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ORIGINAL ARTICLE

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Wortmannin inhibits the growth of mammary tumors despite the existence of a novel wortmannin-insensitive phosphatidylinositol-3-kinase

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Abstract *Purpose*: Phosphatidylinositol (PtdIns) 3-kinase is an important mediator of many cellular functions. The study of PtdIns 3-kinase has been facilitated by the existence of the potent irreversible inhibitor of p110 PtdIns 3-kinase, wortmannin. The purpose of the study was to investigate the relationship between the cell growth inhibitory activity and antitumor activity of wortmannin and inhibition of PtdIns 3-kinase. *Methods*: PtdIns 3-kinase activity was measured in cells and tumors and the effects of wortmannin investigated. *Results*: Wortmannin inhibited the growth of murine C3H and human MCF-7 mammary tumors in vivo. However, the ability of wortmannin to inhibit C3H tumor growth was not related to inhibition of tumor PtdIns 3-kinase activity. The existence of wortmannin-insensitive PtdIns 3-kinase activity was demonstrated in C3H and MCF-7 cell culture lysates and solid tumors, and normal mouse tissue homogenates. In addition to being resistant to inhibition by wortmannin, MCF-7 cell lysate total PtdIns 3-kinase activity was also resistant to five additional known inhibitors of p110 PtdIns 3-kinase. Partial purification of wortmannin-insensitive PtdIns 3-kinase from MCF-7 cell lysate showed the activity to be independent of the PtdIns 3-kinase p85 regulatory subunit. *Conclusion*: The results of the current study demonstrate that wortmannin can inhibit the growth of murine and human mammary tumors despite the presence of novel wortmannin-insensitive PtdIns 3-kinases in these tissues suggesting that some other target is responsible for wortmannin's antitumor activity.

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Introduction

Phosphatidylinositol (PtdIns) 3-kinase was originally identified as an enzyme that phosphorylates the D-3-OH position of the *myo*-inositol ring of the minor cell membrane phospholipid, PtdIns [19]. The most studied member of the PtdIns 3-kinase family is a heterodimer consisting of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) [21]. All of the known isoforms of p110 are capable of phosphorylating both PtdIns and PtdIns(4)phosphate in vitro, but PtdIns-(4,5)bisphosphate is the preferred substrate in vivo [21]. PtdIns 3-kinase is activated by a wide range of growth factor receptors and oncogene protein tyrosine kinases [4] as well as by $p21^{Ras}$ [18]. PtdIns 3-kinases belong to an extended family of high molecular mass kinases containing the PtdIns 3-kinase-related C-terminal catalytic domain which consists of the protein kinase subdomain VIB and VII motifs DXXXXN and DFG, respectively (where X represents any amino acid) [9]. Members of this family include yeast rad3p, Mec1p, Tel1p, Tor1p, Tor2p and the *Drosophila mei*-41 gene product and the mammalian proteins mTor, DNA-dependent protein kinase and the *Ataxia telangiectasia* gene product, ATM [1, 11]. Except for the smaller PtdIns 3-kinases themselves, these family members do not possess lipid kinase activity but are serine/threonine protein kinases [1].

Interest in PtdIns 3-kinase as a target for anticancer drug development comes from situations where mutated oncogenic tyrosine kinases fail to associate with and activate PtdIns 3-kinase [13, 15]. For example, polyoma middle T mutants which associate with and activate $pp60^{c\text{-}src}$ tyrosine kinase but which fail to activate PtdIns 3-kinase are nontransforming [10]. The levels of cellular PtdIns 3-phosphates are elevated by transforming mutants of middle T but not by transformation defective

mutants [17]. PtdIns 3-kinase also has an important role in preventing apoptosis and is necessary for the inhibition of apoptosis by nerve growth factor in PC12 pheochromocytoma cells [23] and by interleukin-3 and -4 in hematopoietic cells [21].

The fungal metabolite wortmannin was identified by us as a potent inhibitor of PtdIns 3-kinase [14]. Nucleophilic attack by C-20 of the furan ring of wortmannin at Lys^{802} of p110 PtdIns 3-kinase results in irreversible inhibition of the enzyme [22] with an IC_{50} of 4 n*M* [14]. Wortmannin has been used extensively as a pharmacological probe of the many cellular functions of PtdIns 3-kinase [8]. More recently it has been shown that wortmannin inhibits other serine/threonine kinases of the PtdIns 3-kinase family, for example mTOR and DNA-dependent protein kinase with IC_{50} values of 2 to 4 n*M*, respectively [2, 3]. When tested against a panel of murine tumors and human tumor xenografts in mice, wortmannin has also been shown to inhibit the growth of C3H murine mammary carcinoma and human BxPC-3 pancreatic carcinoma xenografts [16], although a link to inhibition of PtdIns 3-kinase was not investigated.

In the study reported here we investigated the ability of wortmannin to inhibit the growth of both murine and human mammary tumors in mice and the inhibition of PtdIns 3-kinase in relation to this inhibition of tumor growth.

