Published in final edited form as:

J Med Chem. 2013 March 28; 56(6): 2695–2699. doi:10.1021/jm301782e.

Development of Dual PLD1/2 and PLD2 Selective Inhibitors From a Common 1,3,8-Triazaspiro[4.5]decane Core: Discovery of ML298 and ML299 that Decrease Invasive Migration in U87-MG Glioblastoma Cells

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Abstract

An iterative parallel synthesis effort identified a PLD₂ selective inhibitor, ML298 (PLD₁ IC₅₀ >20,000 nM, PLD₂ IC₅₀ = 355 nM) and a dual PLD_{1/2} inhibitor, ML299 (PLD₁ IC₅₀ = 6 nM, PLD₂ IC₅₀ = 20 nM). SAR studies revealed a small structural change (incorporation of a methyl group) increased PLD₁ activity within this classically PLD₂-preferring core, and that the effect was enantiospecific. Both probes decreased invasive migration in U87-MG glioblastoma cells.

Keywords

Phospholipase D; PLD₁; PLD₂; ML299; ML298; MLPCN probe

INTRODUCTION

Phospholipase D (PLD) is a lipid signaling enzyme that catalyzes the hydrolysis of phosphatidylcholine into choline and phosphatidic acid (PA), an important lipid second messenger that is central to a number of critical metabolic and signaling pathways.^{1–3} The PLD protein is characterized by the presence of a conserved histidine, lysine, aspartate (HKD) amino acid domain that forms the catalytic site, as well as conserved phox homology

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Author Contributions

Professors Lindsley directed and designed the chemistry, Dr. Daniels designed the pharmacokinetic studies, Dr. Brown and Dr. Thomas directed the molecular pharmacology. Mr. O'Reilly and Mr. Brown performed the synthetic chemistry and Mr. Morrison performed the bioanalytical DMPK work. Dr. Scott and Mr. Oguin performed the molecular pharmacology.

Supporting Information. Experimental procedures and spectroscopic data for selected compounds, detailed pharmacology and DMPK methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

(PX) and pleckstrin homology (PH) regulatory domains at the *N*-terminus.^{1–3} There are two mammalian isoforms of PLD, designated PLD₁ and PLD₂, with ~ 53% sequence identity, and subject to different regulatory mechanisms and distinct physiological roles.^{1–3} Both genetic and biochemical studies implicate dysregulated PLD function and/or expression as having a role in cancer (*e.g.*, breast, renal, colorectal and glioblastoma)^{4–7} and CNS disorders (*i.e.*, Alzheimer's disease⁸ and stroke⁹). The tools available to inhibit PLD activity have been limited to genetic/biochemical approaches, unselective small molecules and *n*-butanol (which competes with water in a transphosphatidylation reaction); however, none of these represent viable therapeutic options or allows the role of individual PLD isoforms to be dissected.¹

In 2007, haloperamide **1**, a classical atypical antipsychotic agent (D₂ IC₅₀ = 7 nM) that was successfully evaluated in five human clinical trials, was reported to be a PLD inhibitor; importantly, the exposure of **1** in those trials was sufficient to inhibit both PLD₁ and PLD₂, suggesting PLD inhibition was not overtly toxic in humans.^{10,11} Subsequent work in our lab demonstrated that **1** was a direct, potent dual PLD₁/PLD₂ inhibitor (PLD₁ IC₅₀ = 21 nM, PLD₂ IC₅₀ = 300 nM).² A diversity-oriented synthesis (DOS) effort around **1** led to the discovery of the first isoform selective PLD inhibitors **2–4**, with unprecedented isoform selectivity for either PLD₁ or PLD₂ (Figure 1).^{2,12–14} Our lab is highly interested in selective inhibition of PLD₂, and while **4** is 75-fold PLD₂ selective, the selectivity is driven by potency at PLD₂ (IC₅₀ = 20 nM), but still inhibits PLD₁ (IC₅₀ = 1,500 nM) at modest concentrations.¹⁵ Thus, in this manuscript, we describe the further optimization of **4** to afford an improved PLD₂ inhibitor, a dual PLD₁/PLD₂ inhibitor (based on an enantiospecific 'molecular switch')¹⁵ with interesting activity as a metastatic agent in cellular assays.

