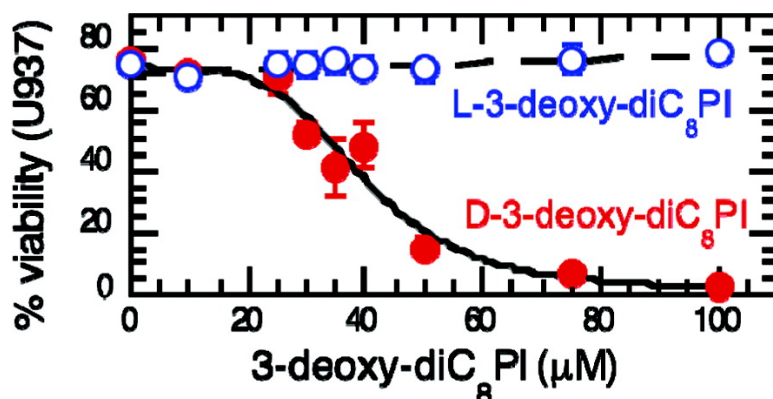


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## Insights into the Structural Specificity of the Cytotoxicity of 3-Deoxyphosphatidylinositols

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**Abstract:** D-3-Deoxyphosphatidylinositol (D-3-deoxy-PI) derivatives have cytotoxic activity against various human cancer cell lines. These phosphatidylinositols have a potentially wide array of targets in the phosphatidylinositol-3-kinase (PI3K)/Akt signaling network. To explore the specificity of these types of molecules, we have synthesized D-3-deoxydioctanoylphosphatidylinositol (D-3-deoxy-diC<sub>8</sub>PI), D-3,5-dideoxy-diC<sub>8</sub>PI, and D-3-deoxy-diC<sub>8</sub>PI-5-phosphate and their enantiomers, characterized their aggregate formation by novel high-resolution field cycling <sup>31</sup>P NMR, and examined their susceptibility to phospholipase C (PLC), their effects on the catalytic activities of PI3K and PTEN against diC<sub>8</sub>PI and diC<sub>8</sub>PI-3-phosphate substrates, respectively, and their ability to induce the death of U937 human leukemic monocyte lymphoma cells. Of these molecules, only D-3-deoxy-diC<sub>8</sub>PI was able to promote cell death; it did so with a median inhibitory concentration of 40 μM, which is much less than the critical micelle concentration of 0.4 mM. Under these conditions, little inhibition of PI3K or PTEN was observed in assays of recombinant enzymes, although the complete series of deoxy-PI compounds did provide insights into ligand binding by PTEN. D-3-Deoxy-diC<sub>8</sub>PI was a poor substrate and not an inhibitor of the PLC enzymes. The in vivo results are consistent with the current thought that the PI analogue acts on Akt1, since the transcription initiation factor eIF4e, which is a downstream signaling target of the PI3K/Akt pathway, exhibited reduced phosphorylation on Ser209. Phosphorylation of Akt1 on Ser473 but not Thr308 was reduced. Since the potent cytotoxicity for U937 cells was completely lost when L-3-deoxy-diC<sub>8</sub>PI was used as well as when the hydroxyl group at the inositol C5 in D-3-deoxy-diC<sub>8</sub>PI was modified (by either replacing this group with a hydrogen or phosphorylating it), both the chirality of the phosphatidylinositol moiety and the hydroxyl group at C5 are major determinants of the binding of 3-deoxy-PI to its target in cells.

### Introduction

The phosphatidylinositol 3-kinase (PI3K)/Akt (or protein kinase B) signaling pathway is critical for cell survival and upregulated in a variety of human cancer cell lines<sup>1</sup> and solid tumors.<sup>2–4</sup> A key step in this pathway is specific phosphorylation of the 3-hydroxyl group of the inositol ring in phosphatidylinositols (PIs) by PI3K enzymes. These lipid products affect cell growth by binding specifically to enzymes such as Akt and recruiting them to the membrane for activation by phosphorylation.<sup>5–7</sup>

The activated Akt survival signal in cells stems from its phosphorylation and inactivation of pro-apoptosis proteins. Counterbalancing PI3K is the lipid phosphatase PTEN, which specifically dephosphorylates the 3-phosphate group and in so doing inhibits PI3K/Akt signaling.<sup>8</sup> Indeed, mutations of PTEN have been reported in an array of human tumors.<sup>9,10</sup>

Attempts to inhibit the PI3K/Akt pathway led to the synthesis of D-3-deoxy-phosphatidylinositol (D-3-deoxy-PI) molecules, which can no longer be phosphorylated by PI3K (for a review, see ref 11). Many of these molecules have antiproliferative properties.<sup>12,13</sup> The first of these, 3-deoxydipalmitoyl-PI, was

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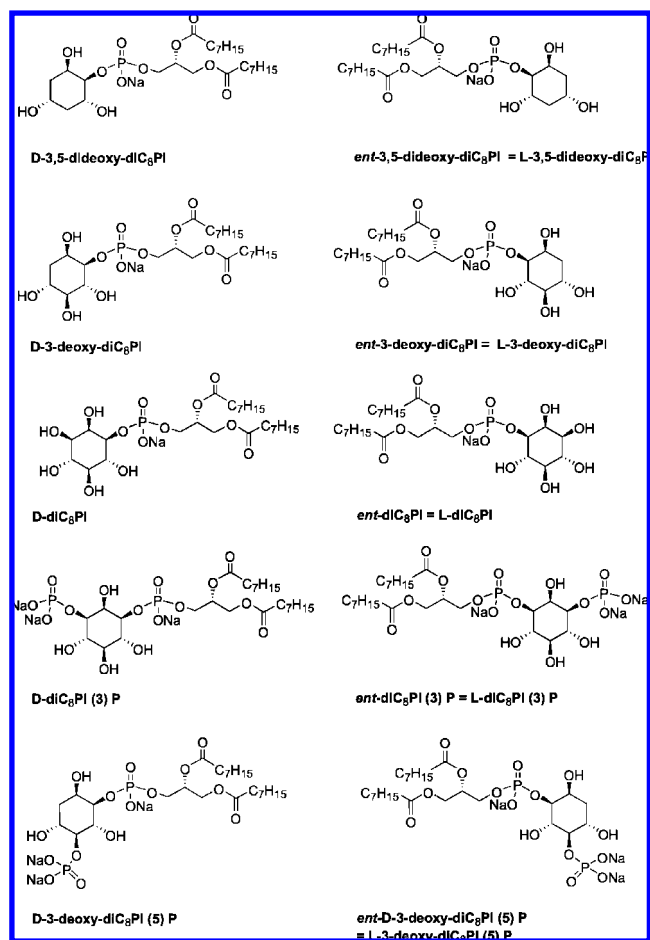
- (1) Vivanco, I.; Sawyers, C. L. *Nat. Rev. Cancer* **2002**, *2*, 489–501.
- (2) Seufferlein, T. *Int. J. Gastrointest. Cancer* **2002**, *31*, 15–21.
- (3) Ghosh, P. M.; Malik, S.; Bedolla, R.; Kreisberg, J. L. *Curr. Drug Metab.* **2003**, *4*, 487–496.
- (4) Barnett, S.; Bilodeau, M.; Lindsley, C. *Curr. Top. Med. Chem.* **2005**, *5*, 109–125.
- (5) Kandel, E. S.; Hay, N. *Exp. Cell Res.* **1999**, *253*, 210–229.
- (6) Qiao, L.; Nan, F.; Kunkel, M.; Gallegos, A.; Powis, G.; Kozikowski, A. P. *J. Med. Chem.* **1998**, *41*, 3303–3206.
- (7) Meuille, E. J.; Mahadevan, D.; Vankayalapati, H.; Berggren, M.; Williams, R.; Coon, A.; Kozikowski, A. P.; Powis, G. *Mol. Cancer Ther.* **2003**, *2*, 389–399.

- (8) Kim, D.; Dan, H. C.; Park, S.; Yang, L.; Liu, Q.; Kaneko, S.; Ning, J.; He, L.; Yang, H.; Sun, M.; Nicosia, S. V.; Cheng, J. Q. *Front. Biosci.* **2005**, *10*, 975–987.
- (9) Li, J.; Yen, C.; Liaw, D.; Podsypanina, K.; Bose, S.; Wang, S. I.; Puc, J.; Millaresis, C.; Rodgers, L.; McComble, R. *Science* **1997**, *275*, 1943–1947.
- (10) Steck, P. A.; Pershouse, M. A.; Jasser, S. A.; Ung, W. K.; Lin, H.; Ligon, A. *Nat. Genet.* **1997**, *15*, 356–362.
- (11) Gills, J. J.; Dennis, P. A. *Expert Opin. Invest. Drugs* **2004**, *13*, 787–797.
- (12) Kozikowski, A. P.; Kiddle, J. J.; Frew, T.; Berggren, M.; Powis, G. *J. Med. Chem.* **1995**, *38*, 1053–1056.
- (13) Kozikowski, A. P.; Sun, H.; Brognard, J.; Dennis, P. A. *J. Am. Chem. Soc.* **2003**, *125*, 1144–1145.

shown to inhibit growth of HT-29 human colon carcinoma cancer cells *in vitro* with a median inhibitory concentration ( $IC_{50}$ ) of  $35 \mu\text{M}$ .<sup>12</sup> Recent syntheses of ether-linked (rather than ester-linked) alkyl chains, e.g., D-3-deoxymyoinositol-1-[(R)-3-(hexadecyloxy)-2-hydroxypropyl hydrogen phosphate], have generated a newer class of PI analogues that should have greater stabilities *in vivo* and may also have slightly better delivery properties since they are more like lysophospholipids.<sup>14</sup> 3-Deoxy-PIs have also been shown to reduce drug resistance in human leukemia cell lines.<sup>15</sup> Thus, these PI analogues may have a future in the treatment of a variety of cancers.