Materials and methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise stated. [^{32}P]-ATP (3000 Ci/mmol) was purchased from NEN Life Science Products, Boston, Mass. Wortmannin was provided by Dr. Steadman Harrison of Eli Lilly and Co. (Indianapolis, Ind.). Dr. Alan Kozikowski (Georgetown University) synthesized and kindly provided the D-3′-deoxy-PtdIns. Antibodies used were rabbit antirat PtdIns 3-kinase p85 (Upstate Biotechnology, Lake Placid, N.Y.) and donkey antirabbit horseradish peroxidase-labeled IgG (Amersham, Arlington Heights, Hl.). Human recombinant PtdIns 3-kinase was a gift from Dr. Stan Barnett (Merck, West Point, Pa.).

Cell culture

MCF-7 human breast cancer cells (American Type Culture Collection, Rockville, Md.) were grown in Dulbecco's minimum Eagle's medium with 10% fetal bovine serum (Gibco BRL, Grand Island, N.Y.) and 1% penicillin/streptomycin at 37 °C under an atmosphere containing 5% CO_2. The C3H mammary tumor cell line (provided by Dr. Richard M. Schultz, Eli Lilly and Co., Indianapolis, Ind.) was cultured in RPMI-1640 medium supplemented with 1% L-glutamine (Fischer, Phoenix, Ariz), 25 m*M* Hepes, pH 7.4, and 10% fetal bovine serum at 37 °C under an atmosphere containing 5% CO_2. The cell lines were tested to be mycoplasma-free with an Elisa kit (Boehringer Mannheim, Indianapolis, Ind.).

Antitumor activity studies

Scid mice were implanted s.c. with a 0.25-mg 21-day release 17-estradiol pellet (Innovative Research of America, Toledo, Ohio), and 24 h later inoculated with the C3H and MCF-7 hormone-dependent breast cancer cell lines. Additional 17-estradiol pellets were implanted every 21 days for the duration of the study. Murine mammary C3H tumor fragments were implanted s.c. in the right mammary fat pad of 20-g female C3H mice as previously described [16]. MCF-7 cells (10^7) were injected s.c. into the hind quarters of the mice. Tumor burdens were assessed from two-dimensional measurements taken three times a week during the course of study. Tumor volumes were calculated with the formula:

$$\text{Tumor volume (ml)} = \text{Tumor length (cm)} \times [\text{Tumor width (cm)}]^2/2$$

In studies where only final masses were reported, tumors were excised and weighed after the completion of the study period. Wortmannin was administered to mice by oral gavage as a 0.1 mg ml^{-1} solution in 0.1% Tween 20 with 10% ethanol at daily doses of 1 or 1.5 mg kg^{-1} for up to 14 days. Animal studies were approved by the Institutional Animal Care and Use Committee.

In vitro PtdIns 3-kinase assays

C3H tumor, brain, liver and kidney were harvested from control and wortmannin-treated mice. The tissues were homogenized using a polytron homogenizer (Kinematica, Switzerland) in three volumes of lysis buffer (20 m*M* Tris-HCl, pH 8.0, 137 m*M* NaCl, 10% glycerol, 0.1% Triton X-100, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1 m*M* sodium orthovanadate, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 2 μg ml^{-1} leupeptin and 2 μg ml^{-1} aprotinin) at 4 °C. Cells were washed with phosphate-buffered saline (1 m*M* KH_2PO_4, 10 m*M* Na_2HPO_4, 137 m*M* NaCl, 2.7 m*M* KCl pH 7.0) and incubated at 4 °C for 20 min in 3 ml lysis buffer. Tissue homogenates and cell lysates were centrifuged at 16 000 *g* for 5 min and the supernatant diluted to a final protein concentration of 0.01 to 0.1 μg protein μl^{-1} in 20 m*M* Tris-HCl, pH 8.0, 137 m*M* NaCl and 1 m*M* EDTA.

Prior to mixing reaction components, PtdIns 3-kinase inhibitors were dried into the reaction tubes from stocks prepared in 10 μl dimethylsulfoxide. Incubations contained 30 μl of diluted crude, partially purified or recombinant PtdIns 3-kinase preparations with 10 μl PtdIns or D-3-deoxy-PtdIns (0.5 mg ml^{-1} in 20 m*M* HEPES, pH 7.6) as substrate. Reactions were initiated by the addition of 10 μ*M* [^{32}P]-ATP (1 Ci/mmol). Samples were incubated at 37 °C for 45 min, quenched by the addition of 100 μl 1 *N* HCl and extracted with 400 μl of 1:1 chloroform/methanol then centrifuged at 2000 *g* for 1 min. For each sample, 25 μl of the lower organic phase was spotted onto the preabsorbent strip of an individual lane in a multichannel thin-layer chromatography Silica gel 60 plate (Whatman, Hillsboro, Ore.). Plates were developed in 65% *n*-propyl alcohol/35% 2 *M* acetic acid. [^{32}P]-Labeled product was quantitated by phosphorimager analysis (Molecular Dynamics, Sunnyvale, Calif.). The absence of a hydroxyl group at the 3′ position of PtdIns in 3′-deoxy-PtdIns prevents 3′-phosphorylation and allows the measurement of non-PtdIns 3-kinase activity [12]. PtdIns 3-kinase activity was determined by subtracting the activity detected in D-3-deoxy-PtdIns-containing samples from that in corresponding PtdIns-containing samples. Values expressed as PtdIns-kinase activity signify phosphorylations at all possible positions of PtdIns. Values expressed in PtdIns 3-kinase activity signify phosphorylation only at the 3′-OH position of PtdIns. Percent inhibition of enzyme activity was calculated from the quotient of the mean treated value divided by the mean control value.

