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Wortmannin, a Potent and Selective Inhibitor of Phosphatidylinositol-3-kinase[1]

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ABSTRACT

Phosphatidylinositol-3-kinase is an important enzyme for intracellular signaling. The microbial product wortmannin and some of its analogues have been shown to be potent inhibitors of phosphatidylinositol-3-kinase. The 50% inhibitory concentration for inhibition by wortmannin is 2 to 4 nM. Kinetic analysis demonstrates that wortmannin is a noncompetitive, irreversible inhibitor of phosphatidylinositol-3-kinase, with inactivation being both time- and concentration-dependent. Wortmannin has previously been reported to be an inhibitor of myosin light chain kinase but with an inhibitory concentration of 0.2 μM. Wortmannin was found not to be an inhibitor of phosphatidylinositol-4-kinase, protein kinase C, or protein tyrosine kinase. Wortmannin inhibited the formation of phosphatidylinositol-3-phosphates in intact cells. The results of the study suggest that wortmannin and its analogues may have utility as pharmacological probes for studying the actions of phosphatidylinositol-3-kinase.

INTRODUCTION

PtdIns-3-kinase[3] exists as a tightly associated heterodimer of an M_r 85,000 regulatory subunit and a M_r 110,000 catalytic subunit (1). PtdIns-3-kinase is found in cellular complexes with almost all ligand-activated growth factor receptor and oncogene protein tyrosine kinases (2). PtdIns-3-kinase is also associated with $p21^{ras}$ (3). The M_r 85,000 regulatory subunit probably acts as an adaptor protein that allows the M_r 110,000 catalytic subunit of PtdIns-3-kinase to interact with growth factor receptors and tyrosine phosphorylated proteins (4). The bovine PtdIns-3-kinase M_r 110,000 catalytic subunit has sequence homology to a yeast PtdIns-3-kinase that is involved in vacuolar protein sorting (5, 6), suggesting there may be a similar role for mammalian PtdIns-3-kinase.

PtdIns-3-kinase is an important enzyme for mitogenesis, cell transformation, and other cellular events involving protein tyrosine kinases. Cells transfected with mutant PDGF receptors that retain protein tyrosine kinase activity but that do not associate with or activate PtdIns-3-kinase fail to show a mitogenic response to PDGF, unlike cells transfected with the wild-type PDGF receptor (7). Specific mutation-restoration of PtdIns-3-kinase binding to a tyrosine-mutated PDGF receptor is sufficient to restore a mitogenic response to PDGF (8). Polyoma middle T mutants that activate $pp60^{c\text{-}src}$ tyrosine kinase but that fail to activate PtdIns-3-kinase are nontransforming (9). The levels of cellular PtdIns-3-phosphates are elevated by transforming mutants of polyoma middle T but not by transformation-defective mutants (10). Transformation-defective $pp60^{v\text{-}src}$ with mutations in the *src*-homology-3 (SH3) domain show decreased association with PtdIns-3-kinase (11). A mutant CSF-1 receptor with a kinase-insert deletion shows a significantly reduced association with PtdIns-3-kinase, and while it is capable of conferring CSF-1-dependent transformation to some cells, it has lost the ability in other cells (12, 13). Transforming *neu*/HER2 is found constitutively coupled to PtdIns-3-kinase, whereas nontransforming kinase-defective or carboxyl-terminal deleted versions show no constitutive association with PtdIns-3-kinase (14).

We have tested wortmannin, a microbial secondary metabolite found in a variety of fungal species and previously reported to be an inhibitor of myosin light chain kinase (15), as an inhibitor of PtdIns-3-kinase. We have found wortmannin and some of its analogues to be highly potent and selective inhibitors of the enzyme.

MATERIALS AND METHODS

Cells. Swiss mouse 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD), and v-*sis*-transformed NIH mouse 3T3 cells were obtained from Dr. D. S. Aaronson (National Cancer Institute, Bethesda, MD). Cells were maintained in bulk culture in DMEM supplemented with 10% fetal calf serum and were passaged using 0.025% trypsin and 0.02% EDTA.