The proposed mechanism for inhibition of cell growth by deoxy-PIs is rather curious. Previous work suggested that rather than inhibiting PI3K, the 3-deoxy-PI compounds inhibit the serine/threonine (Ser/Thr) protein kinase Akt by binding tightly to its PH domain [which normally binds PI(3,4)P<sub>2</sub> (i.e., PI-3,4-diphosphate) or PI(3,4,5)P<sub>3</sub>] and trapping it in the cytoplasm, thereby preventing phosphorylation by effector kinases.<sup>7</sup> The spectrum of changes in cells caused by these 3-deoxy-PI molecules differs from those of other widely studied cell-growth inhibitors.<sup>16</sup> Interestingly, in their patterns of activity they most resemble other lipid-based compounds (e.g., miltefosine and perifosine), which do not contain inositol moieties and also appear to inhibit Akt translocation and phosphorylation,<sup>17,18</sup> and are distinct from other compounds known to inhibit the PI3K/Akt pathway.

Significant synthetic effort has been directed toward modifying the D-inositol ring in order to improve its cytotoxicity,<sup>19</sup> but little has been done to explore broader changes in the stereochemistry of the inositol ring or to systematically employ further deoxygenation<sup>20,21</sup> in order to assess specific interactions of 3-deoxy-PI molecules with targets. Modulating inhibitor solubility by shortening the acyl chains, so that the inhibitor can exist as monomers, may also contribute to understanding the mechanisms of action of these lipids, since this modification is likely to alter uptake and localization in the cell. To this end, we have synthesized a series of 3-deoxydioctanoylphosphatidylinositol (3-deoxy-diC<sub>8</sub>PI) derivatives (Figure 1) with altered chirality of the inositol ring and/or additional modification (deoxygenation or phosphorylation) at the inositol C5 atom. Addition of phosphate at C5 was motivated by the possibility of producing an activator of PTEN, since other groups have shown that PIs with a phosphate group at that position kinetically activate PTEN.<sup>22</sup> These 3-deoxy-diC<sub>8</sub>PI molecules have been examined as substrates/inhibitors of PI3K, PI-specific phospholipase C $\delta$ 1, and PTEN (enzymes that could be inhibited by or degrade deoxy-diC<sub>8</sub>PI molecules in cells) and then screened as inhibitors of the U937 human leukemia cell line. Few of the 3-deoxy-PI compounds affected kinase or phospholipase activi-



**Figure 1.** Structures of the 3-deoxy-diC<sub>8</sub>PI compounds synthesized in this work.

ties at low concentrations relative to substrate. However, interesting trends were observed for PTEN inhibition by the D and L series of lipids that help delineate how this enzyme is likely to bind substrate molecules. Of all the compounds examined, only D-3-deoxy-diC<sub>8</sub>PI was cytotoxic to U937 cells, with an  $IC_{50}$  of  $40 \mu\text{M}$ , which is much less than the critical micelle concentration (CMC) of  $0.4 \text{ mM}$  and low enough that the compound had no effect on the recombinant enzymes examined. This very specific cytotoxicity profile for U937 cells is discussed in terms of the proposed target for this type of molecule.

## Experimental Section

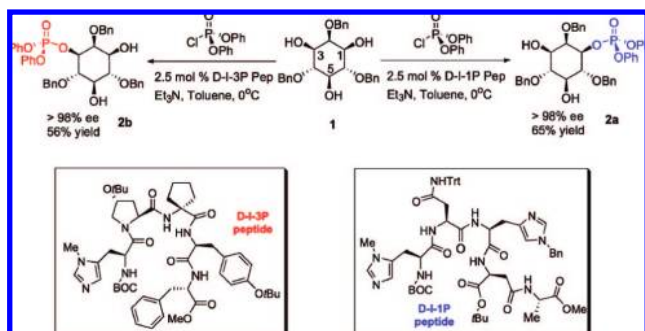
**Deoxy-diC<sub>8</sub>PI Lipids.** Peptide-catalyzed asymmetric phosphorylation (Scheme 1) was previously used in the total synthesis of PI-3-phosphate [PI(3)P] analogues.<sup>23</sup> This methodology in combination with a monodebenzylation–Mitsunobu sequence has also been applied in syntheses of some deoxygenated PI analogues in both enantiomeric series.<sup>24</sup> The 3-deoxygenated analogue of PI(5)P and its enantiomer [D- and L-3-deoxy-diC<sub>8</sub>PI(5)P, respectively] were prepared following the same strategy (Scheme 1) using protected D- and L-3-deoxy-diC<sub>8</sub>PI (obtained as a pair of diastereomers via steps a–g in Scheme 2) as the respective starting materials.

**Protected D-3-Deoxy-diC<sub>8</sub>PI(5)P.** To a stirred solution of benzyl-protected D-3-deoxy-diC<sub>8</sub>PI ( $0.020 \text{ g}$ ,  $0.022 \text{ mmol}$ ) in

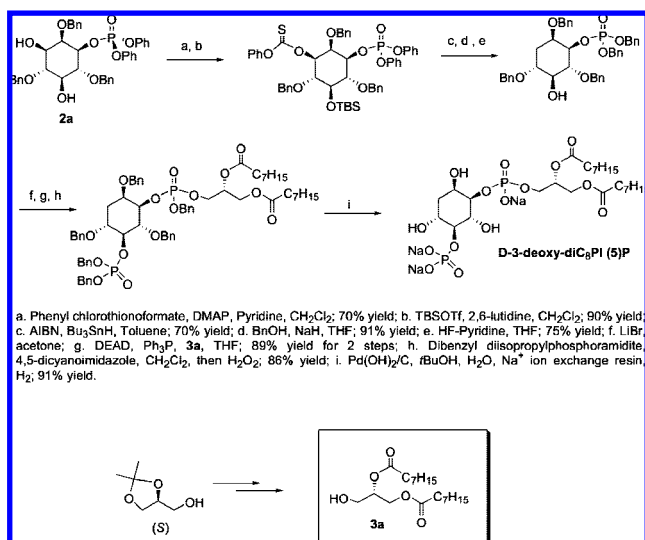
- (14) Andresen, T. L.; Skytte, D. M.; Madsen, R. *Org. Biomol. Chem.* **2004**, *2*, 2951–2957.  
 (15) Tabellini, G.; Tazzari, P. L.; Bortul, R.; Billi, A. M.; Cocco, L.; Martelli, A. M. *Br. J. Haematol.* **2004**, *126*, 574–582.  
 (16) Gills, J. J.; Holbeck, S.; Hollingshead, M.; Hewitt, S. M.; Kozikowski, A. P.; Dennis, P. A. *Mol. Cancer Ther.* **2006**, *5*, 713–722.  
 (17) Ruitter, G. A.; Zerp, S. F.; Bartelink, H.; van Bitterswijk, W. J.; Verheij, M. *Anti-Cancer Drugs* **2003**, *14*, 167–173.  
 (18) Kondapaka, S. B.; Singh, S. S.; Dasmahapatra, G. P.; Sausville, E. A.; Roy, K. K. *Mol. Cancer Ther.* **2003**, *2*, 1093–1103.  
 (19) Castillo, S. S.; Brognard, J.; Petukhov, P. A.; Zhang, C.; Tsurutani, J.; Granville, C. A.; Li, M.; Jung, M.; West, K. A.; Gills, J. J.; Kozikowski, A. P.; Dennis, P. A. *Cancer Res.* **2004**, *64*, 2782–2792.  
 (20) Lemieux, R. U. *Acc. Chem. Res.* **1996**, *29*, 373–380.  
 (21) Tor, Y. *ChemBioChem* **2003**, *4*, 998–1007.  
 (22) Campbell, R. B.; Liu, F.; Ross, A. H. *J. Biol. Chem.* **2003**, *278*, 33617–33620.

- (23) Sculimbrene, B. R.; Xu, Y.; Miller, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 13182–13183.  
 (24) Xu, Y.; Sculimbrene, B. R.; Miller, S. J. *J. Org. Chem.* **2006**, *71*, 4919–4928.

**Scheme 1.** General Scheme for the Synthesis of Different Enantiomers of Inositol Phosphates for Use in Generating diC<sub>8</sub>PI Derivatives



**Scheme 2.** Synthetic Scheme for Generation of D-3-Deoxy-diC<sub>8</sub>PI(5)P



CH<sub>2</sub>Cl<sub>2</sub> (4.0 mL) was added dibenzyl diisopropylphosphoramidite (0.14 mL, 0.43 mmol) followed by 4,5-dicyanoimidazole (0.063 g, 0.54 mmol). The reaction mixture was stirred at room temperature for 14 h and then cooled to 0 °C. An aliquot (2 mL) of 30% H<sub>2</sub>O<sub>2</sub> was added, and the mixture was stirred at 0 °C for an additional 1 h. The reaction was then quenched with saturated Na<sub>2</sub>SO<sub>3</sub> solution (10 mL) and the mixture extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 30 mL). The organic layers were combined, dried over sodium sulfate, and then concentrated under reduced pressure to afford a clear oil. The crude product was purified using silica gel flash chromatography [with Silica Gel 60 Å (32–63 μm)], eluting with a gradient of 0–55% ethyl acetate in hexanes to afford the pure product as a clear oil (0.022 g, 86% yield). <sup>1</sup>H NMR [CDCl<sub>3</sub>, 400 MHz, referenced to tetramethylsilane (TMS)]: δ 7.37–7.05 (m, 30H), 5.07 (m, 1H), 4.99–4.84 (m, 6H), 4.75 (m, 2H), 4.58–4.38 (m, 4H), 4.34–4.27 (m, 1H), 4.14–3.92 (m, 5H), 3.84 (m, 2H), 3.74 (dd, *J* = 5.6 and 6.0 Hz, 1H), 2.19 (m, 5H), 1.62 (m, 1H), 1.54 (m, 4H), 1.27 (m, 16H), 0.87 (m, 6H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz, referenced to TMS): δ 173.1, 173.0, 172.7, 138.4, 138.3, 138.2, 136.4, 136.3, 136.2, 136.1, 135.7, 135.6, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.4, 128.2, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 127.4, 82.4, 80.4, 78.8, 74.9, 74.4, 72.6, 72.5, 72.3, 69.8, 69.7, 69.6, 69.6, 69.5, 69.4, 69.3, 61.8, 34.4, 34.3, 32.0, 30.3, 29.4, 29.3, 29.2, 25.2, 22.9, 14.4. <sup>31</sup>P NMR (CDCl<sub>3</sub>, 121 MHz, referenced to an 85% H<sub>3</sub>PO<sub>4</sub> external standard): δ -0.28, -0.75, -0.85. IR (film) ν (cm<sup>-1</sup>): 2927, 2855, 1742, 1455, 1270, 1213, 1155, 1012, 737, 697. TLC (50% ethyl acetate in hexanes): *R*<sub>f</sub> 0.12. ESI<sup>+</sup>: calcd for [C<sub>67</sub>H<sub>84</sub>O<sub>15</sub>P<sub>2</sub> + H]<sup>+</sup>, *m/z* 1191.5364; found, 1191.5448. Optical rotation (recorded using a Rudolf Research

Analytical Autopol IV automatic polarimeter at the sodium D line with a path length of 50 mm): [α]<sub>D</sub> -3.4 (*c* 4.0, CHCl<sub>3</sub>).