RESULTS AND DISCUSSION

Chemistry

After the large DOS effort that identified the 1,3,8-triazaspiro[4.5]decane core as a PLD₂-preferring motif,² a subsequent 4×6 matrix library led to the discovery of **4**, possessing a key 3-fluorophenyl moiety; however, this exercise only produced four amide analogs of **4**.¹⁶ Thus, we held the 3-fluorophenyl moiety constant, and explored a diverse array of 28 amides in an effort to identify a PLD₂ inhibitor with little or no inhibition of PLD₁ (Scheme 1).

The library was first evaluated in a cell-based assay (single point screen at 200 nM concentration) against both PLD₁ (Calu-1) and PLD₂ (293-PLD₂).² As with other allosteric ligands, SAR was 'flat', and the library afforded few PLD inhibitors (Figure 2).^{2,12–16} In general, aliphatic, cycloalkyl and heteroaryl amides were inactive. However, the library did identify CID53393915 (**7g**), a potent >53-fold selective PLD₂ inhibitor (IC₅₀ = 355 nM) with no measurable inhibition of PLD₁ (IC₅₀ >20,000 nM). Comparable data was generated in the biochemical assay with purified PLD proteins, indicating that **7g** acted directly on PLD₂. Based on the PLD inhibitory profile, **7g** was declared an MLPCN probe and assigned as ML298.¹⁷ ML298 does not inhibit PLD₁ up to 20 μM, which makes it a better tool for *in vitro* and *in vivo* work as compared to **4**, which while more potent at PLD₂, also inhibits PLD₁ at 1.5 μM concentrations. Thus, at standard *in vitro* concentrations and *in vivo* plasma exposures (above 5 μM), ML298 only inhibits PLD₂.

Within the piperidine benzimidazolone-based PLD inhibitors, such as (**2**)^{2,12} the introduction of a chiral methyl group α to the amide dramatically increased PLD₁ inhibitory activity, but interestingly, both the (*R*)- and (*S*)- enantiomers were equipotent/selective.¹² Preliminary data with a lone example indicated that introduction of a methyl group into the

ethyl linker α to the amide of the 1,3,8-triazaspiro[4.5]decane-based PLD inhibitors also enhanced PLD₁ inhibitory activity.¹⁴ As ancillary pharmacology was improved in the 1,3,8-triazaspiro[4.5]decane series relative to the piperidine benzimidazolone series, we opted to explore the impact of both (*R*)- and (*S*)-chiral methyl group introduction into analogs of **7**, as SAR between the series is highly divergent.^{12–14} The synthesis of analogs **9** followed scheme 2 and employed either (*R*)- or (*S*)-*tert*-butyl 1-propan-2-ylcarbamate. As shown in Table 1, this exercise provided the first examples of enantioselective PLD inhibition and PLD isoform selectivity induced by a simple ‘molecular switch’.¹⁵ Within the 1,3,8-triazaspiro[4.5]decane series, the (*S*)-enantiomer was uniformly more potent than the (*R*), enhancing PLD inhibitory activity >230-fold in some cases, while also enhancing PLD₂ activity 2- to 10-fold, leading to a novel series of dual PLD₁/PLD₂ inhibitors **9**. This effort identified **9b**, a potent dual PLD₁ (IC₅₀ = 6 nM) and PLD₂ (IC₅₀ = 20 nM), with equivalent data in the biochemical assay with purified PLD proteins (PLD₁ IC₅₀ = 48 nM and PLD₂ IC₅₀ = 84 nM), indicating that ML299 is a direct inhibitor. Based on this profile, **9b** was declared an MLPCN probe, and designated ML299.¹⁷

