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Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part I: Impact of alternative halogenated privileged structures for PLD1 specificity

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ABSTRACT

This Letter describes the synthesis and structure–activity-relationships (SAR) of isoform-selective PLD inhibitors. By virtue of the installation of alternative halogenated piperidinyl benzimidazolone privileged structures, in combination with a key (*S*)-methyl group, novel PLD inhibitors with low nM potency and unprecedented levels of PLD1 isoform selectivity (~1700-fold) over PLD2 were developed.

Phospholipase D (PLD) isozymes mediate the parallel reactions of phospholipid hydrolysis and transphosphatidylation. There are two mammalian isoforms of PLD, coined PLD1 and PLD2, and despite conserved regulatory and catalytic domains, studies indicate distinct modes of activation and functional roles for PLD1 and PLD2.[1] From a therapeutic perspective, PLD has been implicated in a human cancer cell progression (breast, renal, gastric and colorectal) as well as actin cytoskeleton reorganization and cell motility.[2–8] Thus, small molecules that selectively inhibit PLD1 or PLD2 could represent a novel approach for the treatment of cancer. The lack of isoform selective and direct-acting inhibitors has hindered the PLD field for decades. Instead, the study of PLD has been facilitated for decades by the use of *n*-butanol or indirect, non-selective inhibitors such as *trans*-diethylstilbestrol, resveratrol, honokiol and SCH420789, or non-selective, direct-acting inhibitors such as raloxifene and tamoxifen.[9–15]

Recently, Monovich and co-workers reported that halopemide **1** and some related congeners, identified in a PLD high throughput screen (HTS) inhibited PLD2.[16] As we have recently reported, this work did not discuss activity for these compounds on PLD1, and in fact, we found that these compounds are a combination of dual PLD1/2 inhibitors and modestly PLD1-preferring inhibitors—none

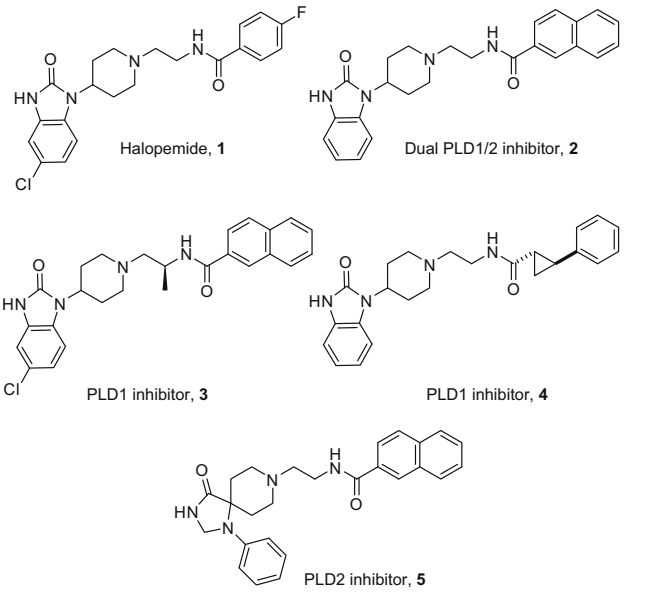

Figure 1. Halopemide **1**, and our recently reported isoform-selective PLD inhibitors: dual PLD1/2 inhibitor **2**, PLD1-selective (>100-fold) inhibitors **3** and **4**, and PLD2 preferring (>9-fold) inhibitor **5**.

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of the analogs disclosed showed any PLD2-preferring inhibition.[17] In the course of our initial investigation of this report, we developed a series of small molecule, isoform-selective PLD inhibitors which included a dual PLD1/2 inhibitor **2**, two PLD1 selective (>100-fold) inhibitors **3** and **4** (>100-fold), and the only known PLD2 preferring (>9-fold) inhibitor **5** (Figure 1). Inhibition of PLD with these direct-acting inhibitors leads to decreased invasive migration in breast cancer cell lines (i.e., MDA-231, 4T1 and PMT), and siRNA confirmed the role of PLD in this response.[17] Thus, PLD inhibitors represent a new class of antimetastatic agents. However, to further probe PLD and the function and role of the individual PLD isoforms, more potent inhibitors with a greater degree of PLD isoform specificity are required.