**Protected L-3-Deoxy-diC<sub>8</sub>PI(5)P.** Synthesis and spectral data matched that given above for protected D-3-deoxy-diC<sub>8</sub>PI(5)P. Optical rotation: [α]<sub>D</sub> +3.1 (*c* 4.0, CHCl<sub>3</sub>).

**D-3-Deoxy-diC<sub>8</sub>PI(5)P.** To a stirred solution of protected D-3-deoxy-diC<sub>8</sub>PI(5)P (0.020 g, 0.017 mmol) in 5:1 *t*-BuOH/H<sub>2</sub>O (3 mL) was added sodium ion-exchange resin (Chelex 100 sodium form, 50–100 dry mesh, washed with H<sub>2</sub>O) followed by Pd(OH)<sub>2</sub>/C (20 mg, washed with H<sub>2</sub>O). The reaction mixture was then stirred at a H<sub>2</sub> pressure of 1 atm for 32 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated under reduced pressure and lyophilized to afford a white solid (0.011 g, 91% yield). <sup>1</sup>H NMR (D<sub>2</sub>O, 300 MHz): δ 5.12 (m, 1H), 4.26 (m, 1H), 4.10 (m, 2H), 3.90 (m, 2H), 3.82 (m, 1H), 3.74 (m, 1H), 3.63 (m, 2H), 2.22 (m, 4H), 1.93 (m, 1H), 1.42 (m, 5H), 1.11 (m, 16H), 0.68 (m, 6H). <sup>31</sup>P NMR (D<sub>2</sub>O, 121 MHz): δ 4.3, 0.5. ESI<sup>-</sup>: calcd for [C<sub>25</sub>H<sub>48</sub>O<sub>15</sub>P<sub>2</sub> - H]<sup>-</sup>, *m/z* 649.2390; found, 649.2360. Optical rotation: [α]<sub>D</sub> +3.4 (*c* 1.0, H<sub>2</sub>O at pH 9).

**L-3-Deoxy-diC<sub>8</sub>PI(5)P.** Synthesis and spectral data matched that given above for 3-deoxy-diC<sub>8</sub>PI(5)P. Optical rotation: [α]<sub>D</sub> -5.2 (*c* 1.0, H<sub>2</sub>O at pH 9).

All of the purified lipids were treated with Chelex to remove contaminating paramagnetic ions introduced via the Pd catalyst used in generating the final product. Concentrations of stock solutions were measured using <sup>31</sup>P NMR spectroscopy (202.3 MHz) by comparing phosphorus peak integrals (in the absence of <sup>1</sup>H decoupling) with the integral of a standard inorganic phosphate (Pi) peak.

**<sup>31</sup>P NMR Characterization of Phospholipids.** Most of the <sup>31</sup>P NMR spectra (202.3 MHz) were obtained using a Varian INOVA 500 spectrometer. Variation of the <sup>31</sup>P NMR line width of the synthetic PI as a function of lipid concentration (0.25–4 mM) was used to estimate CMCs for several of these molecules in 50% D<sub>2</sub>O containing 100 mM Tris HCl (pH 8.0) and 2 mM EDTA. In this concentration range, the <sup>31</sup>P line widths of most of these compounds typically decreased 3–4 Hz to a constant value when only monomers were present. The CMC was estimated from the intersection of the micelle/monomer line (negative slope) and the monomer-only line (zero slope) on a plot of line width versus reciprocal PI concentration. Assays of recombinant enzyme activities were also performed using <sup>31</sup>P NMR on the Varian INOVA 500 (details for each enzyme are given below).

High-resolution <sup>31</sup>P NMR field cycling at magnetic fields of 0.004–11.74 T was carried out using a home-built shuttling system on a Varian Unity Plus 500 spectrometer.<sup>25,26</sup> This technique was used to characterize two deoxy-diC<sub>8</sub>PI micelles and to explore whether 0.5 mM D-diC<sub>8</sub>PI(3)P formed detectable micelles when dispersed in the PTEN assay buffer. The sample, which was sealed in a standard 10 mm tube, was pneumatically shuttled to a higher position within or just above the magnet, where the magnetic field was between 0.06 and 11.7 T. Lower fields (down to 0.004 T for these samples) were accessible by shuttling the sample to a region outside the magnet, in the middle of a small Helmholtz coil located just above the top of the superconducting magnet. The spin-lattice relaxation rate, *R*<sub>1</sub>, at each field strength was measured using 6–8 programmed delay times.

**Recombinant Enzymes.** Recombinant p110α/p85α PI3K was purchased from Upstate. A chimeric rat phospholipase C (PLC) with the catalytic domain of PLCδ1 and the N-terminal PH domain of PLCβ1 was the gift of Dr. Suzanne Scarlata (State University of New York at Stony Brook). Recombinant *Listeria monocytogenes* PI-specific PLC without its signal sequence<sup>27,28</sup> was expressed and purified using the IMPACT-CN expression system (New England

(25) Roberts, M. F.; Redfield, A. G. *J. Am. Chem. Soc.* **2004**, *126*, 13765–13777.

(26) Roberts, M. F.; Redfield, A. G. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17066–17071.

(27) Goldfine, H.; Kolb, C. *Infect. Immun.* **1992**, *60*, 4059–4067.



Biolabs). The construction of pET28a PTEN has been described previously;<sup>29</sup> *Escherichia coli* BL21 (DE3) cells were used as the host for protein expression. PTEN protein was purified from the bacterial extracts using a Qiagen Ni<sup>2+</sup>-nitrilotriacetic acid agarose column according to the manufacturer protocol. Fractions of pure PTEN protein (as judged using SDS-PAGE) were combined and dialyzed against 100 mM Tris-HCl (pH 8.0). Protein concentration was determined by Lowry assay.<sup>30</sup>

**PI3K Assay.** PI3K (2  $\mu$ g) was added to a reaction mixture (400  $\mu$ L) containing 50 mM Tris-HCl (pH 7.5), 0.1 mg/mL bovine serum albumin (BSA) (Sigma), 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 2 mM ATP, 1 mM D-diC<sub>8</sub>PI and 0–3 mM 3-deoxy-diC<sub>8</sub>PI analogue. The mixture was incubated at 22 °C for 3 h, and then the reaction was stopped by addition of 10 mM EDTA. Phosphorylation of PI and PI analogues was monitored by <sup>31</sup>P NMR spectroscopy using the phosphodiester resonance ( $\delta_P$  0.23 at pH 7.5) as the standard for integration of the PI3K phosphomonoester product. The acquisition conditions followed those previously used for PLC assays.<sup>31,32</sup> The product yield was quantified by comparing the phosphomonoester resonance for D-diC<sub>8</sub>PI(3)P to the phosphodiester peak in the absence of <sup>1</sup>H decoupling.

**PLC Assay.** PLC activities toward diC<sub>8</sub>PI and the 3-deoxy-diC<sub>8</sub>PI analogues were measured using <sup>31</sup>P NMR spectroscopy after incubation for fixed times, as described for other PI-specific PLC enzymes.<sup>31,33</sup> The assay buffer for PLC $\delta$ 1 contained 50 mM Tris-HCl, 2 mM substrate PI, and 0.5 mM CaCl<sub>2</sub> (pH 7.5); the incubation temperature was 28 °C. For the recombinant *L. monocytogenes* PLC, the assay buffer was 50 mM Tris-HCl containing 0.5 mg/mL BSA (pH 7.0), and the incubation temperature was 25 °C. Enzyme (6.9–27.6  $\mu$ g of PLC $\delta$ 1 or 0.04–176  $\mu$ g of *L. monocytogenes* PLC) was added to each 200  $\mu$ L sample containing 2 mM substrate, and the incubation time was chosen to give less than 20% PI cleavage. The reaction was stopped by addition of 200  $\mu$ L of CHCl<sub>3</sub> to the sample. The *cyclo*-inositol-1,2-phosphate (cIP) and inositol-1-phosphate [(1)P] contents in the aqueous phase were quantified from the <sup>31</sup>P NMR spectrum using added glucose-6-phosphate as an internal standard.

**PTEN Assay.** Phosphatase assays were carried out in 50  $\mu$ L assay buffer [100 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 10 mM dithiothreitol (DTT)]. The phosphatase reaction was initiated by adding ~25  $\mu$ g of purified PTEN (10  $\mu$ M) and quenched by adding malachite green reagent containing 1 M HCl after a 20 min incubation at 37 °C.<sup>34</sup> Comparison of observed A<sub>660</sub> changes with those for standard Pi samples was used to calculate the reaction rate. In most of the assays, 0.5 mM D-diC<sub>8</sub>PI(3)P was used as the substrate, and various concentrations (0.05–2 mM) of synthetic short-chain PIs were added to test their effect on phosphatase activity. For estimation of the K<sub>m</sub> value for D-diC<sub>8</sub>PI(3)P, assays were carried out in 100  $\mu$ L aliquots containing six different concentrations (0.05–1.6 mM) of substrate. Most of the assays were done at least in duplicate.

