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Communications to the Editor

Synthesis and Biology of 1D-3-Deoxyphosphatidylinositol: A Putative Antimetabolite of Phosphatidylinositol-3-phosphate and an Inhibitor of Cancer Cell Colony Formation

Alan P. Kozikowski,*,[†] James J. Kiddle,[§] Timothy Frew,[‡] Margareta Berggren,[‡] and Garth Powis[‡]

Neurochemistry Research, 12 Mershon Drive, Princeton, New Jersey 08540, Mayo Clinic, Neurochemistry Research, 4500 San Pablo Road, Jacksonville, Florida 32224, and Arizona Cancer Center, 1515 North Campbell Avenue, Tucson, Arizona 85742

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Ligand-activated growth factor and mitogen receptors activate phosphatidylinositol (PtdIns) phospholipase C which hydrolyzes PtdIns 4,5-bisphosphate to form inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] and diacylglycerol.[1] Ins(1,4,5)P_3 releases Ca^{2+} from nonmitochondrial stores to increase the cytoplasmic-free Ca^{2+} concentration whereas diacylglycerol is an activator of protein kinase C (PKC).[2] The increase in cytoplasmic-free Ca^{2+} concentration and the increased activity of PKC lead to a sequence of events that culminate in DNA synthesis and cell proliferation.[3] Recently, another PtdIns signaling pathway has been identified and linked to the action of some growth factors and oncogenes. Phosphatidylinositol-3-kinase (PtdIns-3-kinase) is found associated with a number of protein tyrosine kinases, including the ligand-activated receptors for insulin, platelet-derived growth factor, epidermal growth factor, and colony-stimulating factor, as well as oncogene tyrosine kinases.[4] The PtdIns-3-kinase phosphorylates the D-3 position of the *myo*-inositol ring of PtdIns to yield a class of PtdIns-3-phosphates that are not substrates for hydrolysis by PtdIns phospholipase C,[5] and therefore appear to exert their signaling action independently of the inositol phosphate pathway.

PtdIns-3-kinase is found in cellular complexes with ligand-activated growth factor receptor, with oncogene protein tyrosine kinases,[6] and with $p21^{ras}$.[7] The PtdIns-3-kinase is a heterodimer consisting of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit.[8] The 85 kDa subunit acts as an adaptor protein whose *src*-homology (SH2) domains interact with specific tyrosine phosphorylated residues on the tyrosine kinase receptors and other tyrosine phosphorylated proteins.[9] This brings the PtdIns-3-kinase p110 kDa catalytic subunit from the cytoplasm to the plasma membrane where substrates, PtdIns and its phosphates, are located.[10] Recently a guanine nucleotide binding (G) protein-dependent PtdIns-3-kinase activity has been described that is inactive in the absence of added $G_{\beta\gamma}$ subunits.[11] The mechanism by which PtdIns-3-phosphates stimulate cell growth is not known. They have been suggested to act by reorganizing cytoskeletal elements,[12] by activating protein kinase C-ζ,[13] or by affecting vesicular trafficking in cells.[14]

The PtdIns-3-kinase signaling pathway provides an attractive target for the development of antiproliferative drugs because it is important for both cell growth and transformation.[15] PtdIns-3-kinase takes on added importance in view of the recent finding that it is the direct target for the Ras proteins, key regulators of cell growth.[16] As part of our studies to synthesize analogues of PtdIns as potential antimetabolites[17] of the PtdIns-3-kinase signaling pathway, we have synthesized 1D-3-deoxyphosphatidylinositol (DPI). We report the synthesis of this compound together with its ability to inhibit cancer cell growth *in vitro*. Additionally, DPI is shown to be an important tool to measure the activity of PtdIns-3-kinase and other PtdIns kinases in cell lysates from breast cancer cells.

Chemistry. As shown in Scheme 1, the synthesis of DPI began with the previously prepared intermediate **1** available from the synthesis of 1D-3-deoxy-*myo*-inositol 1,4,5-triphosphate.[18] As illustrated, **1** was transformed into **4** by a sequence of conventional steps. The diol **4** was protected preferentially at the 1-position via a cyclic stannylene[19] and subsequently benzylated to give the

[†] Neurochemistry Research.
[‡] Arizona Cancer Center.
[§] Mayo Clinic.