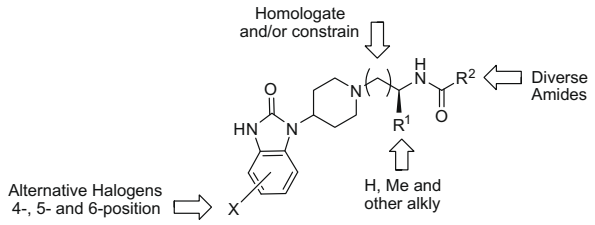
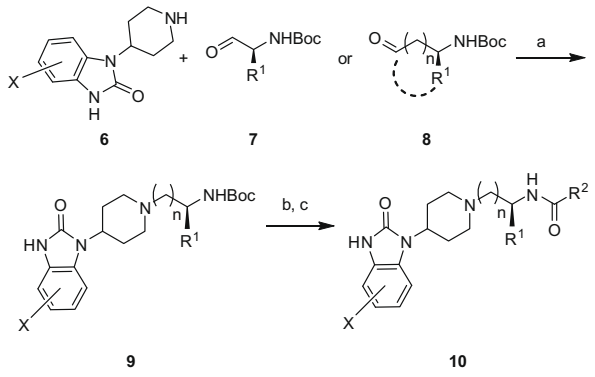


Figure 2. Library strategy to refine PLD inhibitors to improve potency and PLD isoform selectivity.

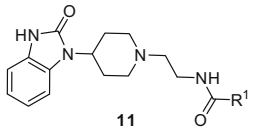


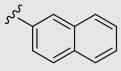
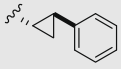
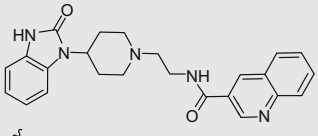
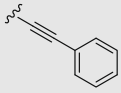
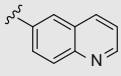
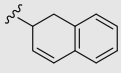
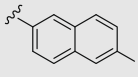
Scheme 1. Reagents and conditions: (a) MP-B(OAc)$_3$, DCE, rt, 16 h (77–97%); (b) 4 N HCl/dioxane, MeOH (98%); (c) R_2COCl, DCM, DIEA, rt (65–95%) or (i) R_2COH, PS-DCC, HOBt, DCM, DIEA; (ii) MP-CO_3^{2-} (58–90%).

Our initial library was based on a diversity-oriented approach utilizing commercial (1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one, and the analogous 5-chloro congener, as key scaffolds which afforded inhibitors **2**–**4**, but was limited in scope. This screen also identified the (*S*)-methyl group on the ethyl diamine linker as a PLD1-inhibition enhancing moiety.[17] In order to refine these inhibitors, we employed our iterative parallel synthesis approach,[18] and synthesized libraries to address the potential SAR depicted in Figure 2.

The alternative halogenated (4-F, 5-F, 6-F, 5-Br) (1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-ones **6** were not commercially available and were synthesized as previously described.[19] The remaining monomers were readily available and the libraries were prepared according to the general route depicted in Scheme 1. In the event, a halogenated (4-F, 5-F, 6-F, 5-Br) or unsubstituted (1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one **6** underwent a reductive amination with either *N*-Boc glycinal, a functionalized alinal **7** or a homologated/cyclic constrained *N*-Boc amino aldehyde **8** to yield **9**. Subsequent removal of the Boc group with 4 N HCl and standard acylation chemistry provides analogs **10**. All compounds were then purified to >98% purity by mass-directed preparative HPLC.[20]

Table 1
Structures and activities of analogs **11**



Cmpd	R^1	PLD1 IC_{50}[a] (nM)	PLD2 IC_{50}[b] (nM)	Fold PLD1-selective
2		21	380	18
4		35	3900	111
11a		8	42	5.1
11b		40	730	18
11c		70	740	10.5
11d		110	860	7.8
11e		120	850	7

[a] Cellular PLD1 assay with Calu-1 Cells.
[b] Cellular assay with HEK293-gfp PLD2 cells. Each IC50 was determined in triplicate. For details see Ref. 17.

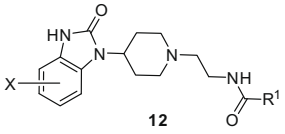
Robust, tractable SAR was observed in the 10 libraries (~250 compounds) synthesized in an iterative fashion over several months—refining library design with new biochemical data.[18] All of the halogenated (4-F, 5-F, 6-F, 5-Br) (1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one provided PLD inhibitors, and a diverse array of alternative amides were also tolerated. In contrast, the ethyl diamino linker was essential—homologation to the corresponding 3- and 4-carbon tethers were inactive, as were cyclic constraints. Only H or (*S*)-methyl substitution was tolerated on the ethyl diamino linker. All library members were evaluated for their ability to inhibit PLD1 and PLD2 in a cellular assay (Calu-1 and HEK293-gfpPLD2, respectively) as well as a biochemical assay with recombinant PLD1 and PLD2 enzymes.[17] The cellular assays were the 'workhorse' assays that drove the SAR, with routine confirmation in the in vitro biochemical assay. Table 1 highlights unsubstituted (X = H) **6** congeners **11** without the (*S*)-methyl group and examines only alternative amides.