**U937 Cell Growth and Incubation with PI Analogues.** The U937 human leukemic monocyte lymphoma cell line was obtained from the American Type Culture Collection (Manassas, VA). U937 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100  $\mu$ g/mL), 2-mercaptoethanol (50  $\mu$ M), and glutamine (2 mM) at cell densities of 0.5–1  $\times$  10<sup>6</sup> cells/mL at 37 °C in a 5% CO<sub>2</sub>

atmosphere at 95% humidity. Cells were cultured in the presence or absence of the short-chain diC<sub>8</sub>PI compounds at the indicated concentration. Cells were also incubated with 10  $\mu$ M LY294002 and/or 20 nM wortmannin. At the appropriate time, 2–5  $\times$  10<sup>5</sup> cells were collected by centrifugation at 400g for 8 min, washed in FACS buffer [1 $\times$  phosphate-buffered saline (PBS) containing 0.5% BSA and 0.01% sodium azide], and resuspended in FACS buffer containing 5  $\mu$ g/mL propidium iodide. Samples were incubated on ice for 10 min and then analyzed by flow cytometry using a BD FACSCanto flow cytometer with BD FACS Diva software (BD Biosciences, San Jose, CA).

**Preparation of Cell Extracts and Western Blot Analysis.** Cells were washed twice in PBS, incubated for 20 min at ~10<sup>8</sup> cells/mL in RIPA buffer containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride protein inhibitor cocktail, 1 mM okadaic acid, 1 mM sodium fluoride, and 10 mM  $\beta$ -glycerophosphate, and subjected to three freeze/thaw cycles in dry ice. Lysates were centrifuged at 14000g for 15 min to remove insoluble material, and then protein (20  $\mu$ g/lane) was separated by polyacrylamide–SDS gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked for 1 h in TBS-T [20 mM Tris (pH 7.6), 137 mM NaCl, and 0.05% Tween-20] containing 5% nonfat dry milk and then incubated at 4 °C overnight in TBS-T with 1  $\mu$ g/mL primary antibody (Ab). The membrane was washed several times in TBS-T, incubated for 60 min with a 1:2500 dilution of anti-rabbit IgG-coupled horseradish peroxidase Ab, and developed using enhanced chemiluminescence. Bands for the different phosphorylated proteins were quantified by densitometric analysis using the band corresponding to an extract from cells incubated with L-3-deoxy-diC<sub>8</sub>PI as a control, since this compound had no effect on the U937 cells.

## Results

**Characterization of diC<sub>8</sub>PI Compounds.** Three target enzymes that might be inhibited by (PI3K and PTEN) or degrade (PLC) 3-deoxy-PI molecules are soluble enzymes whose natural substrates are membrane constituents. However, in detailed kinetic studies with synthetic short-chain phospholipid substrates, these types of enzymes typically display a preference for molecules aggregated into a micelle as opposed to monomers in solution, a phenomenon termed “interfacial activation”. Both PLC and PI3K exhibit this enhanced activity with interfacial substrates/inhibitors.<sup>35,36</sup> Since the short-chain 3-deoxy-diC<sub>8</sub>PI molecules, unlike the previously examined synthetic 3-deoxy-PI molecules with longer acyl chains,<sup>7,14</sup> can exist as both monomers and micelles in solution, we require information on their physical states, in particular, values of the CMC (the concentration above which micelles form) and rough estimates of the sizes of the micelles they form. This information is also critical for determining the distribution of the 3-deoxy-diC<sub>8</sub>PI species in cells at concentrations where they may cause cell death. All of the diC<sub>8</sub>PI derivatives used in this work have CMC values between 0.4 and 0.7 mM in 100 mM Tris HCl (pH 8), as measured by <sup>31</sup>P line-width changes as a function of lipid concentration (Table 1).

To get a sense of the sizes and dynamics of these micelles, two of the synthetic PIs, L-3-deoxy-diC<sub>8</sub>PI and L-3,5-dideoxy-diC<sub>8</sub>PI, were examined at 3 mM using high-resolution <sup>31</sup>P field cycling. This is a novel technique that is very sensitive to the aggregation state of the micelles.<sup>25,26</sup> The L-isomers of the deoxy-diC<sub>8</sub>PIs were chosen because they had CMC values

(28) Ryan, M.; Zaikova, T. O.; Keana, J. F.; Goldfine, H.; Griffith, O. H. *Biophys. Chem.* **2002**, *101–102*, 347–358.

(29) Das, S.; Dixon, J. E.; Cho, W. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7491–7496.

(30) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.

(31) Zhou, C.; Wu, Y.; Roberts, M. F. *Biochemistry* **1997**, *36*, 347–355.

(32) Feng, J.; Bradley, W. D.; Roberts, M. F. *J. Biol. Chem.* **2003**, *278*, 24651–24657.

(33) Wu, Y.; Perisic, O.; Williams, R. L.; Katan, M.; Roberts, M. F. *Biochemistry* **1997**, *36*, 11223–11233.

(34) Itaya, K.; Ui, M. *Clin. Chim. Acta* **1966**, *14*, 361–366.

(35) Lewis, K. A.; Garigapati, V. R.; Zhou, C.; Roberts, M. F. *Biochemistry* **1993**, *32*, 8836–8841.

(36) Barnett, S. F.; Ledder, L. M.; Stirdivant, S. M.; Ahern, J.; Conroy, R. R.; Heimbrook, D. C. *Biochemistry* **1995**, *34*, 14254–14262.

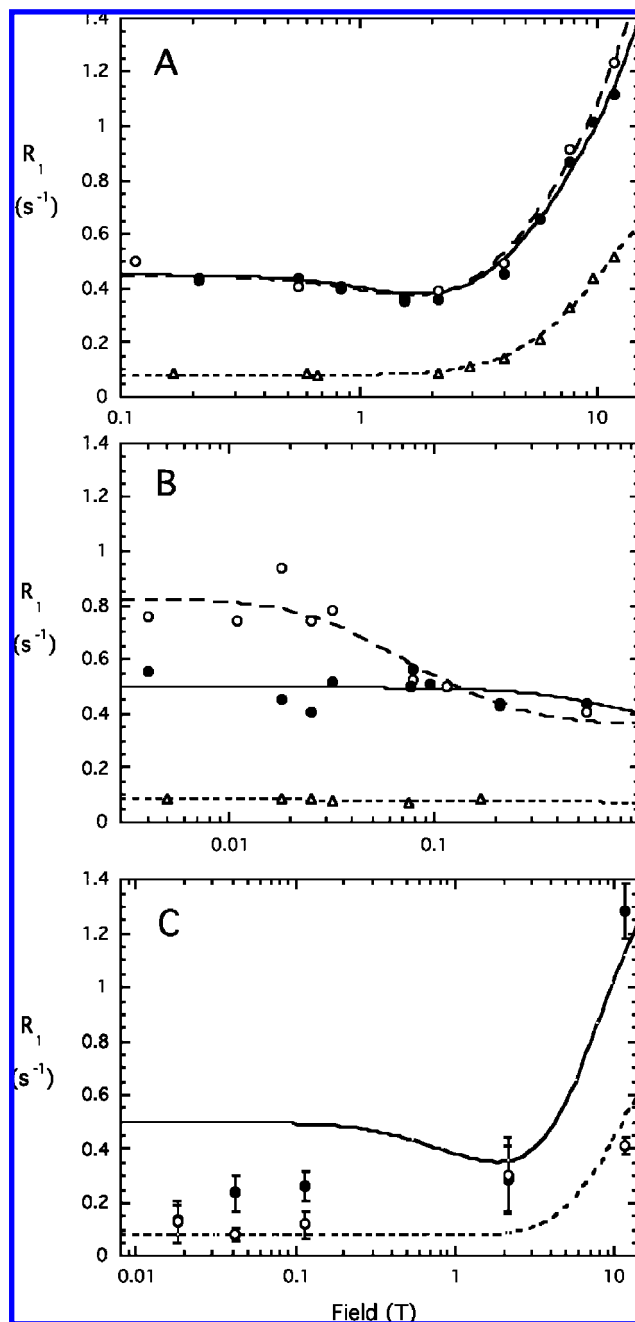
**Table 1.** CMC Values and  $IC_{50}$  Values in Recombinant PTEN Hydrolysis of D-3-Deoxy-diC<sub>8</sub>PI(3)P for diC<sub>8</sub>PI Compounds

phospholipid	CMC (mM) <sup>a</sup>	PTEN $IC_{50}$ (mM) <sup>b</sup>
D-diC <sub>8</sub> PI	0.5 ± 0.1	0.43
L-diC <sub>8</sub> PI	0.5 ± 0.1	>2.5
D-diC <sub>8</sub> PI(3)P	0.7 ± 0.2	—
D-3-deoxy-diC <sub>8</sub> PI	0.42 ± 0.08	0.23
L-3-deoxy-diC <sub>8</sub> PI	0.40 ± 0.07	1.5
D-3,5-dideoxy-diC <sub>8</sub> PI	0.47 ± 0.05	0.86
L-3,5-dideoxy-diC <sub>8</sub> PI	0.47 ± 0.07	0.38
D-3-deoxy-diC <sub>8</sub> PI(5)P	0.61 ± 0.15	0.47
L-3-deoxy-diC <sub>8</sub> PI(5)P	—	>2.5

<sup>a</sup> Determined by analysis of <sup>31</sup>P NMR line widths in D<sub>2</sub>O. <sup>b</sup> Determined using a concentration of 0.5 mM for the D-3-deoxy-diC<sub>8</sub>PI(3)P substrate.

