PLD inhibitors.

Halopemide (**8**, Fig. 1b) and 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one analogs were recently described as PLD2 inhibitors²⁵. PLD1 inhibition was not mentioned in the previous report, and there was little detail regarding the method by which the reported IC₅₀ values were obtained, resulting in the possible misinterpretation that these compounds are PLD2 specific. Compounds re-synthesized from the initial report inhibit both PLD1 and PLD2. *In vitro* experiments show less than tenfold preference for one isoform over the other, and all compounds are slightly more potent for PLD1 inhibition compared with PLD2. Nonetheless, the identification of halopemide as a PLD inhibitor is an important contribution to the field.

The goal of this work was to initiate a comprehensive study to develop selective PLD1 and PLD2 inhibitors. Replacement of the *p*-fluorophenyl ring in lead compound **8** with a naphthyl ring led to the development of the potent dual-specificity PLD1 and PLD2 inhibitor, compound **56**. The addition of a chiral (S)-methyl group in the linker region of inhibitor **56** led to the development of a potent and selective PLD1 inhibitor, compound **69**, showing an IC₅₀ difference of > 10-fold in the *in vitro* assay and > 100-fold in the cellular assays. Other PLD1-preferring compounds were synthesized by substitution of the *p*-fluorophenyl moiety in halopemide with variously substituted phenyl rings (compounds **68** and **55**) and novel ring systems (compounds **58**, **60**, **61** and **64**). SAR was robust and tractable, with a diversity of halogenated aryl and heteroaryl amides affording potent dual or PLD1-preferring inhibitors. Moreover, the (S)-methyl group proved to be a general strategy to engender PLD1-preferring pharmacology to dual PLD1 and PLD2 inhibitor scaffolds.

Though des-chloro and chlorinated congeners of the 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one scaffold alone or in combination with the key (S)-methyl group afforded potent and selective dual and PLD1-selective inhibitors, PLD2 inhibitors proved elusive. Therefore, we used bioisosteres of the 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one scaffold. One bioisostere proved successful in providing entry into PLD2-preferring inhibitors. Replacement of the 1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one scaffold present in compound **8** with a 1-phenyl-1,3,8-triazaspiro [4,5] decan-4-one scaffold conferred PLD2 preference to compounds **72** and **82**. As can be seen from the structures illustrated in Figure 2c, the PLD2-preferring compounds have a substantially different, although related, structural scaffold. PLD2-preferring inhibitors **72** and **82** were capable of discriminating PLD2 from PLD1 inhibition with a 10-fold to 40-fold difference in IC₅₀ values. These are the only inhibitors that illustrate noteworthy PLD2 preference; most of the other 30 molecules characterized and all previously published inhibitors are either slightly or considerably better at inhibiting PLD1 over PLD2. This serendipitous finding argues well for the application of diversity-oriented synthesis.

After confirmation that these compounds inhibit PLD activity *in vitro*, we demonstrated the direct specificity of these compounds for the enzyme. Using an N-terminally truncated PLD1 (PLD1.d311), we showed that compounds act directly on the truncated protein regardless of Arf stimulation. This suggests that these small molecules do not require regulatory protein binding to illicit their effects and do not require the regulatory (PX-PH) domains of the enzyme, but rather directly interact with the catalytic domain of the enzyme. The discrepancy in potency of the compounds on full-length PLD1 versus this truncated construct suggests that there may be an allosteric binding site in the N terminus, or perhaps that the N terminus may strengthen the binding of the compound on the catalytic domain. The detailed mechanism of inhibition of these compounds is currently not defined, but it is clear that these compounds act directly on the enzyme. Future work in this area will focus on discriminating the inhibitor binding site in each of the isoenzymes to determine the precise site of action and better understand how isoenzyme selectivity is achieved.