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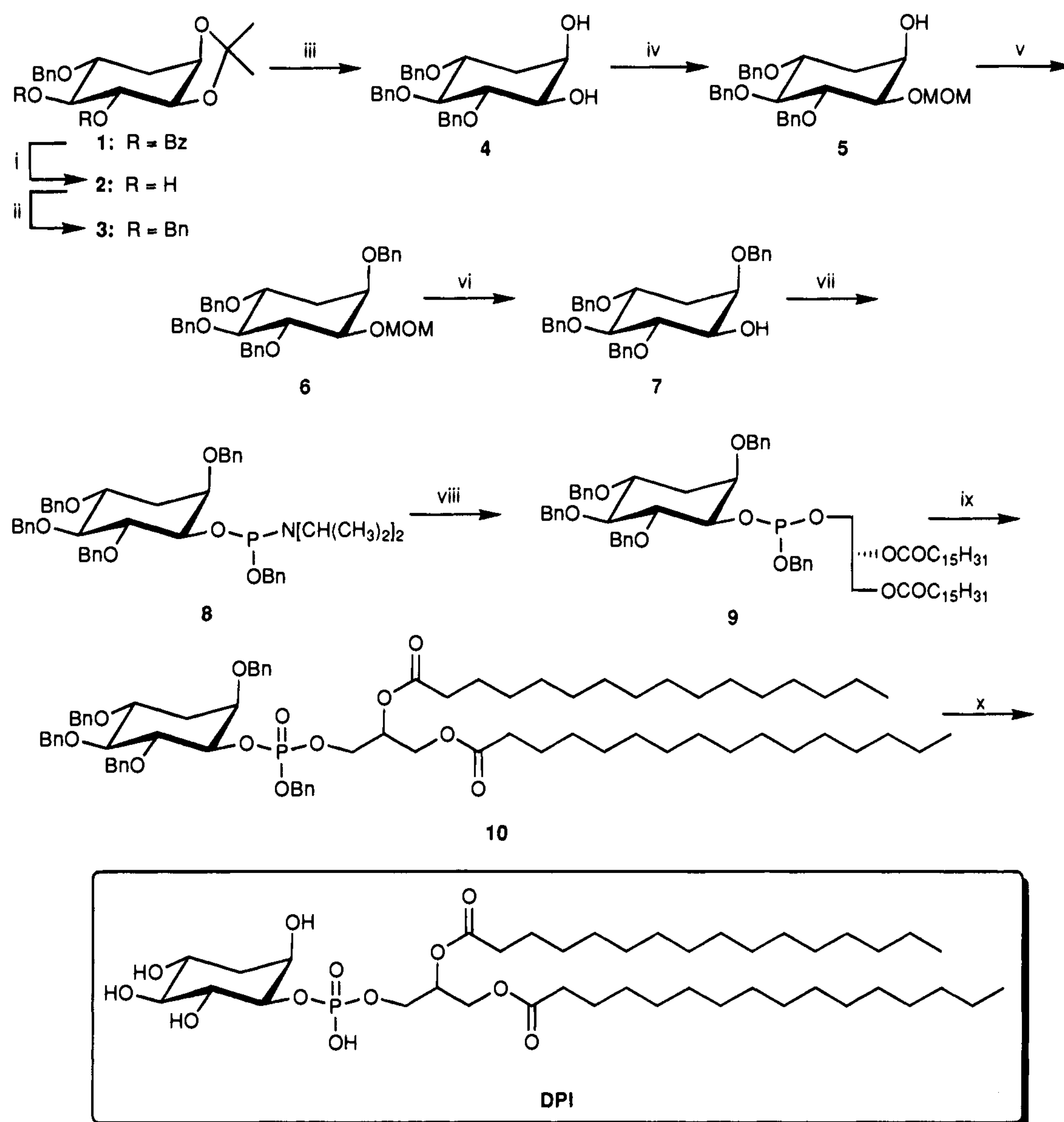
Scheme 1.[a] Synthesis of 1D-3-Deoxyphosphatidylinositol