comparable to those of their enantiomers and greater amounts of them were available. The field dependence of the <sup>31</sup>P  $R_1$  from 0.1 to 11.74 T for phospholipid aggregates can be analyzed in terms of a contribution from three terms: (i) dipolar relaxation associated with  $\tau_c$ , the correlation time for a “slower” motion, and a relaxation rate extrapolated to zero field,  $R_c(0)$ , that is proportional to  $\tau_c$  and inversely proportional to  $r_{PH}^6$ , where  $r_{PH}$  is the effective distance of the phosphorus nucleus from the protons that it; (ii) chemical shift anisotropy (CSA) relaxation associated with the same slower-motion correlation time; and (iii) CSA relaxation due to a “faster” motion whose dipolar counterpart would be negligibly small in the analysis of these data.  $R_1$  is then the sum of these three terms.<sup>25</sup> The value of  $\tau_c$  is determined directly, while that of the faster-motion correlation time,  $\tau_{hf}$ , can be extracted from coefficients in the fits.<sup>25</sup> In samples where phospholipid vesicles or large, rod-shaped micelles form [e.g., diheptanoylphosphatidylcholine (diC<sub>7</sub>PC)], a distinct rise in  $R_1$  is detected at very low fields (<0.06 T).<sup>26</sup> When the data are well-separated from the nanosecond dispersion, yet another correlation time,  $\tau_v$ , which may reflect the contribution of particle tumbling to relaxation, can be extracted. It has been proposed that  $\tau_v$  is equal to the rotational correlation time  $D_r/6$ , where  $D_r$  is the rotational diffusion constant of an individual lipid due to both diffusion of the entire aggregate and translational diffusion of individual lipids relative to the aggregate.<sup>26</sup>

For micelles as opposed to vesicles, a detailed analysis is complicated by the facts that monomers and micelles coexist via a fast-exchange process and that many micelles are rod-shaped rather than spherical. Nonetheless, the line shape of the  $R_1$ -versus-field profile can indicate whether micelles have formed and whether they are large (producing an observable low-field rise in  $R_1$ ) or small (in which case a minimum in  $R_1$  at 1–2 T, suggesting a correlation time of several nanoseconds, is detected instead of a low-field rise). If the compound exists in solution as a monomer or very small aggregates (clusters containing fewer than 10 molecules), a single subnanosecond correlation time is likely to describe the behavior, and  $R_1$  is invariant for fields below 2 T. This type of assay for micelles works well in complex buffers, where the additional components can interfere with other methods for detecting micelle formation (e.g., impurities can skew surface tension measurements, and dyes that fluoresce when partitioned into micelle environments can influence the measured CMC). The control for monomeric phospholipids was 10 mM dibutylphosphatidylcholine (diC<sub>4</sub>PC), whose CMC is greater than 150 mM.<sup>37</sup> This monomer lipid clearly exhibited a rise in  $R_1$  as the field was increased



**Figure 2.** <sup>31</sup>P field cycling profiles for deoxy-diC<sub>8</sub>PI compounds. Dependence of the spin-lattice relaxation rate,  $R_1$ , on magnetic field for 3 mM L-3-deoxy-diC<sub>8</sub>PI (●), 3 mM L-3,5-dideoxy-diC<sub>8</sub>PI (○), and 10 mM diC<sub>4</sub>PC (Δ) in 50 mM HEPES (pH 7.5) over the field ranges (A) 0.1–11.74 and (B) 0.03–1 T. The solid, long-dashed, and short-dashed lines are the respective fits to the data. (C)  $R_1$  for the phosphodiester <sup>31</sup>P (●) and phosphate monoester (○) on the inositol C(3) of 0.5 mM D-diC<sub>8</sub>PI(3)P dissolved in PTEN assay buffer. The solid line indicates the relaxation profile for micellar L-3-deoxy-diC<sub>8</sub>PI while the dashed line indicates field dependence for the diC<sub>4</sub>PC monomer.

above 2 T (Figure 2A). This profile could be fit using a single correlation time of  $0.75 \pm 0.08$  ns. The maximum dipolar contribution extrapolated from the constant low-field contribution was  $R_c(0) = 0.077 \pm 0.006$  s<sup>-1</sup>. Both of the deoxy-diC<sub>8</sub>PI compounds showed higher  $R_1$  values whose profiles could not be well-fit using a single correlation time; this behavior is indicative of aggregate formation (Figure 2A). On the basis of the simplistic but useful model-free analysis described previ-

(37) Bian, J.; Roberts, M. F. J. *Colloid Interface Sci.* **1992**, *153*, 420–428.

ously,<sup>25</sup> L-3-deoxy-diC<sub>8</sub>PI at this concentration was characterized by slower- and faster-motion correlation times of  $\tau_c = 1.6 \pm 0.5$  ns and  $\tau_{\text{hf}} = 290 \pm 170$  ps, respectively; L-3,5-dideoxy-diC<sub>8</sub>PI exhibited similar behavior, with a  $\tau_c = 1.7 \pm 0.5$  ns and  $\tau_{\text{hf}} = 500 \pm 340$  ps. Although the fast motions are not well-defined, they had to be included in order to obtain reasonable fits to the data.

The nanosecond-range values of  $\tau_c$  clearly indicate that micelles formed for these concentrations of L-3-deoxy-diC<sub>8</sub>PI and L-3,5-dideoxy-diC<sub>8</sub>PI. The further-enhanced relaxation rate below 0.1 T can be used to provide an average particle size using high-resolution field cycling. Below 0.1 T,  $R_1$  for L-3-deoxy-diC<sub>8</sub>PI was invariant but that for the dideoxy-PI analogue increased with decreasing field (Figure 2B). The correlation time for the L-3,5-dideoxy-diC<sub>8</sub>PI low-field dispersion was  $\tau_v = 55 \pm 27$  ns. For spherical micelles, this would correspond to a radius of 38 Å. For comparison, rod-shaped diC<sub>7</sub>PC micelles exhibit  $\tau_v$  values of 200–350 ns (depending on concentration) for the low-field dispersion.<sup>38</sup> Clearly, the anionic deoxy-diC<sub>8</sub>PI micelles are considerably smaller. Since L-3-deoxy-diC<sub>8</sub>PI did not exhibit low-field dispersion, its micelles must be even smaller in size and likely are similar to those of diC<sub>6</sub>PC, which contain 20–25 molecules per micelle and do not show a distinct low-field dispersion.<sup>38</sup> Thus, when a second hydroxyl group was removed from the inositol headgroup, the micelles formed were larger but not as large as diC<sub>7</sub>PC micelles.

In the PI3K and PLC assays, the substrate D-diC<sub>8</sub>PI was present at 1 or 2 mM, both of which are concentrations greater than the CMC. Adding more diC<sub>8</sub>PI analogues at 1 mM or higher concentrations should increase the micellar fraction in solution. Given the field-cycling results for the two deoxy-PI compounds, all of the mixed micelles with diC<sub>8</sub>PI are likely to be relatively small compared with short-chain PC micelles. This information on the physical state of the short-chain PI analogues in assay buffers is important for understanding their interactions with enzymes that may show enhanced activity with interfacial substrates/inhibitors.

For PTEN assays, a concentration of 0.5 mM D-diC<sub>8</sub>PI(3)P was optimal for detection of inhibition by the different deoxy-PI compounds. This is close to the CMC for this compound, although the CMC values measured for the phosphorylated lipids D-diC<sub>8</sub>PI(3)P (the substrate for PTEN) and D-3-deoxy-diC<sub>8</sub>PI(5)P were not very accurate because the changes in their line widths with concentration were small (<2 Hz). To assess whether micelles were present in assay mixtures of substrate in the absence of protein, we determined  $R_1$  for 0.5 mM D-diC<sub>8</sub>PI(3)P dispersed in the PTEN assay buffer at several field strengths (Figure 2C). This low concentration taxed the sensitivity of the field-cycling system, so a complete field-dependence profile could not be obtained. Nevertheless, the results still provide insight into the state of D-diC<sub>8</sub>PI(3)P in solution under the conditions used in the PTEN assays. The phosphorus atoms in the phosphodiester group and the inositol C3 phosphate group of D-diC<sub>8</sub>PI(3)P both exhibit significant CSA relaxation at higher fields and decreases in  $R_1$  as the field decreases. The phosphodiester resonance demonstrates behavior intermediate between those of monomeric and aggregated phospholipids, while the low-field data for the C3 phosphomonoester resemble those for a monomer. The higher limiting value of  $R_1$  at low fields

for the phosphodiester compared with the phosphomonoester could reflect the contribution to  $R_1$  of the larger number of protons in the  $-\text{CH}_2\text{O}-\text{PO}_2-\text{OCH}-$  group as opposed to the  $-\text{CHOPO}_3$  group. Of greater importance, direct comparison of the D-diC<sub>8</sub>PI(3)P phosphodiester <sup>31</sup>P profile to that of the phosphodiester in 3-deoxy-diC<sub>8</sub>PI strongly suggests that some small micelles as well as monomers of the substrate may be present at the substrate concentration of 0.5 mM used in the PTEN assay mixture. Since all of the diC<sub>8</sub>PI compounds have similar CMCs, addition of inhibitors to a fixed substrate concentration (0.5 mM) is likely to increase the proportion of micelles. However, all of the inhibitors should have the same effect since their CMCs are essentially the same.

**Effect of PLC Enzymes on 3-Deoxy-diC<sub>8</sub>PI Molecules.** PI-specific PLC enzymes cleave PI molecules in two steps: (i) initial formation of diacylglycerol and the water-soluble product cIP followed by (ii) hydrolysis of cIP to I(1)P.<sup>39–41</sup> The initial 3-deoxy-PI studied as a cell-growth inhibitor, D-3-deoxydi-palmitoyl-PI, did not appear to be a substrate for PLC.<sup>13</sup> However, that phospholipid has a high gel-to-liquid-crystalline phase-transition temperature and may not have been presented in structures accessible to phospholipases in the in vitro assays. By using D-diC<sub>8</sub>PI micelles as the standard substrate, we can quantify the relative extents of cleavage of the 3-deoxy-diC<sub>8</sub>PI substrates and also examine their abilities to inhibit the enzyme, which reflect their abilities to bind to PLC active sites. We examined the 3-deoxy-PI molecules as substrates and inhibitors of two different PI-specific PLC enzymes: Ca<sup>2+</sup>-dependent mammalian PLC $\delta$ 1 (this is a chimera of the PLC $\delta$ 1 catalytic domain and the PLC $\beta$ 1 PH domain and was chosen because it has moderately high activity in vitro<sup>42</sup>) and the Ca<sup>2+</sup>-independent bacterial PLC from *L. monocytogenes*. Mechanistically, PI cleavage occurs in a similar fashion in both types of enzymes, except that an arginine replaces the active-site Ca<sup>2+</sup> in the bacterial enzyme.<sup>39,40</sup> As the data in Table 2 show, removal of the hydroxyl group at C3 generated a very poor substrate for the PLC $\delta$ 1 enzyme, with 0.5–1% of the PI cleavage observed for diC<sub>8</sub>PI. However, in the context of a cell over the time course of several days, these lipids are likely to be hydrolyzed by endogenous PLC enzymes.