Once *in vitro* and cellular PLD inhibition was well established with these inhibitors, we set out to test these compounds' inhibitory activity in cancer cell invasiveness. It is clear from the data presented in three highly metastatic breast cancer cell lines that PLD plays an essential role in invasiveness. Blocking PLD activity with a dualspecificity inhibitor as well as isoform-selective PLD1 and PLD2 inhibitors substantially decreased cancer cell migration in all three cell types tested. To verify that the PLD inhibitors were blocking cellular invasiveness in a PLD-dependent manner, we conducted migration assays on MDA-231 cells using RNA interference to decrease PLD protein levels. This experiment suggests that PLD2 expression is essential to invasive migration and that when the enzyme is knocked down, cells are no longer capable of invading. These data do not demonstrate that PLD1 has no role in the migration of cancer cells, as the most marked decrease in cell migration was observed when PLD1 and PLD2 were knocked down together. It is possible that the cells may be circumventing the absence of PLD1 protein by signaling through a PLD2-dominant mechanism, and that is why both isoenzymes must be knocked down to achieve the most robust inhibition.

The siRNA and small-molecule migration experiments both suggest that PLD activity is required for cellular invasion and that an interaction between PLD1 and PLD2 likely mediates this function.

This report illustrates the development of potent and selective PLD isoform inhibitors. Considering the importance of PLD in the production of mitogenic molecules such as phosphatidic acid, LPA and DAG, it is highly desirable to determine the mechanism of action at the cellular level for an inhibitor of PLD enzymatic activity. It is clear from our studies that these compounds are working directly on PLD proteins. Future work will focus on determining the mechanism by which PLD inhibition is blocking invasive migration in the cancer cells. These selective molecules will serve as specific tools to elucidate the role of PLD in growth deregulation, invasive migration and tumor progression.

Previous reports suggest that PLD-dependent phosphatidic acid is at the center of critical signaling networks implicated in cancer. Multiple protein targets both upstream and downstream of PLD have classically been linked to propagation of survival signals and metastasis in cancer progression. More recently, PLD-generated phosphatidic acid was shown to participate in downstream signal transduction from mitogenic growth factor receptors via Ras and the MAP kinase pathway^{37,38}. PLD activity also regulates mTOR, a serine/ threonine kinase responsible for triggering cell survival pathways^{39,40}. In addition to participating in cell survival pathways, PLD is thought to contribute to cancer metastasis via multiple mechanisms, including cytoskeletal rearrangement and cell migration³¹. Recent reports suggest PLD may also contribute to metastasis through the induction of matrix

metalloproteinase (MMP) secretion and extracellular matrix degradation^{41–45}. PLD has also been suggested to have pro-angiogenic properties via interactions with sphingosine kinase-1 and sphingosine-1-phosphate (S1P)⁴⁶. Previous reports suggest a role for PLD in cancer progression, and the development of small-molecule PLD inhibitors is critical for determining whether PLD is a potential therapeutic target for cancer pharmacology.

PLD participates in a variety of cellular networks including GPCRs, growth factor receptors and integrin signaling pathways. The enzymatic activity plays key roles in regulated secretion, trafficking, cytoskeletal rearrangements and cell survival pathways. It is likely that the effects on invasive migration described in this report involve one or more of these specific cellular functions. Determining the precise site of action of these small-molecule inhibitors on each of the PLD isoenzymes is an important goal. Specifically, it will be useful to ascertain whether there are differences in the binding sites of the dual and more selective inhibitors, or rather whether shared binding sites modulate subtle conformation differences in the structure. The nature of the cross-talk between PLD1 and PLD2 in modulating invasive migration remains a key issue. Such insights will lead to better understanding of the catalytic mechanism and allosteric regulation by G proteins and other regulatory components of PLD. These new inhibitors may facilitate (i) identification of where PLD is localized in cellular signaling cascades and (ii) a better understanding of the roles played by the respective isoenzymes in the generation of the lipid second messengers. These small-molecule inhibitors provide an important new set of tools that will be used in combination with RNA interference and molecular genetic techniques to better define the role of PLD1 and PLD2 in essential biological functions.

METHODS

Chemical synthesis and purification

See Supplementary Methods online.

Cell culture

See Supplementary Methods.

Partial purification of human PLD2

See Supplementary Methods.