Chemicals. Wortmannin, compound 1 (Fig. 1), was produced by the aerobic liquid fermentation of a soil-derived *Penicillium* species designated A24603.1, which has been deposited in the collection of the Agricultural Research Service (Peoria, IL) and given the accession number NRRL 21122. Isolation of wortmannin was accomplished by previously published methods (16–18). Wortmannin analogues 2, 3, 4, and 5 were synthesized from wortmannin using standard procedures (17, 19).

Agarose bead conjugated antiphosphotyrosine monoclonal antibody (IgG2bk) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), and PDGF, a recombinant human BB homodimer, was purchased from Genzyme (Cambridge, MA). [γ-^{32}P]ATP (10 Ci/mmol) was purchased from Dupont New England Nuclear (Boston, MA), and [^{3}H]PtdIns(4,5)bisphosphate (5 Ci/mmol) and carrier free [^{32}P]H_3PO_4 (285 Ci/mg) were puchased from ICN Biomedicals (Irvine, CA).

Preparation of PtdIns-3-kinase Assay. PtdIns-3-kinase was prepared in two ways. In the first method, PtdIns-3-kinase was prepared from confluent Swiss 3T3 cells. Cells (24×10^6) on four 100-mm culture plates were washed with 10 ml HBSS, pH 7.4, and the cells were left in DMEM without fetal calf serum for 1 h before being stimulated for 15 min with 100 ng/ml PDGF. The medium was aspirated, and the cells were washed with 10 ml HBSS before being lysed with 3 ml 137 mM NaCl, 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotonin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. The cells were scraped free from the surface of the dish and centrifuged at $6000 \times g$ for 10 min. The supernatant was mixed with 50 μl washed IgG2bk antiphosphotyrosine antibody beads in 1.5-ml tubes. The tubes were capped and rotated for 2 h at 4°C, and the beads were washed with 2×1 ml HBSS containing 2 μg/ml leupeptin, 4 μg/ml aprotonin, 1 mM phenylmethylsulfonyl fluoride, 200 μM adenosine, and 1 mM sodium orthovanadate. The tyrosine-phosphorylated PtdIns-3-kinase was eluted from the beads with 200 μl/tube of 10 mM sodium phenylphosphate, 10 mM Tris (pH 7.5), 2 M NaCl, 1 mM EDTA, and 200 μM adenosine. In the second method, PtdIns-3-kinase was purified from bovine brain using isoelectric precipitation, QAE-Sepharose anion exchange chromatography, hydroxylapatite adsorption chromatography, MonoS cation exchange chromatography, and monoQ anion exchange chromatography; details

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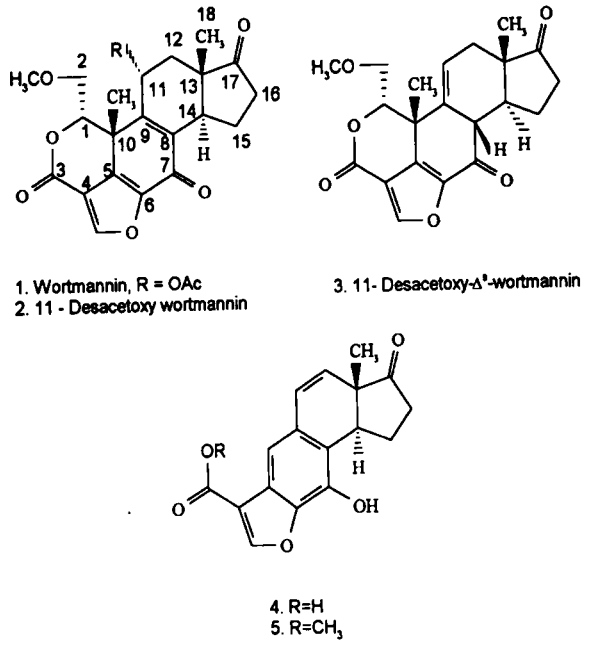
[3] The abbreviations used are: PtdIns, phosphatidylinositol; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; DMSO, dimethylsulfoxide; PKC, protein kinase C; IC_{50}, 50% inhibitory concentration.