DMPK Profiling

The two probes were then profiled in a battery of *in vitro* and *in vivo* DMPK assays to assess their utility as *in vivo* tools (Table 2). Both compounds were stable in PBS buffer up to 48 hours, afforded no GHS conjugates, were soluble in PBS buffer (>20 μ M or >10 μ g/mL) and in a Ricerca radioligand binding panel of 68 GPCRs, ion channels and transporter,^{18,19} displayed significant activity (>50% inhibition @ 10 μ M) at only 3 targets (opiate and hERG) as compared to **1** with significant activity at over 30 targets.¹⁸ Importantly, in follow-up functional assays, neither compound functionally inhibited hERG (IC₅₀ >20 μ M), and there was no agonist activity at the opiate receptors. Both probes were highly cleared in rat and human microsomes, but possessed good free fraction in both rat and human as well as favorable CYP profiles. Thus, *in vivo* PK in mice (due to future oncology PD models) was dosed IP to diminish first pass effects. This route of administration provided excellent plasma levels for both probes, but while ML299 was CNS penetrant (Brain_{AUC}/Plasma_{AUC} of 0.44), ML298 was peripherally restricted (Brain_{AUC}/Plasma_{AUC} of 0.05).¹⁸ Thus, ML298 compliments **4**, which is highly CNS penetrant, providing key tools to dissect selective PLD₂ in the periphery as well as in the CNS.

Efficacy in Cell-Based Assays

In our earlier work with PLD₁ inhibitor **2** and the dual PLD_{1/2} inhibitor **3**, we found that both inhibitors blocked invasive migration in both a triple negative breast cancer cell line (MDA-MB-231) and a U87-MG glioblastoma cell line *in vitro*; however, siRNA studies indicated that PLD₂ played a dominant role.² Now, with ML299, a far more potent dual PLD₁ and PLD₂ inhibitor and ML298, a selective PLD₂ inhibitor, we found that both probes had no effect (relative to DMSO) at concentrations up to 10 μ M on U87-MG cell viability (in the presence of 10% FBS), so cytotoxicity as a driver of decreasing invasive migration can be ruled out.¹⁸ Also, ML299 robustly increased caspase 3/7 activation under serum-free conditions.^{2,18} However, as shown in Figure 3A, inhibition of both PLD₁ and PLD₂ by ML299, provides a dose-dependent decrease (100 nM to 10 μ M) in invasive migration in U87-MG cells, with statistical significance reached at both the 1 μ M and 10 μ M doses.¹⁸ At these doses, both PLD₁ and PLD₂ are inhibited. In Figure 3B, selective inhibition of PLD₂ by ML298, also provides a dose-dependent decrease in invasive migration in U87-MG cells, with statistical significance reached at a 10 μ M dose. At this dose, only PLD₂ is inhibited, suggesting a key role for this PLD isoform in decreasing invasive migration; however, the effects with ML299 are more robust than with ML298 as the potency for PLD₂ inhibition is ~16-fold greater.¹⁸

Conclusion

In summary, we have developed a new, direct acting >53-fold selective PLD₂ inhibitor (ML298), with no inhibition of PLD₁ and an attractive DMPK profile, making it an attractive tool compound to further dissect PLD₂ function in multiple cellular and *in vivo* environments. In the course of these efforts, we also discovered a key enantiospecific 'molecular switch' in the classically PLD₂-preferring 1,3,8-triazaspiro[4.5]decane scaffold, that enhanced PLD₁ inhibition up to 230-fold, and afforded a potent dual PLD₁/PLD₂ probe, ML299, with a good DMPK profile. Both probes decreased invasive migration in U87-MG glioblastoma cells, suggesting the centrally penetrant ML299 as a possible tool compound to assess therapeutic utility in brain cancer. Further *in vivo* studies with these probes are in progress and will be reported in due course.