***In vitro* PLD activity assay**—*In vitro* PLD activity was measured with an exogenous substrate assay as previously described^{26,27}. Briefly, PLD activity was measured as the release of [methyl-³H]choline from [choline-methyl-³H]di-palmitoylphosphatidylcholine. PLD enzyme (PLD1 = 3 nM, PLD2 = 15 nM and PLD1.d311 = 50 nM) was reconstituted with phospholipid vesicle substrates. Lipid solutions were dried and resuspended, and small unilamellar vesicles were prepared by bath sonication. All assays were performed at 37 °C with agitation for 30 min. Reactions were stopped by addition of trichloroacetic acid and bovine serum albumin. Free [methyl-³H]choline was separated from precipitated lipids and proteins by centrifugation and analyzed by liquid scintillation counting. Raw data are normalized and are presented as percent total activity. Experiments performed in triplicate.

Purification of rat PLD1.d311, human PLD1 and myristoyl-ADP-ribosylation factor-1—Rat PLD1b.d311 (amino acids 312–1036) was cloned and purified as previously described²⁷. Partially purified human PLD1b was generated as described⁴⁷. Human myr-Arf-1 was purified as described^{24,27}.

Endogenous PLD activity assay using deuterated 1-butanol incorporation—

Endogenous PLD activity was determined using a modified *in vivo* deuterated 1-butanol PLD assay²⁶. All cell types aside from the stable HEK293-gfpPLD2 cells were serum-starved 18 h before experiment. Cells were pretreated with PLD inhibitor or DMSO for 5 min at room temperature (20 °C). After pretreatment, Calu-1 cells were treated with 1 μ M PMA + 0.3% (v/v) 1-butanol-d₁₀ and either PLD inhibitor or DMSO, or medium alone for 30 min at 37 °C. HEK293-gfpPLD2 cells were treated in the presence of 0.3% 1-butanol-d₁₀ and PLD inhibitor or vehicle. After treatment, samples were extracted and internal standard was added. The resulting lipids were dried and resuspended in MS solvent. Samples were directly injected into a Finnigan TSQ Quantum triple quadrupole MS, and data were collected in negative ion mode. Data were analyzed as a ratio of major phosphatidylbutanol-d₉ lipid products and internal standard. Background signal was subtracted using cells not treated with 1-butanol-d₁₀ as a negative control. The data were then expressed as percent of PMA-stimulated PLD activity or as percent of basal PLD activity. Experiments were performed in triplicate.

siRNA, western analysis and RT-PCR—Calu-1 and HEK293-gfpPLD2 cells were seeded into 6-well plates the day before transfection. Cells were transfected with 100 nM siRNA pool against either PLD1 or PLD2 (On-target Plus siRNA; Dharmacon hPLD1 siRNA target sequences (number is open reading frame position) 605-CAA CAG AGT TTC TTG ATA T-623, 2596-GGT AAT CAG TGG ATA AAT T-2614, 3206-CCA TGG AGG TTT GGA CTT A-3224, 2445-CCG GGT ATA TGT CGT GAT A-2463; hPLD2 siRNA target sequences 1566-GGA CCG GCC TTT CGA AGA T-1584, 2180-CAG CAT GGC GGG ACT ATA T-2198, 1965-CAA GGT GGG CGA TGA GAT T-1983, 1151-ACA TTA TGC TCA AGA GGA A-1169) using DharmaFECT 1 reagent according to the manufacturer's instructions. In PLD1 and PLD2 dual knockdown, 50 nM of each siRNA was used, and a GAPDH siRNA pool (100 nM) was used as a control. 48 h after transfection, Calu-1 cells were serum-deprived for 18 h. PLD activity was measured as described above. In addition, cell lysates were separated by SDS-PAGE, and PLD1 protein was detected by western blot using PLD1-specific antibody (H-160, Santa Cruz). Due to the lack of effective PLD2 antibody, the knockdown level of PLD2 was assessed by qRT-PCR. Briefly, total RNA was extracted using Trizol (Invitrogen), and complementary DNA was synthesized using Superscript II reverse transcriptase (Invitrogen) and random hexamers. Primers for PLD2 RT-PCR were 5'-GGCGATGAGATTGTGGACA-3' and 5'-CTGGAAGAAGTCATCACAGA-3', and primers for β -actin were 5'-ATCCTGCGTCTGGACCTG-3' and 5'-ACGTCACACTTCATGATGGA-3'. Fold change of PLD2 mRNA in each siRNA condition was calculated by normalizing by β -actin mRNA level. Quantification of RT-PCR product bands was performed using QuantityOne software (BioRad). In HEK293-gfpPLD2 cells, GFP-tagged PLD2 knockdown was examined by western blot for GFP using GFP antibody (B-2, Santa Cruz). GAPDH antibody (FL-335, Santa Cruz) was used as a positive control and β -actin antibody (AC-74, Sigma) was used as an equal loading control.