[a] Reagents and conditions: (i) K_2CO_3, MeOH (83%); (ii) NaH, BnBr, DMF (99%); (iii) HCl, MeOH (95%); (iv) (a) *n*-Bu_2SnO, MeOH, reflux; (b) MOMCl, DMF/toluene (59% overall); (v) NaH, BnBr, DMF (76%); (vi) HCl, MeOH/H_2O (98%); (vii) BnOP[N(CH(CH_3)$_2$)$_2$]$_2$, CH_2Cl_2 (82%); (viii) 1,2-dipalmitoyl-*sn*-glycerol, tetrazole, CH_2Cl_2/CH_3CN (31%); (ix) *t*-BuOOH, CH_2Cl_2 (88%); (x) 20% Pd(OH)$_2$/C, H_2, *t*-BuOH (89%).

fully protected intermediate **6**. The MOM protecting group was removed, and the 1-position was phosphitylated with (benzyloxy)bis(diisopropylamino)phosphorus to afford **8**. Next, **8** was coupled with 1,2-dipalmitoyl-*sn*-glycerol, and the phosphite was oxidized to the fully protected phosphate (**10**). Finally, all benzyl groups were removed by hydrogenolysis over Pd(OH)$_2$/C in *t*-BuOH. *t*-BuOH was chosen as the solvent in order to minimize the possibility of transesterification of the palmitoyl groups of the diacylglycerol moiety. Of all of the steps executed in the synthesis of DPI, the hydrogenolysis reaction proved to be the most difficult, and the conditions in this step were arrived at only after considerable experimentation.

Biology. Cell Growth Inhibition. To assay the compound for cell growth inhibition, HT-29 human colon carcinoma cells were exposed to various concentrations of DPI over a 7 day period.[20] DPI was found to inhibit colony formation by these carcinoma cells with an IC_{50} of 35 μM. The PtdIns analogue bearing a fluorine atom in place of the 3-hydroxy group exhibited an IC_{50} of 37 μM in the same assay.[21]

PtdIns-3-Kinase Activity. Phosphatidylinositol-3-kinase was prepared as phosphotyrosine immunoprecipitates from lysates of platelet-derived growth factor stimulated Swiss mouse 3T3 fibroblasts as previously described.[22] PtdIns-3-kinase activity was assayed as described[22] by the [γ^{32}P]ATP-dependent phosphorylation of PtdIns. In marked contrast to 1D-3-deoxy-3-fluoro-PtdIns (FTI) which inhibits this enzyme with an IC_{50} of 30 μM, DPI was found to have no effect on PtdIns-3-kinase activity at concentrations up to 250 μM; additionally it was not a substrate for the kinase, as no phosphorylated product was detected by TLC analysis (Figure 1). Apparently, the electronic nature of the group at the 3-position of these PtdIns analogues is one element essential for recognition by the enzyme. The steric volume of this substituent also plays a role, for 1D-3-chloro-3-deoxy-PtdIns, like DPI, fails to inhibit the PtdIns-3-kinase.

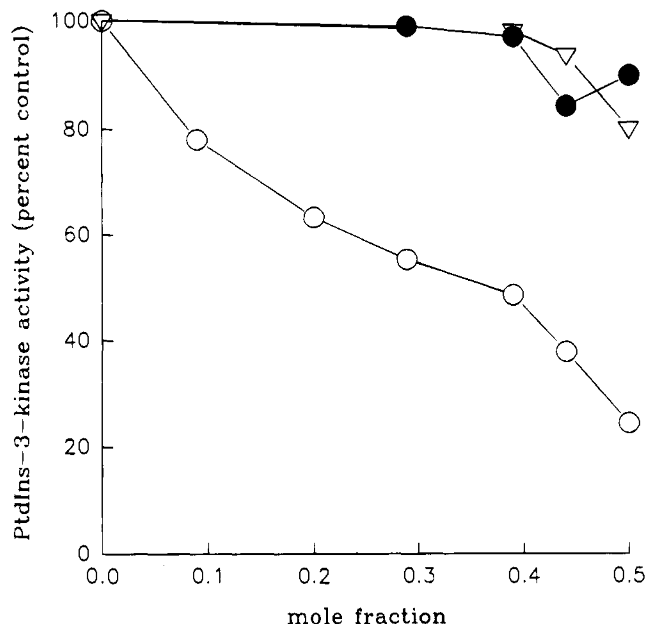


Figure 1. Effects of 1D-3-substituted-PtdIns on PtdIns-3-kinase activity. The ability of (●) 1D-3-deoxy-PtdIns; (○) 1D-3-deoxy-3-F-PtdIns; and (△) 1D-3-deoxy-3-Cl-PtdIns when prepared as mixed vesicles with PtdIns to inhibit the ATP-dependent phosphorylation of PtdIns by immunoprecipitated PtdIns-3-kinase was measured as described in the text. The concentration of PtdIns in the assay was 100 μM, and the mole fraction of 1D-3-substituted-PtdIns was varied as shown.