1. Wortmannin, R = OAc
2. 11 - Desacetoxy wortmannin
3. 11- Desacetoxy-Δ^9-wortmannin
4. R=H
5. $R=CH_3$

Fig. 1. Structures of wortmannin and its analogues.

of the purification will be described in a later publication.[4] The specific activity of the purified bovine brain PtdIns-3-kinase was routinely between 2.5 and 7.2 nmol/min/mg. The isolated enzyme was determined to be completely free from contaminating PtdIns-4-kinase. PtdIns-4-kinase was obtained from the flow-through fractions of the QAE-Sepharose column and used without further purification.

PtdIns Kinase Assay. The incubation for measuring PtdIns-3-kinase activity contained 30 μl of the immunoprecipitated enzyme preparation (representing enzyme from 5×10^6 cells) or 0.1 μg purified bovine brain enzyme and 10 μl PtdIns micelles that were prepared by sonicating 100 μg PtdIns in 0.2 ml 20 mM HEPES buffer (pH 7.6) for 2×10 s at 4°C (Model XL 2020 Sonicator with a microtip probe; Heat Systems, Farmington, NY). PtdIns is the major substrate for PtdIns-3-kinase in intact cells (20) and was chosen as the substrate for the assay rather than PtdIns(4)phosphate or PtdIns(4,5)bisphosphate. The mixture was allowed to stand 10 min at 4°C, and the reaction started with 20 μM [γ-^{32}P]ATP (1 Ci/mmol). After 10 min at 37°C, the reaction was stopped with 0.2 ml 1 N HCl and 0.4 ml methanol:chloroform (1:1 by volume), and the mixture was vortexed for 3 min before centrifuging at $6000 \times g$ for 1 min. The lower chloroform phase was washed with 0.16 ml 1 N HCl:methanol (1:1 by volume) and then evaporated to dryness under N_2. The residue was dissolved in 20 μl chloroform:methanol (2:1 by volume) and applied to a silica gel thin layer chromatography plate (60A; Whatman Maidstone, Kent, United Kingdom); then the residue was developed with 1-propanol:2 N acetic acid (65:35 by volume). The plate was dried and autoradiographed, and the radiolabeled PtdIns-3-phosphate spots were scraped from the plate and counted by liquid scintillation. Inhibitors were dissolved in DMSO and then diluted with 50 mM HEPES buffer (pH 7.5) containing 15 mM $MgCl_2$ and 1 mM EGTA. The final reaction mixture contained 3% DMSO which had no effect on PtdIns-3-kinase activity. Control incubations contained 3% DMSO. For studies of the kinetics of inhibition of PtdIns-3-kinase by wortmannin, bovine brain enzyme was incubated with 0 to 14 nM wortmannin with varying ATP concentrations from 2.5 to 20 μM. For studies of the time course of PtdIns-3-kinase inhibition, wortmannin 0 to 14 nM, enzyme, and PtdIns were incubated for various times at room temperature before adding [γ-^{32}P]ATP to start the reaction.

PtdIns-4-kinase activity was measured in a similar manner to PtdIns-3-kinase activity except using partly purified enzyme except that the incubation contained 0.05% Nonidet P-40. PtdIns-3-kinase is completely inactive in the presence of Nonidet P-40 (21), whereas PtdIns-4-kinase retains full activity (22).