Data and statistical analyses—Data were analyzed using GraphPad Prism v 4.0. Nonlinear curve fitting and statistical analysis was done using built-in functions. Data are plotted as mean $\pm$ s.e.m. unless otherwise stated.

Invasion assays—Transwell invasion assays were performed using BD BioCoat growth factor-reduced matrigel invasion chambers according to the manufacturer's protocol (BD Biosciences). Briefly, cells (2.5×10^4 per well) were plated in medium containing 10% fetal bovine serum, $\pm$ 0.2 μ M, 20 μ M PLD inhibitor or DMSO, in the upper and lower chambers of an 8- μ m-pore matrigel-coated transwell plate. All assays were done in duplicate or

triplicate chambers. After 48 h, cells that migrated to the underside of the transwell filters were fixed and stained using Diff-Quick stain set from Dade Behring AG. Cells in five random fields at 20× magnification were counted. In the siRNA knockdown experiments, cells were transfected with targeted siRNA in 100 mm dishes as described. After 18 h, cells were trypsinized and plated in the transwell chambers, and the remaining cells were plated in 100 mm and 35 mm dishes for RNA and protein extraction. After 48 h, cell lysates and RNA were isolated for western blots and RT-PCR to determine knockdown efficiency. Concomitantly, the migrated cells were stained and counted as described above. Experiments were performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge outstanding support from A. Goodman (mass spectrometry) and D. Myers (data analysis and statistics) as well as R. Bruntz (Vanderbilt University) for assisting with the generation of the HEK293-gfpPLD2 stable cell line. We thank C. Rouzer for helpful discussions. We gratefully acknowledge partial support for this project from the A.B. Hancock Jr. Memorial Laboratory for Cancer Research and the Vanderbilt Institute for Chemical Biology (to H.A.B. and C.W.L.). P.E.S. was supported in part by a US National Research Service Award Training Fellowship (T32 GM007628). T.L.C. was partially supported by the US National Cancer Institute T32 grant CA09592 and the Breast Cancer Specialized Program of Research Excellence (P50-CA98131 to C.L.A.).

References

1. Ponting CP, Kerr ID. A novel family of phospholipase D homologues that includes phospholipid synthases and putative endonucleases: identification of duplicated repeats and potential active site residues. *Protein Sci.* 1996; 5:914–922. [PubMed: 8732763]
2. Xie Z, Ho WT, Exton JH. Association of N- and C-terminal domains of phospholipase D is required for catalytic activity. *J. Biol. Chem.* 1998; 273:34679–34682. [PubMed: 9856987]
3. Brown HA, et al. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell.* 1993; 75:1137–1144. [PubMed: 8261513]
4. Hammond SM, et al. Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. *J. Biol. Chem.* 1995; 270:29640–29643. [PubMed: 8530346]
5. Park SK, et al. Cloning and characterization of phospholipase D from rat brain. *J. Biol. Chem.* 1997; 272:29263–29271. [PubMed: 9361006]
6. Exton JH. Regulation of phospholipase D. *FEBS Lett.* 2002; 531:58–61. [PubMed: 12401203]
7. Hancock JF. PA promoted to manager. *Nat. Cell Biol.* 2007; 9:615–617. [PubMed: 17541412]
8. Wymann MP, Schneider R. Lipid signalling in disease. *Nat Rev. Mol. Cell Biol.* 2008; 9:162–176. [PubMed: 18216772]
9. Noh DY, et al. Overexpression of phospholipase D1 in human breast cancer tissues. *Cancer Lett.* 2000; 161:207–214. [PubMed: 11090971]
10. Uchida N, Okamura S, Nagamachi Y, Yamashita S. Increased phospholipase D activity in human breast cancer. *J. Cancer Res. Clin. Oncol.* 1997; 123:280–285. [PubMed: 9201251]
11. Zhao Y, et al. Increased activity and intranuclear expression of phospholipase D2 in human renal cancer. *Biochem. Biophys. Res. Commun.* 2000; 278:140–143. [PubMed: 11185526]
12. Uchida N, Okamura S, Kuwano H. Phospholipase D activity in human gastric carcinoma. *Anticancer Res.* 1999; 19:671–675. [PubMed: 10216475]
13. Yamada Y, et al. Association of a polymorphism of the phospholipase D2 gene with the prevalence of colorectal cancer. *J. Mol. Med.* 2003; 81:126–131. [PubMed: 12601529]
14. Min DS, et al. Neoplastic transformation and tumorigenesis associated with over-expression of phospholipase D isozymes in cultured murine fibroblasts. *Carcinogenesis.* 2001; 22:1641–1647. [PubMed: 11577003]