The observation that DPI does not inhibit PtdIns-3-kinase would suggest that it can be used in whole cell lysates to measure PtdIns-3-kinase activity as the difference between [γ^{32}P]ATP-dependent phosphorylation of PtdIns and DPI. Previous studies to measure cell PtdIns-3-kinase activity have typically employed immunoprecipitation of the kinase from cell lysates using phosphotyrosine or p85 antibodies.[23] With the recent reports on G protein-dependent forms of PtdIns-3-kinase,[11] the question of whether all the PtdIns-3-kinase activity is being measured can be raised. An alternative method is to measure the formation of PtdIns-3-phosphates in intact cells.[24] TLC separation of PtdIns-3-phosphate and PtdIns-4-phosphate in cell lysates gives relatively poor separation.[25] A specific HPLC method is available but requires high specific activity radiolabeling and derivatization prior to HPLC in order to measure the small amounts of PtdIns-3-phosphates.

Accordingly, DPI and PtdIns were used as substrates to measure PtdIns-3-kinase and other PtdIns kinase activities in whole cell lysates of MCF-7 human breast carcinoma cells (Figure 2). An approximately linear relationship was found between the amount of cell protein in the assay and the amounts of PtdIns and DPI phosphorylation. Since DPI cannot be phosphorylated at the 3-position, its phosphorylation represents other positions on the *myo*-inositol ring. The known biologically relevant positions for phosphorylation of PtdIns are 3, 4, and 5.[26] Additionally, in the presence of 50 nM wortmannin, a specific inhibitor of PtdIns-3-kinase, DPI was shown to be as good a substrate as PtdIns for the other PtdIns kinases in MCF-7 cell lysates.[27] Thus phosphorylation of DPI most likely reflects PtdIns-4- and PtdIns-5-kinase activities. In no case was formation of PtdIns bis- or trisphosphates observed, as these are readily separated from the PtdIns monophosphates by the TLC system used.

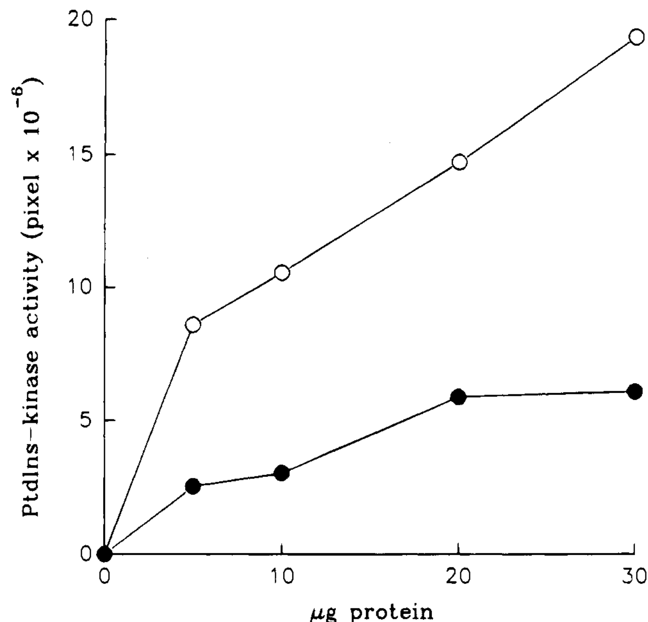


Figure 2. Phosphorylation of PtdIns and 1D-3-deoxy-PtdIns by MCF-7 cell lysate. The [γ^{32}P]ATP-dependent phosphorylation of (○) PtdIns and (●) 1D-3-deoxy-PtdIns by whole cell lysates of MCF-7 human breast carcinoma cells was measured as described in the text. The concentration of the lipids in the assay was 100 μM. There was no detectable phosphorylation of any endogenous lipids in the MCF-7 cell lysates under the assay conditions employed. The results are typical of three separate experiments.