Other Kinase Assays. PKC was assayed as described previously using rat brain PKC (primarily PKCα and PKCβ) and histone as a substrate (23). Phosphatidylserine and phorbol ester but no Ca^{2+} were included in the assay. Protein tyrosine kinase activity was assayed using recombinant pp60$^{c\text{-}src}$. Briefly, the assay mixture consisted of 6 μg enzyme, 10 μg Raytide (Oncogene Science, Long Island, NY), 20 μg bovine serum albumin, and 2 μCi [γ^{32}P]ATP in 40 μl 20 mM 1,4-piperazinediethanesulfonic acid buffer (pH 7.0), 10 mM KCL, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 50 μM ATP. The mixture was incubated at 30°C for 15 min, and the reaction was terminated with 90 μl cold 3.3% trichloroacetic acid. After centrifugation at $6000 \times g$ for 5 min, a 50-μl aliquot of the supernatant was applied to ion exchange paper (Whatman P81; Maidstone, Kent, United Kingdom); the paper was washed four times with 1% phosphoric acid, and the activity remaining bound to the paper was determined by liquid scintillation counting.

PtdIns-3-phosphate Formation by Intact Cells. v-*sis* NIH 3T3 cells were chosen for measuring of PtdIns-3-phosphate levels because, unlike Swiss 3T3 cells, they exhibit constitutive as well as PDGF-stimulated PtdIns-3-kinase activity (24, 25). v-*sis* NIH 3T3 cells in logarithmic growth in a 75-cm^2 culture flask were placed for 2 h in DMEM without fetal calf serum. The cells were washed with phosphate-free DMEM and incubated in the same medium containing 0.1% fatty acid free bovine serum albumin and 0.15 mCi/ml [^{32}P]H_3PO_4 for 70 min. The cells were stimulated with 50 ng/ml PDGF for 10 min. ET-18-OCH_3, when used, was added to the incubation medium 30 min before the addition of PDGF. To measure PtdIns-3-phosphates in the cells, the medium was removed, and the cells were washed once with phosphate-buffered saline before adding 4 ml/flask of HCl:methanol (1:1 by volume). The cells were scraped from the flask, and total lipids were extracted by the method of Folch *et al.* (26). Deacylated lipids were prepared using methylamine as described by Clark and Dawson (27) and separated by HPLC using a 10-cm RAC II Partisil 5 SAX column (Whatman) eluted with an $NH_4H_2PO_4$ gradient at a flow of 0.8 ml/min as described by Auger *et al.* (28). Detection of the eluting peaks was by a radioactive flow detector (Flo-One Beta, Model A515; Radiomatic Instruments, Meriden, CT). The reference compounds used were [^{3}H]PtdIns(3,4,5)bisphosphate and [^{32}P]PtdIns(3,4,5)trisphosphate. [^{32}P]PtdIns(3,4,5)trisphosphate was prepared by the [γ-^{32}P]ATP-dependent phosphorylation of PtdIns(4,5)bisphosphate by PtdIns-3-kinase as described above.

RESULTS

Wortmannin was found to be a potent inhibitor of PtdIns-3-kinase activity in antiphosphotyrosine immunoprecipitates from Swiss 3T3 cells with an IC_{50} of 1.9 nM. The wortmannin analogues 2 and 3 were almost equally active as wortmannin in inhibiting PtdIns-3-kinase (Fig. 2). The wortmannin analogues 4 and 5 inhibited immunoprecipitated PtdIns-3-kinase but could not produce greater than a 50% inhibition of the enzyme.

In order to obtain accurate estimates of the IC_{50}s of wortmannin and its analogues, we used purified bovine brain PtdIns-3-kinase (Fig. 3). The IC_{50}s (determined from triplicate studies) were: wortmannin analogue 1, 1.8 ng/ml (4.2 nM); analogue 2, 6.2 ng/ml (16.7 nM); analogue 3, 20 ng/ml (54 nM); analogue 5, 1500 ng/ml (4.6 μM); and analogue 4 did not inhibit PtdIns-3-kinase at the highest dose tested, 32 μM.

PtdIns-3-kinase activity was assayed at various concentrations of ATP in the presence of increasing concentrations of wortmannin. As indicated in Fig. 4, Lineweaver-Burk analysis suggests that wortmannin decreased V_{max} without affecting K_m, suggesting that the apparent mode of inhibition is noncompetitive with respect to ATP. Since irreversible inhibitors generally show apparent noncompetitive inhibition, the reversibility of inhibition of PtdIns-3-kinase was investigated by preincubating the enzyme with wortmannin prior to the addition of ATP for different times. Fig. 5 shows that inhibition of PtdIns-3-kinase by wortmannin is time dependent. As preincubation time increases, enzyme activity decreases, suggesting that a covalent, irreversible adduct is formed between the kinase and wortmannin.