15. Buchanan EG, et al. Requirement of phospholipase D1 activity in H-RasV12-induced transformation. *Proc. Natl. Acad. Sci. USA*. 2005; 102:1638–1642. [PubMed: 15668389]
16. Bocckino SB, Wilson PB, Exton JH. Ca²⁺-mobilizing hormones elicit phosphatidylethanol accumulation via phospholipase D activation. *FEBS Lett*. 1987; 225:201–204. [PubMed: 3319693]
17. Ktistakis NT, et al. Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. *J. Cell Biol*. 1996; 134:295–306. [PubMed: 8707816]
18. Tou JS, Urbizo C. Diethylstilbestrol inhibits phospholipase D activity and degranulation by stimulated human neutrophils. *Steroids*. 2008; 73:216–221. [PubMed: 18036628]
19. Tou J, Urbizo C. Resveratrol inhibits the formation of phosphatidic acid and diglyceride in chemotactic peptide- or phorbol ester-stimulated human neutrophils. *Cell Signal*. 2001; 13:191–197. [PubMed: 11282457]
20. Garcia A, et al. Honokiol suppresses survival signals mediated by Ras-dependent phospholipase D activity in human cancer cells. *Clin. Cancer Res*. 2008; 14:4267–4274. [PubMed: 18594009]
21. Puar MS, Barrabee E, Hallade M, Patel M. Sch 420789: a novel fungal metabolite with phospholipase D inhibitory activity. *J. Antibiot. (Tokyo)*. 2000; 53:837–838. [PubMed: 11079806]
22. McDonald LA, et al. 07H239-A, a new cytotoxic eremophilane sesquiterpene from the marine-derived Xylariaceae fungus LL-07H239. *J. Nat. Prod*. 2004; 67:1565–1567. [PubMed: 15387660]
23. Levy BD, et al. Novel polyisoprenyl phosphates block phospholipase D and human neutrophil activation in vitro and murine peritoneal inflammation in vivo. *Br. J. Pharmacol*. 2005; 146:344–351. [PubMed: 16041402]
24. Eisen SF, Brown HA. Selective estrogen receptor (ER) modulators differentially regulate phospholipase D catalytic activity in ER-negative breast cancer cells. *Mol. Pharmacol*. 2002; 62:911–920. [PubMed: 12237338]
25. Monovich L, et al. Optimization of halopemide for phospholipase D2 inhibition. *Bioorg Med. Chem. Lett*. 2007; 17:2310–2311. [PubMed: 17317170]
26. Brown HA, et al. Biochemical analysis of phospholipase D. *Methods Enzymol*. 2007; 434:49–87. [PubMed: 17954242]
27. Henage LG, Exton JH, Brown HA. Kinetic analysis of a mammalian phospholipase D: allosteric modulation by monomeric GTPases, protein kinase C, and polyphosphoinositides. *J. Biol. Chem*. 2006; 281:3408–3417. [PubMed: 16339153]
28. Yang H, Roberts MF. Phosphohydrolase and transphosphatidylations reactions of two *Streptomyces* phospholipase D enzymes: covalent versus noncovalent catalysis. *Protein Sci*. 2003; 12:2087–2098. [PubMed: 12931007]
29. Billah MM, et al. Regulation of phospholipase D in HL-60 granulocytes Activation by phorbol esters, diglyceride, and calcium ionophore via protein kinase-independent mechanisms. *J. Biol. Chem*. 1989; 264:9069–9076. [PubMed: 2498324]
30. Coley WC, et al. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol*. 1997; 7:191–201. [PubMed: 9395408]
31. Aguirre Ghiso JA, et al. A phospholipase D and protein kinase C inhibitor blocks the spreading of murine mammary adenocarcinoma cells altering f-actin and pi-integrin point contact distribution. *Int. J. Cancer*. 1997; 71:881–890. [PubMed: 9180160]
32. Shen Y, Zheng Y, Foster DA. Phospholipase D2 stimulates cell protrusion in v-Src-transformed cells. *Biochem. Biophys. Res. Commun*. 2002; 293:201–206. [PubMed: 12054584]
33. Imamura F, et al. Induction of in vitro tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. *Biochem. Biophys. Res. Commun*. 1993; 193:497–503. [PubMed: 8390242]
34. Pai JK, Frank EA, Blood C, Chu M. Novel ketoepoxides block phospholipase D activation and tumor cell invasion. *Anticancer Drug Des*. 1994; 9:363–372. [PubMed: 7916902]
35. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol. Cell Biol*. 1992; 12:954–961. [PubMed: 1312220]