These compounds were also poor inhibitors of PLC $\delta$ 1. The ratio of cIP to the final water-soluble product I(1)P reflects how well the intermediate is bound to the enzyme. A more tightly binding cIP generated in situ translates to a smaller cIP/I(1)P ratio.<sup>33,33,43</sup> As hydroxyl groups are removed from the inositol ring, the intermediate cIP analogue becomes more hydrophobic, and its release is slow compared to attack by water and production of I(1)P (Table 2). In the case of D-3,5-dideoxy-diC<sub>8</sub>PI, no cIP was observed, suggesting that the enzyme must hold the cyclic intermediate sufficiently long that it is always hydrolyzed (but very slowly). Addition of a phosphate at C5 to produce D-3-deoxy-diC<sub>8</sub>PI(5)P did not generate a better substrate but did bias the enzyme so that cIP became the dominant product

(38) Shi, X.; Shao, C.; Zhang, X.; Zambonelli, C.; Redfield, A. G.; Head, J. F.; Seaton, B. A.; Roberts, M. F. *J. Biol. Chem.*, submitted for publication, 2008.

(39) Griffith, O. H.; Ryan, M. *Biochim. Biophys. Acta* **1999**, *1441*, 237–254.

(40) Hondal, R. J.; Zhao, Z.; Kravchuk, A. V.; Liao, H.; Riddle, S. R.; Yue, X.; Bruzik, K. S.; Tsai, M. D. *Biochemistry* **1998**, *37*, 4568–4580.

(41) Heinz, D. W.; Essen, L. O.; Williams, R. L. *J. Mol. Biol.* **1998**, *275*, 635–650.

(42) Feng, J.; Roberts, M. F.; Drin, G.; Scarlata, S. *Biochemistry* **2005**, *44*, 2577–2584.

(43) Liu, Y.; Bruzik, K. S.; Ananthanarayanan, B.; Cho, W. *Bioorg. Med. Chem.* **2003**, *11*, 2471–2475.



**Table 2.** Recombinant PLC $\delta$ 1 Activity toward diC<sub>8</sub>PI and Deoxy-diC<sub>8</sub>PI Lipids

substrate <sup>a</sup>	inhibitor (c) <sup>b</sup>	cIP/I(1)P	specific activity <sup>c</sup> ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	relative activity
D-diC <sub>8</sub> PI		0.93	3.68	1.00
D-3-deoxy-diC <sub>8</sub> PI		0.22	0.039	0.011
D-3,5-dideoxy-diC <sub>8</sub> PI		0.00	0.018	0.005
D-3-deoxy-diC <sub>8</sub> PI(5)P		3.01	0.022	0.006
D-diC <sub>8</sub> PI	D-3-deoxy-diC <sub>8</sub> PI (2)	1.99	3.01	0.82
D-diC <sub>8</sub> PI	D-3-deoxy-diC <sub>8</sub> PI(5)P (6)	1.29	4.81	1.31
D-diC <sub>8</sub> PI	D-3,5-dideoxy-diC <sub>8</sub> PI (2)	1.77	3.25	0.88
D-diC <sub>8</sub> PI	D-3,5-dideoxy-diC <sub>8</sub> PI (6)	0.99	3.93	1.07
D-diC <sub>8</sub> PI	L-diC <sub>8</sub> PI (2)	1.02	3.08	0.84

<sup>a</sup> Substrates present at 2 mM. <sup>b</sup> c is the concentration of inhibitor used (mM). <sup>c</sup> Errors in specific activities were typically <20%.

**Table 3.** Recombinant *L. monocytogenes* PI–PLC activity toward D-diC<sub>8</sub>PI Molecules and Inhibition by Deoxy-diC<sub>8</sub>PI Lipids

substrate <sup>a</sup>	inhibitor (c) <sup>b</sup>	specific activity ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ) <sup>c</sup>	relative activity
D-diC <sub>8</sub> PI		489	1.00
D-3-deoxy-diC <sub>8</sub> PI		6.44	0.013
D-3,5-dideoxy-diC <sub>8</sub> PI		0.18	$3.7 \times 10^{-4}$
D-diC <sub>8</sub> PI	D-3-deoxy-diC <sub>8</sub> PI (2)	522	1.07
D-diC <sub>8</sub> PI	D-3-deoxy-diC <sub>8</sub> PI(5)P (6)	452	0.92
D-diC <sub>8</sub> PI	D-3,5-dideoxy-diC <sub>8</sub> PI (2)	522	1.07
D-diC <sub>8</sub> PI	D-3,5-dideoxy-diC <sub>8</sub> PI (6)	539	1.10
D-diC <sub>8</sub> PI	L-diC <sub>8</sub> PI (2)	441	0.90

<sup>a</sup> Substrates present at 2 mM. <sup>b</sup> c is the concentration of inhibitor used (mM). <sup>c</sup> Errors in specific activities were  $\leq 15\%$ .

(compared with hydrolysis of D-3-deoxy-diC<sub>8</sub>PI). This result is an interesting contrast to that for PLC $\delta$ 1 hydrolysis of phosphorylated glycerophosphoinositols,<sup>33</sup> where the phosphorylated substrate was more efficiently hydrolyzed and I(1)P became the major product, and strongly indicates that the 3-hydroxy group of the inositol ring must make important hydrogen-bonding contacts with the enzyme that stabilize binding of PI analogues to the protein in a configuration that is not optimal for PI cleavage.

Mammalian PLC enzymes have multiple domains that could complicate and/or mask the effects of the deoxy-diC<sub>8</sub>PI compounds. Therefore, for comparison we also examined the effect of these compounds on the PLC from *L. monocytogenes*. This bacterial PLC is essentially the catalytic domain of the mammalian enzymes and serves to assess the effects of the deoxy-PI compounds on catalysis only. The 3-deoxy-diC<sub>8</sub>PI compounds were also poor substrates and poor inhibitors for *L. monocytogenes* PLC (Table 3). The specific activities toward D-3-deoxy-diC<sub>8</sub>PI and D-3,5-dideoxy-diC<sub>8</sub>PI were 1.3 and 0.037%, respectively, of that toward diC<sub>8</sub>PI. Addition of 2–6 mM deoxy-PI compounds had only minor effects on the activity of *L. monocytogenes* PLC toward 2 mM D-diC<sub>8</sub>PI. For all of the deoxy compounds, the decrease in specific activity was less than 10%, which was comparable to the effect of L-diC<sub>8</sub>PI. As with the mammalian enzyme, removal of the 3-hydroxyl group generates a compound that binds very poorly to PLC and does not compete well with substrate.

**Effect of 3-Deoxy-diC<sub>8</sub>PI Molecules on PI3K Activity.** Removal of the 3-hydroxyl group from PI generates a molecule that should inhibit PI3K phosphorylation of D-diC<sub>8</sub>PI. Previous work<sup>7</sup> has suggested that 3-deoxy-PI species are not very potent inhibitors of PI3K, although this can be misleading if the substrate and inhibitor have different chain lengths or solubilities. In an attempt to keep the substrate and the inhibitors in the same physical state, we investigated the activity of the

**Table 4.** Recombinant PI3K (p110 $\alpha$ /p85 $\alpha$ ) Activity toward D-diC<sub>8</sub>PI and Inhibition by L- and D-Deoxy-diC<sub>8</sub>PI Lipids<sup>a</sup>

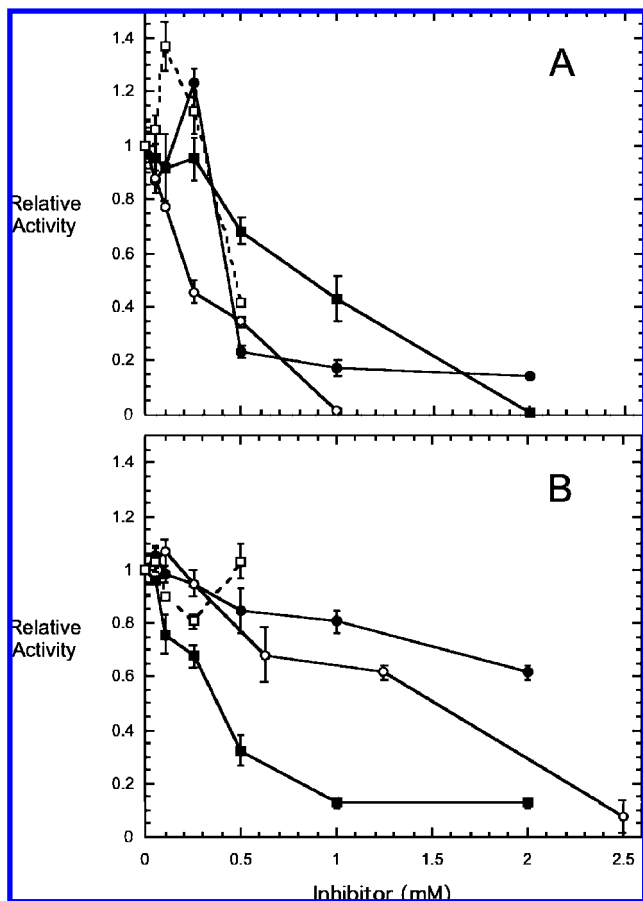
inhibitor	specific activity <sup>b</sup> ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	relative activity
none	0.60	1.00
D-3-deoxy-diC <sub>8</sub> PI	0.09	0.15
D-3-deoxy-diC <sub>8</sub> PI(5)P	0.49	0.81
L-diC <sub>8</sub> PI	0.60	1.00
L-3-deoxy-diC <sub>8</sub> PI	0.11	0.18