Wortmannin and its analogues were not inhibitors of PtdIns-4-kinase at concentrations up to about 10 μg/ml (32 μM) (Fig. 6).

[4] C. J. Vlahos, W. F. Matter, K. Y. Hui, and R. F. Brown, submitted for publication.

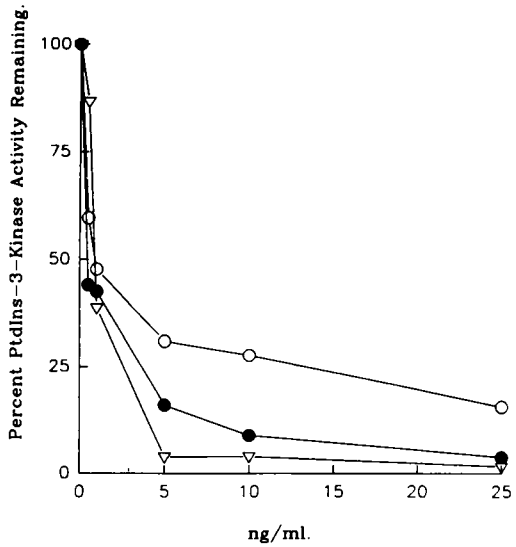


Fig. 2. Inhibition of antiphosphotyrosine immunoprecipitated PtdIns-3-kinase by wortmannin and analogues. PtdIns-3-kinase was immunoprecipitated from PDGF-stimulated Swiss 3T3 cells and activity measured as described in the text. ○, wortmannin *1*; ●, analogue *2*; and ▽, analogue *3*.

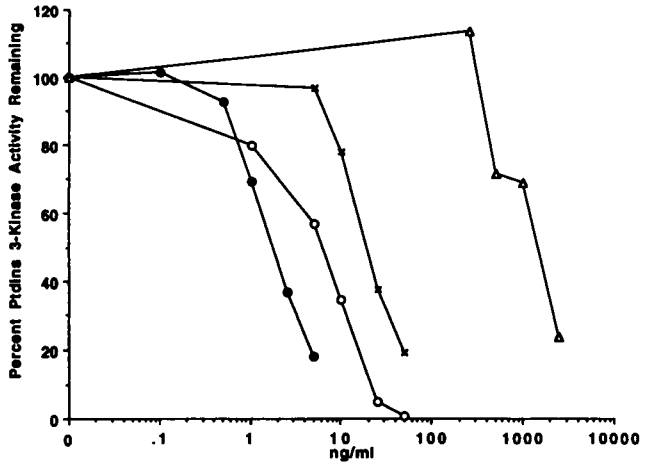


Fig. 3. Determination of IC_{50}s for inhibition of purified PtdIns-3-kinase by wortmannin and its analogues. Methods are as described in the text. ●, wortmannin *1*; ○, analogue *2*; (X) analogue *3*; △, analogue *5*.

Wortmannin at a concentration of 1.3 μM almost completely inhibited the formation of PtdIns-3-phosphates by PDGF-stimulated v-*sis* NIH 3T3 cells (Fig. 7). Treatment of these cells with wortmannin (Fig. 7*B*) had no effect on the pattern of phospholipids in resting cells (Fig. 7*A*). PDGF stimulation of the v-*sis* 3T3 cells caused formation of PtdIns-3,4,5-P_3 (Fig. 7*C, arrow*), which is not present in PDGF-stimulated cells treated with wortmannin (Fig. 7*D*). Levels of PtdIns-4,5-P_2 were not affected by wortmannin in resting or PDGF-stimulated cells.