36. Bi K, Roth MG, Ktistakis NT. Phosphatidic acid formation by phospholipase D is required for transport from the endoplasmic reticulum to the Golgi complex. *Curr. Biol.* 1997; 7:301–307. [PubMed: 9133344]
37. Rizzo MA, et al. Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem.* 1999; 274:1131–1139. [PubMed: 9873061]
38. Zhao C, et al. Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. *Nat. Cell Biol.* 2007; 9:706–712. [PubMed: 17486115]
39. Fang Y, et al. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science.* 2001; 294:1942–1945. [PubMed: 11729323]
40. Chen Y, Zheng Y, Foster DA. Phospholipase D confers rapamycin resistance in human breast cancer cells. *Oncogene.* 2003; 22:3937–3942. [PubMed: 12813467]
41. Williger BT, Ho WT, Exton JH. Phospholipase D mediates matrix metalloproteinase-9 secretion in phorbol ester-stimulated human fibrosarcoma cells. *J. Biol. Chem.* 1999; 274:735–738. [PubMed: 9873009]
42. Kato Y, et al. Acidic extracellular pH induces matrix metalloproteinase-9 expression in mouse metastatic melanoma cells through the phospholipase D-mitogen-activated protein kinase signaling. *J. Biol. Chem.* 2005; 280:10938–10944. [PubMed: 15657063]
43. Kang DW, et al. Phorbol ester up-regulates phospholipase D1 but not phospholipase D2 expression through a PKC/Ras/ERK/NFkappaB-dependent pathway and enhances matrix metalloproteinase-9 secretion in colon cancer cells. *J. Biol. Chem.* 2008; 283:4094–4104. [PubMed: 18084005]
44. Reich R, Blumenthal M, Liscovitch M. Role of phospholipase D in laminin-induced production of gelatinase A (MMP-2) in metastatic cells. *Clin. Exp. Metastasis.* 1995; 13:134–140. [PubMed: 7882615]
45. Williger BT, et al. Release of gelatinase A (matrix metalloproteinase 2) induced by photolysis of caged phosphatidic acid in HT 1080 metastatic fibrosarcoma cells. *J. Biol. Chem.* 1995; 270:29656–29659. [PubMed: 8530350]
46. Wang L, et al. Involvement of phospholipases D1 and D2 in sphingosine 1-phosphate-induced ERK (extracellular-signal-regulated kinase) activation and interleukin-8 secretion in human bronchial epithelial cells. *Biochem. J.* 2002; 367:751–760. [PubMed: 12149127]
47. Walker SJ, Wu WJ, Cerione RA, Brown HA. Activation of phospholipase D1 by Cdc42 requires the Rho insert region. *J. Biol. Chem.* 2000; 275:15665–15668. [PubMed: 10747870]

Figure 1.