<sup>a</sup> Assay conditions: 1 mM D-diC<sub>8</sub>PI and 2 mM ATP as substrates, 3 mM inhibitor, and 5 mM Mg<sup>2+</sup> in 50 mM Tris HCl (pH 7.5). <sup>b</sup> For several of the samples run in duplicate, the errors in determining the specific activities were <10%.

p110 $\alpha$ /p85 $\alpha$  complex toward 1 mM D-diC<sub>8</sub>PI in the absence and presence of 3 mM 3-deoxy-diC<sub>8</sub>PI analogues (the high deoxy-PI concentration was chosen to maximize any observation of inhibition). Since PI3K has been shown to work in a scooting mode with PI dispersed in vesicles,<sup>36</sup> the concentrations of PIs were chosen so that the substrate (and in most cases the inhibitor) was micellar. In fact, attempts to study the kinetics with 0.3 or 0.5 mM substrate and comparable concentrations of the deoxy-PI molecules exhibited either no effect or an increase in activity (along with a high degree of error), likely as a result of micelle formation and better presentation of substrate. As shown in Table 4, both D- and L-3-deoxy-diC<sub>8</sub>PI inhibited the phosphorylation of diC<sub>8</sub>PI; removing a second hydroxyl group (data not shown) or adding a phosphate to the 5-hydroxyl group reduced the potency of these compounds as inhibitors, so very little inhibition was observed under these conditions of excess inhibitor. For comparison, we also examined the inhibitory effect of L-diC<sub>8</sub>PI on p110 $\alpha$ /p85 $\alpha$  activity toward D-diC<sub>8</sub>PI. The substrate enantiomer had no effect on D-diC<sub>8</sub>PI phosphorylation, indicating that the observed inhibition by the 3-deoxy-diC<sub>8</sub>PI molecules is not just surface-dilution inhibition but represents the effect of real binding of the enzyme with PI3K. Deoxygenation at C3 of the inositol ring of diC<sub>8</sub>PI not only improved the binding of 3-deoxy-diC<sub>8</sub>PI compounds to PI3K (compared with diC<sub>8</sub>PI) but also eliminated the inositol ring stereoisomer selectivity for ligand binding. A phosphorylated 3-deoxy-diC<sub>8</sub>PI [D-3-diC<sub>8</sub>PI(5)P] was also examined for inhibition of diC<sub>8</sub>PI phosphorylation by PI3K. Enzyme activity decreased only 20% with an excess of D-3-diC<sub>8</sub>PI(5)P. These in vitro assays suggest that the 3-deoxy-diC<sub>8</sub>PI molecules, especially when monomeric, are unlikely to have a large effect on PI3K in vivo. They also suggest that if PI3K is involved in vivo, both D- and L-3-deoxy-diC<sub>8</sub>PI should have similar effects as growth inhibitors.

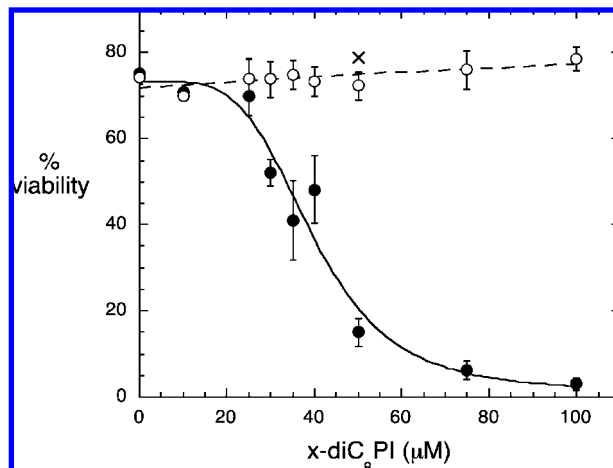
**Effect of 3-Deoxy-diC<sub>8</sub>PI Molecules on PTEN Activity.** Although PTEN is mutated in some tumor cells, in others it could be a potential target for inhibition by the short-chain





**Figure 3.** Effect of (A) D and (B) L enantiomers of diC<sub>8</sub>PI derivatives on the activity of PTEN toward 0.5 mM D-diC<sub>8</sub>PI(3)P: diC<sub>8</sub>PI (●), 3-deoxy-diC<sub>8</sub>PI (○), 3,5-dideoxy-diC<sub>8</sub>PI (■), and 3-deoxy-diC<sub>8</sub>PI(5)P (□).

3-deoxy-diC<sub>8</sub>PI molecules. This would not be productive if the aim of the treatment is to induce death of the tumor cells. To check this, we examined all of the different 3-deoxy-PI molecules as inhibitors of PTEN (Figure 3). The substrate in this case is D-diC<sub>8</sub>PI(3)P at 0.5 mM, a concentration in these assay mixtures at which there is a mixture of monomers and some small micelles. The dependence of the specific activity on the concentration of this substrate exhibited an apparent  $K_m$  of  $0.20 \pm 0.07$  mM and a  $V_{max}$  of  $14 \pm 1$  nmol min<sup>-1</sup> mg<sup>-1</sup> at 37 °C. The different 3-deoxy-PI compounds as well as diC<sub>8</sub>PI at concentrations of 0.05–2.5 mM were examined as inhibitors of PTEN, and the measured IC<sub>50</sub> values are given in Table 1. At inhibitor concentrations greater than 0.5 mM, all of the synthetic PI molecules were inhibitory, although the L enantiomers (Figure 3B) usually were poorer inhibitors than the D enantiomers (Figure 3A). The compound D-3-deoxy-diC<sub>8</sub>PI was the most potent inhibitor toward 0.5 mM substrate, with an IC<sub>50</sub> of 0.23 mM under these conditions; this is consistent with tighter binding than that reflected in the apparent  $K_m$  of the substrate. Two very interesting kinetic trends were observed: (i) as the 3- and 5-hydroxy groups were replaced with hydrogen, the L-diC<sub>8</sub>PI derivatives became more potent inhibitors, with L-3,5-dideoxy-diC<sub>8</sub>PI being more inhibitory than its enantiomer at concentrations below 1 mM; and (ii) at 0.1–0.3 mM, two of the compounds, D-diC<sub>8</sub>PI and D-3-deoxy-diC<sub>8</sub>PI(5)P, activated PTEN by 20–40% for D-diC<sub>8</sub>PI(3)P hydrolysis. The L enantiomers of these compounds had no activating effect in this concentration range. Activation of PTEN by PI(4,5)P<sub>2</sub> has been

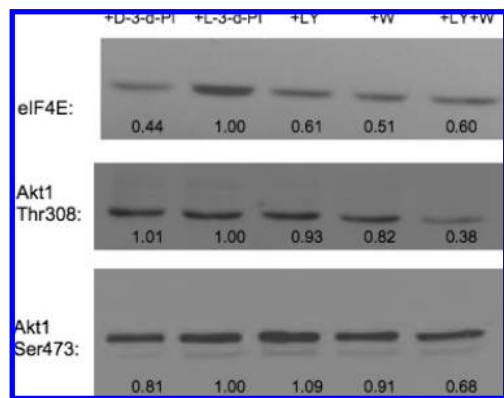


**Figure 4.** Viability of U937 cells after incubation for 24 h with different concentrations of 3-deoxy-diC<sub>8</sub>PI compounds: D-3-deoxy-diC<sub>8</sub>PI (●), L-3-deoxy-diC<sub>8</sub>PI (○), and D-3-diC<sub>8</sub>PI(5)P (×). Data for dideoxy-diC<sub>8</sub>PI compounds were the same as those for L-3-deoxy-diC<sub>8</sub>PI.

reported previously.<sup>22</sup> In our system, where all of the PIs have similar CMC values, we can suggest that the nonsubstrate D-3-deoxy-diC<sub>8</sub>PI(5)P must be binding as a monomer in a stereospecific manner to PTEN to enhance its hydrolysis of diC<sub>8</sub>PI(3)P, since activation is seen at low total PI concentrations. Even if some substrate aggregation occurs, the other nonactivating PI molecules are likely to have the same effect on the physical distribution of substrate between monomer and very small micelles. Since they do not activate PTEN, the effect must be due to a specific PTEN/D-3-deoxy-diC<sub>8</sub>PI(5)P interaction. However, extrapolating these results to the in vivo situation, we would predict that at 3-deoxy-PI concentrations less than 0.05 mM, there should be little inhibition of PTEN; any activation by diC<sub>8</sub>PI compounds is also likely to be small under these conditions.

**Effect of 3-Deoxy-diC<sub>8</sub>PI Compounds on the Growth of the U937 Human Lymphoma Cell Line.** Long-chain D-3-deoxy-PI analogues are thought to inhibit cell growth by competing with PI(3)P for binding to the Akt1 PH domain and reducing Akt1 translocation to the plasma membrane.<sup>7</sup> Short-chain 3-deoxy-diC<sub>8</sub>PI compounds could affect cell survival in a similar fashion. The difference is that at low concentrations, the short-chain PI analogues are likely to be monomeric in the cell rather than partitioned into membranes (as expected for the long-chain PI analogues). There may also be specificities among the different deoxy-PI compounds that would shed light on key interactions of these modified PIs with diverse targets. To explore this, we incubated the U937 human leukemic monocyte lymphoma cell line with the compounds D-3-deoxy-diC<sub>8</sub>PI, L-3-deoxy-diC<sub>8</sub>PI, D-3,5-dideoxy-diC<sub>8</sub>PI, and L-3,5-dideoxy-diC<sub>8</sub>PI at various concentrations and assessed the viability of the cells at different time points. Of all the compounds tested, only D-3-deoxy-diC<sub>8</sub>PI had any significant effect on cell viability, with an IC<sub>50</sub> of 40 μM (Figure 4). The other compounds had no measurable effect on viability at concentrations up to 200 μM.