EXPERIMENTAL SECTION

Chemistry

The synthesis of **ML298** is described below. The general chemistry, experimental information, and syntheses of all other compounds are supplied in the Supporting Information. Purity of all final compounds was determined by HPLC analysis is >95%.

3,4-Difluoro-N-(2-(1-(3-fluorophenyl)-4-oxo-1,3,8-triazaspiro[4.5]decan-8-yl)ethyl)benzamide, 7g (ML298)—8-(2-aminoethyl)-1-(3-fluorophenyl)-1,3,8-triazaspiro[4.5] decan-4-one dihydrochloride **6** (146.5 mg, .5 mmol), DMF (5 mL), triethylamine (.257 mL, 2.55 mmol, 5 eq.) followed by 3,4 difluoro benzoyl chloride **29** (136.3 mg, 0.772 mmol, 1.5 eq.) were added to this stirred mixture and the reaction was quenched in less than 30 minutes, determined by consumption of starting material seen via LC-MS. The reaction was quenched with water/brine and was extracted 3X with ethyl acetate. The organic extract was concentrated and the product was purified via reverse phase HPLC eluting with MeCN/H₂O/TFA to afford the product ML298 as a white solid (177 mg, 0.41 mmol, 80%). ¹H NMR (400.1 MHz, DMSO-*d*₆) δ (ppm): 8.73 (s, 1H); 8.59 (t, *J* = 5.4 Hz, 1H); 7.90-7.83 (m, 1H); 7.76-7.70 (m, 1H); 7.56-7.48 (m, 1H); 7.10 (q, *J* = 8.0 Hz, 1H); 6.65 (dd, *J*₁ = 8.0 Hz, *J*₂ = 1.9, 1H); 6.58- 6.52 (m, 1H); 6.48 (td, *J*₁ = 8.5 Hz, *J*₂ = 2.3 Hz, 1H); 4.57 (s, 2H); 3.44 (q, *J* = 5.7, 2H); 2.99-2.91 (m, 2H); 2.90-2.79 (m, 2H); 2.68-2.55 (m, 4H); 1.60 (d, *J* = 13.7). ¹³C NMR (100.6 MHz, CDCl₃) δ (ppm): 176.08, 164.45, 162.30, 151.62 (dd, *J*₁ = 250.5 Hz, *J*₂ = 12.9 Hz); 149.50 (dd, *J*₁ = 246.3, *J*₂ = 13.0); 145.3 (d, *J* = 11.4); 132.43-132.28 (m); 130.63 (d, *J* = 10.6); 124.95 (dd, *J*₁ = 7.3, *J*₂ = 3.3); 118.01 (dd, *J*₁ = 91.4, *J*₂ = 17.5); 117.38 (dd, *J*₁ = 93.15, *J*₂ = 17.9); 109.69, 103.68 (d, *J* = 21.22); 100.54 (d, *J* = 27.46); 59.14, 58.28, 56.83, 49.56, 37.21, 28.17. HRMS (TOF, ES+) C₂₂H₂₄N₄O₂F₃ [M+H]⁺ calc. mass 433.1851, found 433.1855.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work was generously supported by the NIH/MLPCN U54 MH084659 (C.W.L.) and the McDonnell Foundation. M.C.O. acknowledges funding from a Predoctoral ACS Medicinal Chemistry Fellowship (2011–2012).

Dr. Lindsley thanks the Warren family for support of the research in his laboratory. Vanderbilt is a member of the MLPCN and houses the Vanderbilt Specialized Chemistry Center for Accelerated Probe Development, and the probes ML298 and ML99 are freely available upon request.