PLD and phosphatidic acid in the cell. (a) PLD signaling pathway from GPCR and receptor tyrosine kinase (RTK) to cellular responses. PA, phosphatidic acid; PC, phosphatidylcholine. (b) Previously published indirect and direct PLD inhibitors.

Figure 2. Design and synthetic strategy for PLD inhibitor development. **(a)** Diversity-oriented modular design strategy. **(b)** General synthetic approach. **(c)** Selected compounds from small-molecule PLD inhibitor library.

Figure 3.

In vitro inhibition of recombinant PLD with select compounds. Graphs demonstrate CRCs for three classes of inhibitors on recombinant human PLD1 (■) and PLD2 (▲) normalized to total myr-Arf-1-stimulated activity $\pm$ s.e.m. (representative data from a single 30-min experiment done in triplicate), (a–c) Shown are examples of compounds demonstrating dual PLD1 and PLD2 inhibition (a), PLD1-selective inhibition (b) and PLD2-selective inhibition (c) with data from the most potent compounds shown in the lower panels.

Figure 4.

Direct small-molecule inhibition of PLD. **(a)** Schematic representation of truncated PLD1.d311 construct, **(b)** *In vitro* concentration response curves for two dual-isoform compounds, **56** (left) and **64** (right), demonstrating direct inhibition of purified PLD1.d311 basal (○) or myr-Arf-1-stimulated (●) activity. Error bars, s.e.m. **(c)** Colloidal-stained Tris-glycine 4–20% gradient SDS-PAGE gel of purified PLD1.d311 and myr-Arf-1 shows that both proteins are of high purity.

Figure 5.

Development of distinct PLD isoform cell systems. **(a)** Calu-1 cells were transfected with siRNA against PLD1, PLD2, PLD1+PLD2 or GAPDH control and then stimulated with 100 nM PMA. Data are plotted as a percent of PMA-stimulated PLD activity with siRNA GAPDH as the 100% control. PLD1 protein knockdown showed a significant decrease in PLD activity following PMA stimulation, whereas PLD2 knockdown had no effect. $**P < 0.005$. **(b)** Western blot of siRNA transfected Calu-1 cells shows protein levels decrease in response to siRNA transfection. **(c)** RT-PCR of PLD2 message in response to siRNA transfection. mRNA levels were decreased upon transfection with PLD2 siRNA. **(d)** HEK293-gfpPLD2 stable cells transfected with siRNA against PLD1, PLD2, PLD1+PLD2 or GAPDH control and basal PLD activity are shown. Data are plotted as percent of basal PLD activity with siRNA GAPDH as the 100% control. PLD2 knockdown showed a significant decrease in PLD activity ($*P < 0.05$), whereas PLD1 knockdown showed no effect. **(e)** Western blot of HEK293-gfpPLD2 confirms siRNA protein knockdown in these samples. Error bars, s.e.m.

Figure 6.

Small molecules potently inhibit cellular PLD activity. CRCs were obtained for multiple small-molecule PLD inhibitors for both PMA-stimulated Calu-1 cells (■) and a basal HEK293-gfpPLD2 stable cell line (▲), (a) Dual-specificity PLD1+PLD2 inhibitors. Compounds **56** and **64** inhibit PLD activity in both Calu-1 and HEK293-gfpPLD2 cells. Data are representative of the CRC of each compound as percent of total PLD activity in the respective cell line with mean $\pm$ s.e.m. (b) PLD1-selective compounds. **58** and **69** selectively inhibited Calu-1 PLD activity over HEK293-gfpPLD2 PLD activity. (c) PLD2-prefering inhibitors. **72** and **82** preferentially inhibited HEK293-PLD2 PLD activity over Calu-1 PLD activity.

Figure 7.