While a number of amide caps afforded good PLD1 inhibition, the 2-naphthyl analog **11a** provided single digit nanomolar levels of PLD1 inhibition, but poor selectivity versus PLD2 (~5.1-fold). The *trans*-phenyl cyclopropane congener **4** provided a 35 nM PLD1 inhibitor with excellent selectivity against PLD2 (111-fold).[17]

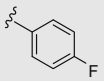
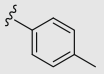
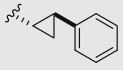
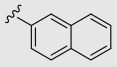
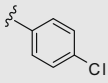
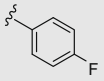
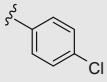
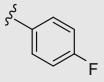
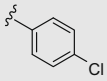
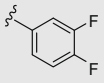
Next, we evaluated halogenated (5-F, 6-F, 5-Cl and 5-Br) (1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one analogs **12** without the (*S*)-methyl group and explored alternative amides (Table 2). Robust, tractable SAR was observed, with this exploration identifying a combination of dual PLD1/2 inhibitors (**12e** and **12i**) with ~3-fold PLD1 selectivity to an inhibitor **12b** (X = 5-Cl, *trans*-phenyl cyclopropane amide) with improved PLD1 selectivity (250-fold) compared to **4**. In general, halogen substitution increased PLD1 inhibition to the low nanomolar range irrespective of the halogen (F, Cl or Br) or the position (5- or 6-substitution). However, the degree of PLD2 inhibition also increased relative to analogs **11**. Thus, efforts now centered on installation of the key PLD1 enhancing (S)-methyl group in an attempt to further improve PLD1 inhibition while diminishing activity towards PLD2 inhibition.

The combination of halogenated (4-F, 5-F, 6-F, 5-Cl and 5-Br) (1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-ones **6** with the (*S*)-methyl group and optimized amides led to some phenomenal, synergistic results (Table 3). With only two exceptions (**13b** and **13l**) PLD1 selectivity for analogs **13** exceeded 140-fold. Importantly, the combination of halogen substitution on the benzimidazolone ring and the (*S*)-methyl group on the diamino ethyl linker performed as we had hypothesized, and increased PLD1 activity into

Table 2
Structures and activities of halogenated analogs **12**

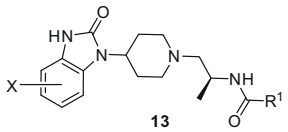


12

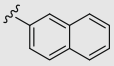
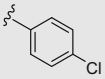
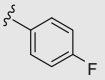
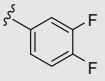
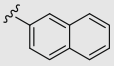
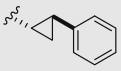
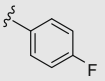
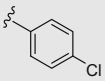
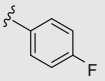
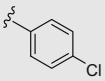
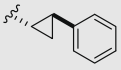
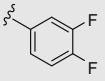
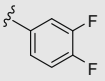
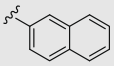
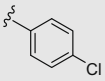
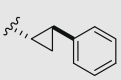
Cmpd	X	R^1	PLD1 IC_{50}[a] (nM)	PLD2 IC_{50}[b] (nM)	Fold PLD1-selective
1	5-Cl		21	300	14.2
12a	5-Cl		10	240	24
12b	5-Cl		2	520	250
12c	5-F		4	140	35
12d	5-F		18	61	3.3
12e	5-F		12	375	31
12f	5-Br		3	97	32
12g	5-Br		4	76	19
12h	6-F		7	42	6
12i	6-F		4	14	3.2

[a] Cellular PLD1 assay with Calu-1 Cells.
[b] Cellular assay with HEK293-gfp PLD2 cells. Each IC_50 was determined in triplicate. For details see Ref. 17.