To determine if the 3-deoxy-diC<sub>8</sub>PI compounds inhibited endogenous signaling via the PI3K pathway, we assessed phosphorylation of Akt1, a downstream target of PI3K, and compared the effects of the short-chain PI compounds (50 μM) to those of LY294002 (10 μM) and wortmannin (20 nM), which are known inhibitors of signaling via the PI3K/Akt pathway (Figure 5). Since L-3-deoxy-PI had no effect on cell growth,



**Figure 5.** Western blots used to monitor phosphorylation of Akt1 (at both Ser473 and Thr308) as well as eIF4E (at Ser209) after 24 h incubation with 50  $\mu$ M D-3-deoxy-diC<sub>8</sub>PI (D-3-d-PI), 50  $\mu$ M L-3-deoxy-diC<sub>8</sub>PI (L-3-d-PI), 10  $\mu$ M LY294002 (LY), 20 nM wortmannin (WM), or a combination LY294002 and wortmannin (LY+W). The number under each lane represents the fractional phosphorylation in the presence of the indicated compound compared to phosphorylation in the presence of L-3-deoxy-diC<sub>8</sub>PI, which has no effect on the cells.

the intensity of the band from cells incubated with that compound was used as a phosphorylation control. Phosphorylation of the transcriptional initiation factor eIF4E was clearly reduced after incubation of the cells with D-3-deoxy-diC<sub>8</sub>PI for 24 h. However, reduction in Akt phosphorylation appeared to show interesting specificity. Phosphorylation of Thr308 did not appear to be affected by D-3-deoxy-diC<sub>8</sub>PI; however, LY294002 and wortmannin led to some reduction in phosphorylation over the same time period, and the combination of the two was extremely effective. In contrast, Ser473 phosphorylation was reduced by a greater amount at this time point when the cells were incubated with D-3-deoxy-diC<sub>8</sub>PI than with the two other known inhibitors of the PI3K/Akt pathway. In previous work with D-3-deoxy-PI molecules,<sup>7</sup> Akt Ser473 phosphorylation was significantly reduced (to 65% of the maximum phosphorylation when D-3-deoxy-diC<sub>16</sub>PI was used). Here we show that the more soluble short-chain D-3-deoxy-diC<sub>8</sub>PI can also reduce Akt1 phosphorylation on Ser473. Whether it does so by preventing Akt translocation or by altering the interaction of the protein with the membrane remains to be seen. That this soluble specific enantiomer but not further-deoxygenated compounds is cytotoxic and affects Akt1 phosphorylation is an exciting observation.

## Discussion

Compounds based on D-3-deoxy-PI have been examined as cell growth inhibitors for a variety of cancer cells.<sup>12–14</sup> The first analogue examined, D-3-deoxy-diC<sub>16</sub>PI, inhibited the growth of NIH3T3 mouse fibroblasts with an IC<sub>50</sub> of 17.6  $\mu$ M.<sup>7</sup> Modifications of the 3-deoxy-PI to increase potency included replacing acyl chains with ether-linked chains and shortening the chain attached to the glycerol sn-2 position.<sup>14</sup> The ether bonds should make the 3-deoxy-PI compounds stable to phospholipase A<sub>2</sub> (PLA<sub>2</sub>)-type activities, although most of the mammalian PLA<sub>2</sub> enzymes are not selective for PI headgroups. Work by Tabellini et al.<sup>15</sup> showed that at 5  $\mu$ M, the ether-linked 3-deoxy-PI analogues (2-substituted 3-deoxy-PIs) had small effects by themselves but enhanced the induction of apoptosis when administered with either etoposide or cytarabine. At this concentration, they were not toxic to human umbilical cells, although doubling the concentration of the deoxy-PI caused a pronounced increase in cell apoptosis. Shortening of the sn-2

chain length may also make a more significant contribution to molecule potency. This creates a more lysolike phospholipid that has a higher solution concentration than the diacyl long-chain lipids. However, other structural aspects of 3-deoxy-PI that make it cytotoxic have not been studied.

In this work we have used synthetic diC<sub>8</sub>PI compounds both as substrates for different target enzymes and to screen for 3-deoxy-PI inhibition in vitro. Knowing the CMCs for these lipids allows us to carefully interpret the in vitro assays. In the case of PI3K and PLC enzymes, the assays used mixed micelles to assess analogue inhibitory potency. For PTEN, the assays had a mixture of monomers and some small micelles. We have examined how inositol stereochemistry as well as specific hydroxyl groups at C3 and C5 on the inositol ring contribute to the cytotoxicity of this class of PIs. The 3-deoxy-diC<sub>8</sub>PI molecules are poor inhibitors of PI3K and only moderate inhibitors of PTEN. They are neither good substrates nor good inhibitors of PI-PLC enzymes, strongly indicating that these enzymes are not the in vivo target(s). The compound D-3-deoxy-diC<sub>8</sub>PI is the only PI in this series that is cytotoxic to U937 cells. These leukemic cells provide a good system for evaluating the effectiveness of the diverse 3-deoxy-PIs because they have an activated PI3K/Akt pathway. Since neither L-3-deoxy-diC<sub>8</sub>PI nor D-3,5-dideoxy-diC<sub>8</sub>PI were cytotoxic, the in vivo target must bind D-3-deoxy-diC<sub>8</sub>PI in a very specific way.

The mechanism of action of the 3-deoxy-PIs has been proposed to be the binding of this lipid to the PH domain of Akt1.<sup>7</sup> This binding is then supposed to prevent targeting of Akt1 to the plasma membrane for phosphorylation and subsequent activation. Indeed, studies have shown that this molecule inhibits growth of cell lines in which Akt1 is the major isoform of this protein kinase.<sup>12–14</sup> Akt1 is phosphorylated on Ser473 and Thr308; treatment of cells with D-3-deoxy-diC<sub>16</sub>PI and the ether-linked long-chain PI analogue DPIEL showed that phosphorylation of the Ser sites was reduced.<sup>7</sup> Other lipid-based antitumor compounds have been shown to alter Thr308 phosphorylation,<sup>15</sup> so it is not clear whether one or both phosphorylation sites are critical for Akt1 activation. In fact, one can ask whether the reduced phosphorylation of Akt1 really represents the mechanism by which 3-deoxy-PI molecules inhibit cell growth. All of the previously examined modified phospholipids are relatively hydrophobic and presumably could be integrated into different intracellular membranes, attracting the Akt1 to a membrane devoid of its activating kinase. By using short-chain synthetic 3-deoxy-PI compounds, which should be monomeric in the cell at concentrations less than 0.1 mM, and showing that only D-3-deoxy-diC<sub>8</sub>PI is inhibitory in vivo, we have provided data indicating that a very specific interaction of the 3-deoxy-PI with its target occurs. The stereochemistry of the attachment of the inositol ring to the glycerol backbone (only the D enantiomer is inhibitory) plays a critical role, as does the C5 hydroxyl group. The observation that an Akt1 downstream target (eIF4E) exhibited reduced phosphorylation strongly points to Akt1 as the likely in vivo target of D-3-deoxy-diC<sub>8</sub>PI. Furthermore, since Akt1 phosphorylation on Ser473 was reduced while that on Thr308 was unaffected, we can propose that the primary effect of this lipid analogue is modulation of Ser phosphorylation. Interestingly, recent work also suggests that PI ether lipid analogues activate the proapoptotic stress kinase p38 $\alpha$  that is a subgroup of the MAPK family and activated via phosphorylation by MAPKKs.<sup>16</sup> This particular interaction could also contribute to the cytotoxicity of 3-deoxy-PIs. With the in vivo specificity profile for the soluble, synthetic 3-deoxy-diC<sub>8</sub>PI

analogues in hand, one might further explore that kinase in vitro to assess the direct effects of the 3-deoxy-PIs.

Although there is no direct evidence for an Akt1 PH domain complex with 3-deoxy-PI, there is a crystal structure of the PH domain with a bound polyphosphorylated inositol. The crystal structure of the Akt PH domain to which I(1,3,4,5)P<sub>4</sub> is bound<sup>44,45</sup> shows strong interactions between the protein and the 3- and 4-phosphate groups but not the 5-phosphate group, which was poised toward solvent and not tightly held. Given the importance of the phosphate monoester interactions in this structure, it might be surprising that the 3-deoxy-PIs bind to the PH domain at all. The interaction of the Akt1 PH domain with the inositol ring of the 3-deoxy compounds is likely to be quite different than its interaction with I(1,3,4,5)P<sub>4</sub>. Indeed, previous studies modeling 3-deoxy-PI binding to the Akt1 PH domain showed strong H bonds between the inositol 4- and 5-hydroxyl groups and the protein.<sup>7</sup> In accord with these modeling results, we have shown that while D-3-deoxy-diC<sub>8</sub>PI inhibits U937 cell growth, D-3,5-dideoxy-diC<sub>8</sub>PI is not cytotoxic. Given the strongly cationic nature of the Akt1 PH domain binding site, one might expect a phosphate at C5 in D-3-deoxy-diC<sub>8</sub>PI to enhance analogue binding. However, we observed no

growth inhibition by D-3-deoxy-diC<sub>8</sub>PI(5)P. It is possible that such a compound was not taken up by the U937 cells, since intracellular phosphatases would have been expected to remove the phosphate and generate D-3-deoxy-diC<sub>8</sub>PI, which is inhibitory. D-3-Deoxy-diC<sub>8</sub>PI, unlike the long-chain analogues studied previously, is monomeric in the tumor cells at the concentrations where it was found to be cytotoxic. This observation decouples membrane partitioning of the modified PI from specific target/3-deoxy-PI interactions. The increased solubility without a decrease in tumor potency (compared with the ester-linked long-chain 3-deoxy-PI) may also be important for reducing toxic effects in normal cells. In support of this tantalizing possibility, preliminary experiments involving incubation of 0.2 mM D-3-deoxy-diC<sub>8</sub>PI with primary B cells (which have all three Akt forms) have shown little loss of cell viability.<sup>46</sup> Clearly, this area is ripe for further investigation using well-defined soluble 3-deoxy-PI molecules.

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(44) Thomas, C. C.; Deak, M.; Alessi, D. R.; van Aalten, D. M. F. *Curr. Biol.* **2002**, *12*, 1256–1262.

(45) Kumar, C. C.; Madison, V. *Oncogene* **2005**, *24*, 7493–7501.

JA710348R

(46) Pu, M.; Blair, D.; Chiles, T. Unpublished work, 2008.