Inhibition of PLD leads to decreased invasive migration in breast cancer cell lines. **(a)** Cells were plated in medium, $\pm$ 20 μ M **56**, in the upper chamber of 8- μ m-pore matrigel-coated transwell filters (scale bar, 50 μ m). The lower chamber contained medium and inhibitor containing 10% fetal bovine serum. Cells migrating to the underside of the filter were stained and counted 48 h later. Each bar, mean of 5 fields of 2 wells $\pm$ s.d. **(b)** Different cell types were plated in medium, $\pm$ the indicated dose of PLD inhibitors **82**, **72**, **58** or **69**, in the upper chamber of transwell filters. The lower chamber contained medium and inhibitor containing 10% fetal bovine serum. Cells migrating to the underside of the filter were stained and counted 48 h later. Each bar, mean of 5 fields of 2 wells $\pm$ s.d. **(c)** siRNA knockdown of PLD proteins in MDA-231 cells decreases invasive migration. MDA-231 cells were transfected with siRNA against GAPDH, PLD1, PLD2 and PLD1+PLD2 and plated in the upper chamber of transwell filters. Cells migrating to the underside of the filter were stained and counted 48 h later. Each bar represents the mean of 5 fields of 3 wells, $n = 3$. **(d)** Western blot shows that PLD1 protein level was substantially decreased upon targeted siRNA treatment. **(e)** PLD2 mRNA level was measured by RT-PCR, and data show that message was significantly decreased in samples transfected with siRNA against PLD2 and PLD1+PLD2. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

Table 1

In vitro and *in vivo* IC₅₀ values for PLD inhibitors

Name	<i>In vitro</i> IC ₅₀ (nM)			Cellular IC ₅₀ (nM)			<i>In vitro</i> IC ₅₀ (nM)			Cellular IC ₅₀ (nM)	
	PLD1	PLD2	d.311	Calu-1	293-PLD2	Name	PLD1	PLD2	d.311	Calu-1	293-PLD2
Raloxifene (6)	4,300	3,400	>20,000	8,500	10,000	52	780	1,100	1,700	32	210
Tamoxifen (7)	ST	ST	>20,000	NE	ST	63	360	1,400	790	22	450
4-OH tamoxifen	ST	74,000	28,000	4,800	6,500	53	540	1,900	1,900	79	1,400
Halopemide (8)	220	310	11	21	300	61	316	1,200	1,500	37	340
14	9.5	17	33	1	44	49	400	1,900	3,500	85	1,100
15	100	340	1,000	14	210	55	59	690	780	51	79
56	81	240	1,000	21	380	68	1,200	12,000	15,000	870	>20,000
64	79	170	2,900	8.9	220	58	660	6,800	7,600	35	3,900
47	480	560	5,000	17	130	60	7,400	>20,000	>20,000	380	>20,000
57	260	600	1,100	20	1,000	46	3,200	NE	NE	>20,000	NE
48	46	250	910	19	220	69	46	933	331	11	1,800
50	55	130	910	6.3	140	70	>20,000	4,300	>20,000	NE	NE
51	15,000	>20,000	>20,000	1,000	1,100	82	>20,000	2,500	>20,000	3,400	1,200
59	300	1,000	7,200	330	260	72	5,100	140	>20,000	1,000	110
66	1,400	2,200	870	48	470	45	NE	NE	NE	6,000	>2,000

In vitro CRCs (from 200 pM to 20 μM) produced IC₅₀s for 30 compounds with myr-Arf-1-stimulated mammalian hPLD1, hPLD2 and rat PLD1.d311. Cell-based assays were used to develop CRCs (from 200 pM to 2 μM) and determine IC₅₀s for 30 compounds in Calu-1 or HEK293-gfpPLD2 cell lines. NE, no effect; ST, stimulatory. The geometric mean of the standard errors of the log(IC₅₀) values from the curve fits of all 30 compounds were computed and compared to the IC₅₀s themselves. There were levels of ~30% error for Calu-1 and ~70% for HEK293-gfpPLD2 IC₅₀s. The exogenous assays had CRCs with somewhat higher scatter: PLD1 standard errors corresponded to a factor of two error, and ± 60% for PLD2. Despite the variance in the absolute values over a large number of assays, the reproducibility of the effects and relative potency of the inhibitors were found to be robust.