Table 3
Structures and activities of halogenated analogs **13**

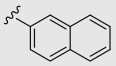
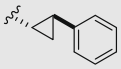


13

Cmpd	X	R^1	PLD1 IC_{50}[a] (nM)	PLD2 IC_{50}[b] (nM)	Fold PLD1-selective
3	5-Cl		46	933	163
13a	4-F		43	12,000	280
13b	4-F		130	10,000	77
13c	4-F		38	14,000	370
13d	4-F		66	13,000	200
13e	4-F		100	>20,000	>200
13f	5-F		11	3100	180
13g	5-F		7.4	1040	140
13h	5-Cl		10	1400	140
13i	5-Cl		6.4	1200	190
13j	5-Cl		8	1150	140
13k	5-Cl		3	730	240
131	5-Br		3.5	187	53
13m	5-Br		5.5	3900	700
13n	5-Br		4	890	222
13o	5-F,		3.7	6400	1700

(*continued on next page*)

Table 3 (*continued*)

Cmpd	X	R^1	PLD1 IC_{50}[a] (nM)	PLD2 IC_{50}[b] (nM)	Fold PLD1-selective
13p	6-F		2	360	180
13q	6-F		12	3800	320

[a] Cellular PLD1 assay with Calu-1 Cells.
[b] Cellular assay with HEK293-gfp PLD2 cells. Each IC_{50} was determined in triplicate. For details see Ref. 17.

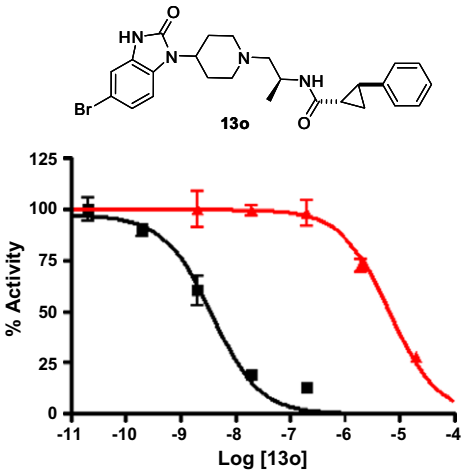


Figure 3. CRCs for cellular PLD1 ■ (Calu-1) assay and PLD2 ▲ (HEK293-gfpPLD2) assay, highlighting the unprecedented PLD1 versus PLD2 selectivity (1700-fold).

the single digit nanomolar range, while dramatically decreasing PLD2 inhibition into the micromolar range. Without question, the *trans*-phenyl cyclopropane amide was the optimal amide moiety, uniformly providing 140- to 1700-fold PLD1 versus PLD2 selectivity. In terms of potency and selectivity, the 5-Br (**13l**–**m**) and 6-F (**13p** and **q**) substitutions offered the best results, providing PLD1 IC_{50}s ranging from 2 nM to 12 nM and with high PLD1 selectivity. Of all of these analogs, **13o** was the standout molecule and possesses unprecedented selectivity for PLD1 inhibition. Compound **13o**, with the 5-Br-(1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one, the (*S*)-methyl group linker and the racemic *trans*-phenyl cyclopropane amide is the most potent (IC_{50} = 3.7 nM) and selective versus PLD2 (IC_{50} = 6.4 μM, ~1700-fold selective) PLD1 inhibitor described to date. The concentration-response-curves (CRC) for the cellular PLD1 (Calu-1) and PLD2 (HEK293-gfpPLD2) assays[17] are shown in Figure 3.

How can we account for this unprecedented level of PLD1 isoform selectivity? Preliminary evidence suggests that these inhibitors do not interact with the catalytic site of PLD, but may bind, and inhibit PLD via an allosteric site.[21] Further work to confirm this allosteric mode of isoform-selective PLD inhibition is in progress.

In summary, the discovery of these highly potent and selective PLD1 inhibitors highlights the power of an iterative analog library synthesis approach for lead optimization, coupled with biochemical assays and mass spectrometric lipid profiling of cellular responses. PLD inhibitor **13o** (PLD1 IC_{50} = 3.7 nM, PLD2 IC_{50} = 6400 nM, 1700-fold selective) represents the most potent and selective PLD1 small molecule inhibitor reported to date, and will serve as an invaluable tool to study and dissect the role of PLD1 in blocking invasiveness in metastatic breast cancer models and other signaling pathways in which PLD is thought to play key regulatory roles. In order to better understand the role of PLD, a more potent PLD2 selective inhibitor is still required; however, PLD2 preferring inhibitors with the same order of selectivity of those generated for PLD1 remain elusive thus far in this chemical series. Work in this field, as well as additional studies to confirm an allosteric mode of inhibition are ongoing.

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