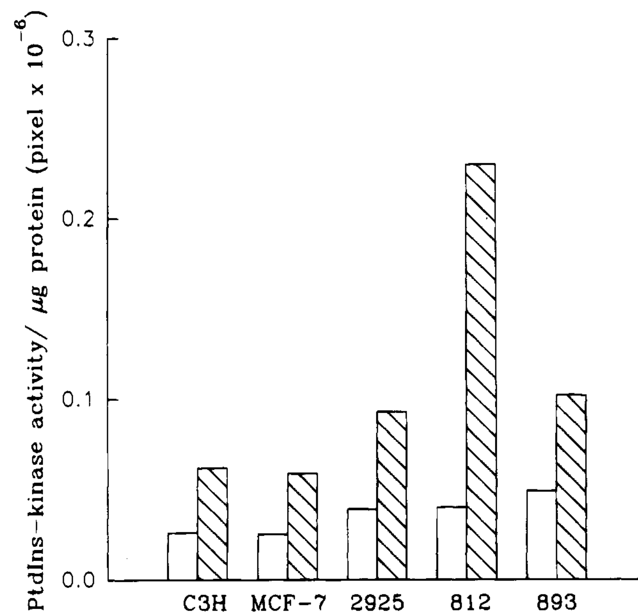


Figure 3. PtdIns-3-kinase and other PtdIns-kinase activities in breast cancer cells. PtdIns-3-kinase (hatched boxes) and other PtdIns-kinase activities (open boxes) were measured in cell lysates from C3H mouse mammary carcinoma, and MCF-7, ACC-2925, ACC-812, and ACC-893 human breast carcinoma cell lines as described in the text. Each value is a mean derived from protein concentration/PtdIns and 1D-3-deoxy-PtdIns kinase activity curves similar to that shown in Figure 2. The results are typical of two separate experiments.

DPI was used to measure PtdIns-kinase activities in a panel of mouse and human breast cancer cell lines (Figure 3). PtdIns-3-kinase activity is approximately 2-fold higher than that of other PtdIns-kinases. How-

ever, in the ACC-812 human breast cancer cell line the PtdIns-3-kinase activity was almost 4-fold that seen in the other breast cancer cell lines, while other PtdIns-kinase activities remained the same. The significance, if any, of the elevated PtdIns-3-kinase activity in the breast cancer cell line remains to be established.

In summary, the colony-inhibiting activity found for DPI in this study is encouraging and suggestive that further structural alterations of PtdIns should be made in the quest for novel anticancer agents. Additionally, DPI is shown to serve as a possible pharmacological tool for characterizing the level of PtdIns-3-kinase activity in diverse cell lines, a feature which should gain added importance as our understanding of the role of the PtdIns-3-kinase signaling pathway in abnormal cell growth continues to evolve.

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Supplementary Material Available: Spectroscopic and analytical data for all new compounds (2 pages). Ordering information is given on any current masthead page.

References

(1) (a) Margolis, B.; Rhee, S. G.; Felder, S.; Mervic, M.; Lyall, R.; Levitzki, A.; Ullrich, A.; Zilberstein, A.; Schlessinger, J. EGF induces tyrosine phosphorylation of phospholipase C: a potential mechanism for EGF receptor signaling. *Cell* **1989**, *57*, 1101–1107. (b) Secrist, P.; Sehgal, I.; Powis, G.; Abraham, R. Selective inhibition of platelet-derived growth factor receptor-mediated protein tyrosine phosphorylation and phospholipase C activation by staurosporine. *J. Biol. Chem.* **1990**, *265*, 20394–20400. (c) Harden, T. K. G Protein-dependent regulation of phospholipase C by cell surface receptors. *Am. Rev. Respir. Dis.* **1990**, *141*, S119–S122.

(2) Berridge, M. J.; Irvine, R. F. Inositol phosphates and cell signalling. *Nature* **1989**, *341*, 197–205.

(3) Whitman, M.; Cantley, L. Phosphoinositide metabolism and the control of cell proliferation. *Biochem. Biophys. Acta* **1988**, *948*, 327–344.