DISCUSSION

Wortmannin and some of its analogues were found to be potent inhibitors of PtdIns-3-kinase with IC_{50}s for wortmannin of 1.8 to 4.0 nM and for other wortmannin analogues tested of 20 nM to 4.3 μM. Wortmannin has previously been reported to be an inhibitor of MLC kinase with an IC_{50} of 0.17 μM but did not inhibit cAMP-dependent protein kinase, cGMP-dependent protein kinase, and calmodulin-dependent protein kinase II (15). We have found that wortmannin and its analogues did not inhibit other intracellular signaling enzyme targets. Wortmannin and its analogues are not inhibitors of PtdIns-4-kinase (at concentrations up to 32 μM), protein kinase C (at concentrations up to 22 μM), c-*src* protein tyrosine kinase (at concentrations up to 5.5 μM), and phosphoinositide-specific phospholipase C (at concentrations up to 0.27 mM). Thus, wortmannin is a highly specific as well as a potent inhibitor of PtdIns-3-kinase.

Few comments can be made at this time regarding the structure-activity relationships between the wortmannins and inhibition of PtdIns-3-kinase. It appears, however, that the A ring (*i.e.*, lactone) may be necessary for effective inhibition. The two poorest inhibitors, analogues 4 and 5, are lacking this structural feature. Interestingly, these analogues also lack an α,β-unsaturated ketone, which may be necessary for formation of a covalent adduct between the enzyme and the inhibitor.

There are relatively few known inhibitors of PtdIns-3-kinase. Quercetin and its analogues are relatively weak inhibitors of PtdIns-3-kinase with IC_{50}s of 4 to 20 μM (29). Other inhibitors of PtdIns-3-kinase, also relatively weak, are CGP-41251 (benzoylstaurosporine; IC_{50}, 9 μM); tyrphostin (IC_{50}, 33 μM); and the ether lipid ET-18-OCH_3 (IC_{50}, 36 μM) (30). Recently, a highly selective inhibitor of PtdIns-3-kinase was reported, LY294002 (2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; Ref. 31); however, this compound is

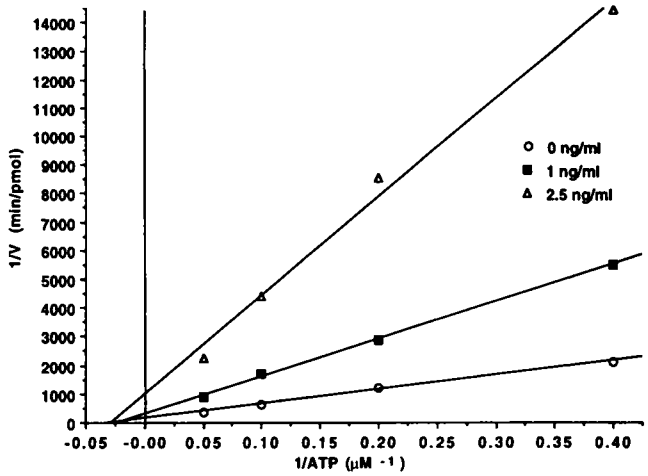


Fig. 4. Lineweaver-Burk plots of the inhibition of PtdIns-3-kinase with wortmannin. Inhibition by (○) 0 ng/ml, (■) 1 ng/ml, or (△) 2.5 ng/ml wortmannin against varying concentrations of ATP. Methods are described in the text.