ABBREVIATIONS USED

PLD	phospholipase D
U87-MG	human glioblastoma cell line
CRC	concentration-response-curve

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17. For information on the MLPCN, please see: <http://mli.nih.gov/mli>
18. See Supporting Information for full details.
19. For information on Ricerca, see: www.ricerca.com

Figure 1. Structure of halopemide (1) and optimized inhibitors after a DOS campaign: PLD₁ selective inhibitor (2), dual PLD₁/PLD₂ inhibitor (3) and PLD₂ selective inhibitor (4).

Figure 2.

A) Single point (200 nM) cell-based screen of amide analogs **7** for their ability to inhibit both PLD₁ and PLD₂. B) Structure and PLD inhibitory activity of **7g** (ML298), a >53-fold PLD₂ selective inhibitor. C) Cell-based PLD₁ and PLD₂ CRCs for **7g**.

Figure 3.

Inhibition of PLD₂ leads to decreased invasive migration in U87-MG cell lines. A) Effect of dual PLD₁/PLD₂ inhibitor ML299 and B) effect of PLD₂ selective inhibitor ML298 on invasive migration.¹⁹ Cells were plated in medium $\pm$ various concentrations of either ML298 or ML299 of the upper chamber of 8 micron-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 were stained/counted 48 hours later. Each bar, mean of 5 fields of 3 wells $\pm$ s.d.

Scheme 1. Synthesis of amide analogs 7

Reagents and conditions: (a) 1. *tert*-butyl 2-oxoethylcarbamate, MPB(OAc)₃H, DCM, RT; 2. 4.0 M HCl/dioxane, DCM, MeOH, RT; (b) RCOCl, DIEA, DMF, RT.

Scheme 2. Synthesis of amide analogs 9

Reagents and conditions: (a) 1. (*R*)- or (*S*)-*tert*-butyl 1-propan-2-ylcarbamate, MP-B(OAc)₃H, DCM, RT; 2. 4.0 M HCl/dioxane, DCM, MeOH, RT; (b) RCOCl, DIEA, DMF, RT.

Table 1Chiral Methyl Groups in Analogs **9** as 'Molecular Switches' Governing PLD Isoform Potency and Selectivity.

Entry	R	PLD1 IC ₅₀ ^a	PLD2 IC ₅₀ ^b
9a		1,540 nM	200 nM
9b (ML299)		6 nM	20 nM
9c		75 nM	800 nM
7g (ML298)		>'; >20,000 nM	355 nM
9d		86 nM	120 nM

Entry	R	PLD1 IC ₅₀ ^a	PLD2 IC ₅₀ ^b
9e		1,400 nM	3,450 nM
9f		11,800 nM	350 nM
9g		20 nM	35 nM
9h		760 nM	1,060 nM

^a Cellular PLD1 assay with Calu-1 cells.

^b Cellular PLD2 assay with HEK293-gfp-PLD2 cells. Cell-based assays were used to develop CRCs (from 200 pM to 20 μM) and determine IC₅₀s.

Table 2DMPK Characterization of *ML298* and *ML299*.¹⁸

<i>Parameter</i>	<i>ML298</i>	<i>ML299</i>
MW	432.44	489.3
TPSA	64.8	64.9
cLogP	3.20	4.11
<i>In Vitro Pharmacology</i>		
<i>IC₅₀ (μM)</i>		
CYP (1A2, 2C9, 3A4, 2D6)	>30, >30, 17.6, 7.9	>30, 13.3, 6.8, 9.4
<i>In Vitro PK</i>		
Rat CL _{HEP} (mL/min/kg)	60.9	61.8
Human CL _{HEP} (mL/min/kg)	17.8	19.1
rPPB (%fu)	5.0	3.0
hPPB (%fu)	3.0	1.0
<i>In Vivo PK, Mouse IP Plasma:lung:brain (10 mg/kg, 0–6 hr)</i>		
Plasma (μM*hr)	1.55	1.08
Lung (μM*hr)	4.61	48.5
Brain (μM*hr)	0.08	0.48
B/P	0.05	0.44