(4) (a) Auger, K. R.; Serunian, L. A.; Soltoff, S. P.; Libby, P.; Cantley, L. C. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **1989**, *57*, 167–175. (b) Bjorge, J. D.; Chan, T.-O.; Antczak, M.; Kung, H.-J.; Fujita, D. J. Activated type I phosphatidylinositol kinase is associated with the epidermal growth factor (EGF) receptor following EGF stimulation. *Proc. Nat. Acad. Sci. U.S.A.* **1990**, *87*, 3816–3820. (c) Coughlin, S. R.; Escobedo, J. A.; Williams, L. T. Role of phosphatidylinositol kinase in PDGF-receptor signal transduction. *Science* **1989**, *243*, 1191–1194. (d) Courtneidge, S. A.; Heber, A. *Cell* **1987**, *50*, 1031–1037. (e) Escobedo, J. A.; Williams, L. T. *Nature* **1988**, *335*, 85–87. (f) Fukui, Y.; Kornbluth, S.; Jong, S.-M.; Wang, L.-H.; Hanafusa, H. *Oncogene Res.* **1989**, *4*, 283–292.

(5) (a) Lips, D. L.; Majerus, P. W. The discovery of a 3-phosphomonoesterase that hydrolyses phosphatidylinositol 3-phosphate in NIH 3T3 cells. *J. Biol. Chem.* **1989**, *264*, 19911–19915. (b) Serunian, L. A.; Haber, M. T.; Fukui, T.; Kim, J. W.; Rhee, S. G.; Lowenstein, J. M.; Cantley, L. C. Polyphosphoinositides produced by phosphatidylinositol-3-kinase are poor substrates for phospholipase C from rat liver and bovine brain. *J. Biol. Chem.* **1989**, *264*, 17809–17815.

(6) Cantley, L. C.; Auger, K. R.; Carpenter, C.; Duckworth, B.; Graziani, A.; Kapeller, R.; Soltoff, S. Oncogenes and signal transduction. *Cell* **1991**, *64*, 281–302.

(7) Sjolander, A.; Yamamoto, K.; Huber, B. E.; Lapetina, E. G. Association of p21*ras* with phosphatidylinositol-3-kinase. *Proc. Nat. Acad. Sci. U.S.A.* **1991**, *88*, 7908–7947.

(8) Shibaski, F.; Homma, Y.; Takenawa, T. Monomers and heterodimer forms of phosphatidylinositol-3-kinase. *J. Biol. Chem.* **1991**, *266*, 8108–8114.

(9) Margolis, B. Transducers in the tyrosine kinase signaling pathway. *Cell Growth Differ.* **1992**, *3*, 73–80.

(10) Varticovski, L.; Harrison-Findik, D.; Keeler, M. L.; Susa, M. Role of PI 3-kinase in mitogenesis. *Biochem. Biophys. Acta* **1994**, *126*, 1–11.

(11) Stephans, L.; Smrcka, A.; Cooke, F. T.; Jackson, T. R.; Sternweis, P. C.; Hawkins, P. T. A novel phosphoinositide 3-kinase activity in myeloid-derived cells is activated by G protein β-γ subunits. *Cell* **1994**, *77*, 83–93.

(12) (a) Traynor-Kaplan, A. E.; Thompson, B. L.; Harris, A. L.; Taylor, P.; Omann, G. M.; Sklar, L. A. Transient increase in phosphatidylinositol 2,3-biphosphate and phosphatidylinositol trisphosphate during activation of human neutrophils. *J. Biol. Chem.* **1989**, *264*, 15668–15673. (b) Eberle, M.; Traynor-Kaplan, A. E.; Sklar, L. A.; Norgauer, J. Is there a relationship between phosphatidylinositol trisphosphate and F-actin polymerization in human neutrophils? *J. Biol. Chem.* **1990**, *265*, 16725–16728.

(13) Nakanishi, H.; Brewer, K. A.; Exton, J. H. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **1993**, *268*, 13–16.

(14) (a) Hilgard, P. Anticoagulants and tumor growth: Pharmacological considerations. In *Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects*; Nicolson, G. L., Milas, L., Eds.; Raven Press: New York, 1984; p 353. (b) Fry, M. J. Waterfield, M. D. Structure and function of phosphatidylinositol 3-kinase: a potential second messenger system involved in growth control. *Philos. Trans. R. Soc. London B* **1993**, *340*, 337–344.

(15) (a) Powis, G. Signalling targets for anticancer drug development. *Trends Pharmacol. Sci.* **1991**, *12*, 188. (b) Matter, W. F.; Brown, R. F.; Vlahos, C. J. The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. *Biochem. Biophys. Res. Commun.* **1992**, *186*, 624–631.