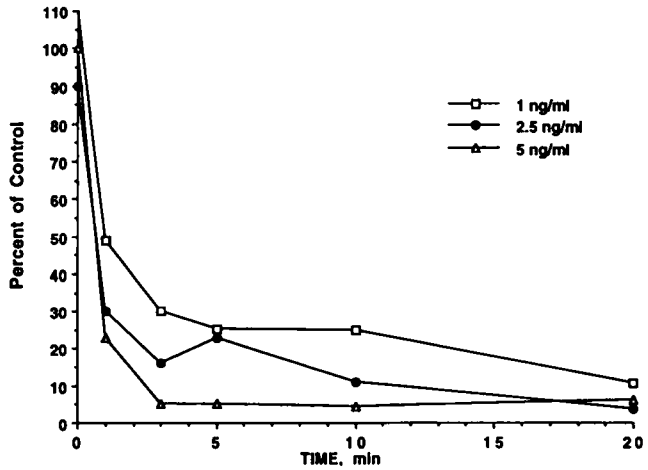


Fig. 5. Wortmannin preincubation time dependence. Bovine brain PtdIns-3-kinase was incubated at various times with (□) 1 ng/ml, (●) 2.5 ng/ml, or (△) 5 ng/ml wortmannin. At indicated times, samples were assayed for PtdIns-3-kinase activity as described in the text.

much less potent than wortmannin (IC_{50}, 1.4 μM) and differs in mechanism, being a competitive inhibitor of the ATP binding site of PtdIns-3-kinase. Thus, wortmannin is considerably more potent at inhibiting the enzyme than other inhibitors so far discovered.

Kinetic analysis and preincubation experiments suggest that wortmannin irreversibly inhibits PtdIns-3-kinase. The activity of the enzyme decreases as time of preincubation with wortmannin increases, suggesting an irreversible, covalent adduct. Kinetic studies show that wortmannin inhibits the enzyme in an apparent noncompetitive manner, which is consistent with an irreversible inhibitor. The structure of wortmannin suggests at least two sites for covalent addition: an α,β-unsaturated ketone and a lactone ring, both of which would be susceptible for addition by a nucleophilic group in the protein. Similar findings were obtained with the inhibition of myosin light chain kinase by wortmannin (15); however, wortmannin is 100-fold more potent against PtdIns-3-kinase as opposed to myosin light chain kinase.

Wortmannin inhibited the formation of PtdIns-3-phosphates in intact cells. However, whether this is related to the cell killing effects of wortmannin remains to be determined. Wortmannin killed cells at much higher concentrations, 1 to 25 μg/ml (2.3–58.4 μM), than required to inhibit purified PtdIns-3-kinase.

Wortmannin has been reported to block agonist-mediated tyrosine phosphorylation and the activation of phosphatidylinositol-specific phospholipase C and phospholipase D in human neutrophils (32, 33). In rat pheochromocytoma PC12 cells, wortmannin enhances the muscarinic stimulation of phospholipase D activity (34). According to our present results, wortmannin does not affect the phospholipases directly (32, 33) and whether inhibition of PtdIns-3-kinase could be involved in these effects is not known.

Wortmannin has been reported to have antiinflammatory activity based on inhibition of neutrophil activation by protein kinase C independent pathways (35). It also suppresses several other cellular responses including IgE-mediated histamine release in basophil and basophilic leukemia cells (36, 37), high K^+ and acetylcholine-induced catecholamine release by adrenal chromaffin cells (38), superoxide formation in neutrophils (35), IgG induced superoxide formation in eosinophils (39) and platelet responses to various stimuli (40). Whether any of these effects is due to inhibition of PtdIns-3-kinase will have to be determined. However, many of the biological effects of wortmannin are seen at concentrations of wortmannin lower than those required to inhibit MLC kinase and closer to those that inhibit PtdIns-3-kinase, so that it is possible that this represents the mechanism of action of the drug.