(16) Rodriguez-Viciana, P.; Warne, P. H.; Dhand, R.; Vanhaesebroeck, B.; Gout, I.; Fry, M. J.; Waterfield, M. D.; Downward, J. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **1994**, *370*, 527–532.

(17) Kozikowski, A. P.; Fauq, A. H.; Malaska, M. J.; Tückmantel, W.; Ognyanov, V. I.; Powis, G. Inositol analogues as potential anticancer therapeutics. *Curr. Med. Chem.* **1994**, *1*, 1–12.

(18) Kozikowski, A. P.; Ognyanov, V. I.; Fauq, A. H.; Nahorski, S. R.; Wilcox, R. A. Synthesis of 1D-3-deoxy-, 1D-2,3-dideoxy-, and 1D-2,3,6-trideoxy-*myo*-inositol 1,4,5-trisphosphate from quebrachitol, their binding affinities and calcium release activity. *J. Am. Chem. Soc.* **1993**, *115*, 4429–4434.

(19) David, S.; Hanessian, S. Regioselective manipulation of hydroxyl groups via organotin derivatives. *Tetrahedron* **1985**, *41*, 643–663.

(20) Alley, M. C.; Powis, G.; Appel, P. L.; Kooistra, K. L.; Lieber, M. M. Activation and inactivation of cancer chemotherapeutic agents by rat hepatocytes cocultured with human tumor cell lines. *Cancer Res.* **1984**, *44*, 549–556.

(21) Kozikowski, A. P.; Powis, G.; Fauq, A. H.; Tückmantel, W.; Gallegos, A. Synthesis and biological activity of the D-3-deoxy-3-fluoro and D-3-chloro-3-deoxy analogues of phosphatidylinositol. *J. Org. Chem.* **1994**, *59*, 963–971.

(22) Vlahos, C. J.; Matter, W. F.; Hui, K. Y.; Brown, R. F. A specific inhibitor of phosphatidylinositol 3-kinase: 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **1994**, *269*, 5241–5248.

(23) (a) Carter, A. N.; Downes, C. P. Phosphatidylinositol 3-kinase is activated by nerve growth factor and epidermal growth factor in PC12 cells. *J. Biol. Chem.* **1992**, *267*, 14563–14567. (b) Zhang, J.; Fry, M. J.; Waterfield, M. D.; Jaken, S.; Liao, L.; Fox, J. E. B.; Rittenhouse, S. E. Activated phosphoinositide 3-kinase associates with membrane skeleton in thrombin-exposed platelets. *J. Biol. Chem.* **1992**, *267*, 4686–4692. (c) Hu, P.; Margolis, B.; Skolnik, E. Y.; Lammers, R.; Ullrich, A.; Schlessinger, J. Interaction of phosphatidylinositol 3-kinase associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol. Cell Biol.* **1992**, *12*, 981–990.

(24) Auger, K. R.; Cantley, L. C. Novel polyphosphoinositides in cell growth and activation. *Cancer Cells* **1991**, *3*, 263–270.

(25) Schu, P. V.; Takegawa, K.; Fry, M. J.; Stack, J. H.; Waterfield, M. D.; Emr, S. D. Phosphatidylinositol 3-kinase encoded by yeast *VPS*34 gene essential for protein sorting. *Science* **1993**, *260*, 88. Kodaki, T.; Woscholski, R.; Emr, S.; Waterfield, M. D.; Nurse, P.; Parker, P. J. Mammalian phosphatidylinositol 3′-kinase induces a lethal phenotype expression in *Schizosaccharomyces pombe*; comparison with the *VPS*34 gene product. *Eur. J. Biochem.* **1994**, *219*, 775.

(26) Downes, C. P.; Carter, A. N. Phosphoinositide 3-kinase: a new effector in signal transduction. *Cell Signal* **1991**, *3*, 501–507.

(27) Powis, G.; Bonjouklian, R.; Berggren, M. M.; Gallegos, A.; Abraham, C.; Zalkow, L.; Matter, W. F.; Dodge, J.; Grindey, G.; Vlahos, C. J. Wortmannin, a potent and selective inhibitor of phosphatidylinositol 3-kinase. *Cancer Res.* **1994**, *54*, 2419–2423.

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