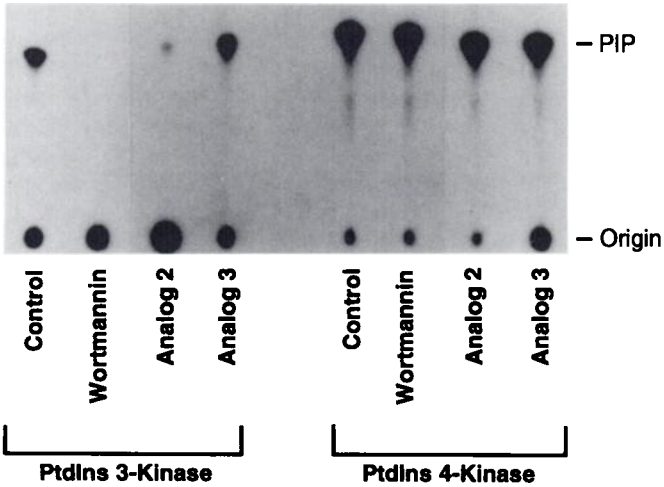


Fig. 6. Thin layer chromatography autoradiogram showing the effects of wortmannin and its analogues on the formation of PtdIns monophosphates (*PIP*) from PtdIns and [α-^{32}P]ATP with PtdIns-3-kinase (*left*) and PtdIns-4-kinase (*right*). Wortmannin and its analogues were present at a concentration of 10 μg/ml.

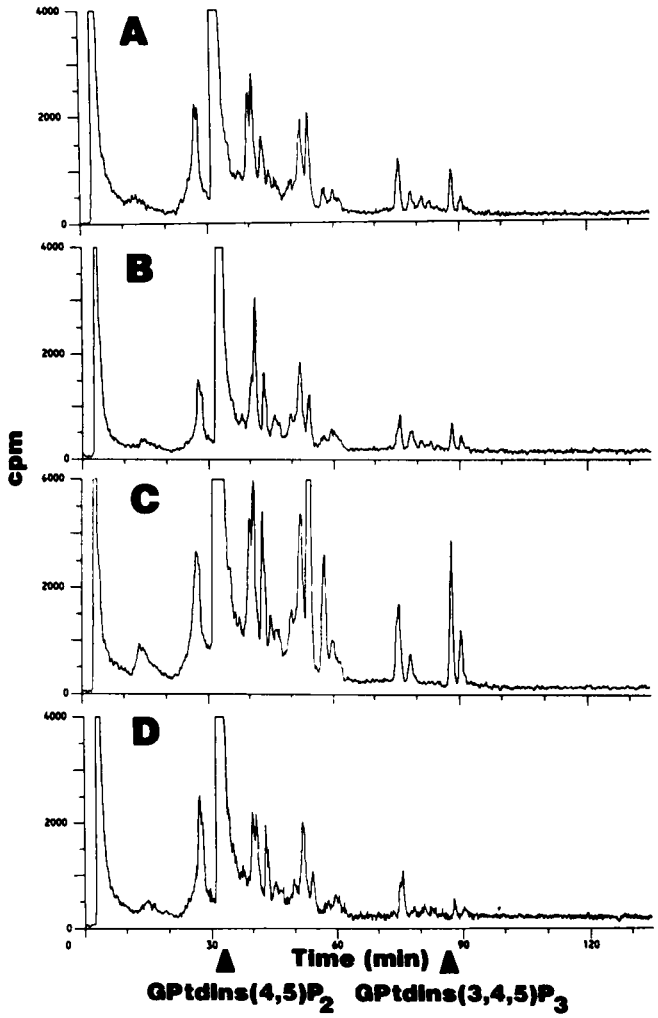


Fig. 7. Anion exchange chromatography of the deacylated products derived from lipid extracts of ^{32}P-labeled v-*sis* NIH 3T3 cells. *A*, nonstimulated cells; *B*, cells exposed to 1.3 μM wortmannin; *C*, cells stimulated with 50 ng/ml PDGF for 10 min; and *D*, cells treated with 1.3 μM wortmannin and stimulated with 50 ng/ml PDGF for 10 min. The position of the deacylated glycero (G) reference compounds are shown by *arrowheads*; GPtdIns(4,5)P_2 derived from PtdIns(4,5)P_2; and GPtdIns(3,4,5)P_3 derived from PtdIns(3,4,5)P_3. The results are typical of three separate studies.

In summary, we have shown that wortmannin and some of its analogues are potent inhibitors of PtdIns-3-kinase. Wortmannin is also a highly specific inhibitor of the enzyme with no effect on PtdIns-4-kinase, c-*src* protein tyrosine kinase, or protein kinase C with a potency up to a 100-fold greater than its previously reported activity as an inhibitor of myosin light chain kinase.

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