Partial purification of PtdIns 3-kinase

Cell lysates used in fractionation studies were prepared as described above except for the use of low-salt lysis buffer (20 m*M* NaCl substituted in lysis buffer). Chromatography was performed on a fast protein liquid chromatography system (Pharmacia Biotech, Piscataway, N.J.) at 4 °C. Solubilized protein was fractionated by

loading onto a 5 ml MonoQ ion exchange column with buffer A (20 m*M* Tris-HCl pH 7.2, 1 m*M* EDTA, 1 m*M* beta-mercaptoethanol, 1 m*M* PMSF and 0.01% Triton X-100). Bound protein was eluted with a gradient to 50% B (buffer A with 1 *M* NaCl) for 70 ml followed by a gradient to 100% B for 15 ml and 3-ml fractions were collected.

Immunoblotting analysis

Western blots were performed by electroblotting proteins resolved on 7.5% acrylamide gel to a polyvinylidene difluoride membrane (Dupont, Boston, Mass.). After protein transfer, 3% bovine serum albumin (BSA) in TBST (20 m*M* Tris-HCl, pH 7.6, 0.5 *M* NaCl, 0.1% Tween 20) was used to block nonspecific binding. Blots were exposed to primary antibody (0.1 to 1.0 μg ml^{-1} in 3% BSA TBST) for 1 h, washed in TBST and incubated for 1 h in the presence of secondary antibody (2000-fold dilution of horseradish peroxidase-labeled antirabbit antibody in 3% BSA TBST). The Renaissance® chemiluminescence detection system (NEN Life Science Products, Boston, Mass.) was used to visualize immunolabeled proteins.

Statistical analysis

Individual sample means were compared using Student's *t*-test and differences were considered statistically significant if the *P*-value was < 0.05 using $n-1$ degrees of freedom. Tumor growth rates were determined from the slopes of the linearized tumor masses and compared by Students *t*-test. Statistical analysis of in vivo and in vitro inhibition curves was performed by the Biometry Department, Arizona Cancer Center, by the method of parallel, intersecting and identical models.

Results

Mammary tumor growth inhibition by wortmannin

A dose of 1 mg kg^{-1} wortmannin administered by oral gavage for 7 days decreased the tumor burdens in mice with established murine C3H mammary tumors by 54% relative to controls ($P < 0.05$; Fig. 1A). The growth rate of the treated tumors was significantly slower during drug administration than that of nontreated tumors ($P = 0.002$) and inhibition of tumor growth was maintained for at least 7 days after treatment was discontinued ($P = 0.0003$). The growth of advanced (1 g) C3H mammary tumor (Fig. 1B) was inhibited by 44% ($P < 0.05$) when wortmannin was administered by oral gavage for 6 days.

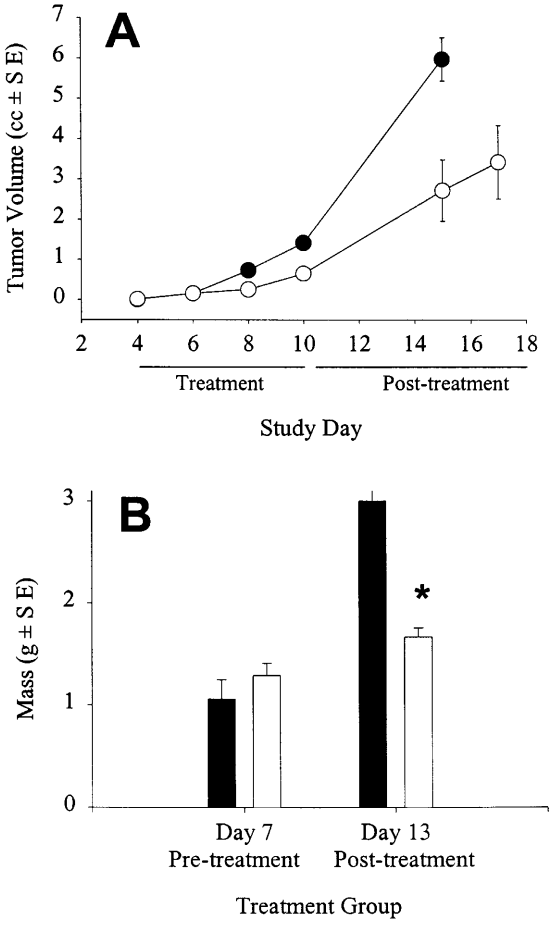

Fig. 1A, B Effect of wortmannin on the growth of C3H mammary tumor. C3H tumor fragments were implanted subcutaneously with a trocar catheter into the axillary region of female C3H mice and allowed to grow for 4 to 7 days. **A** At 4 days, when tumors had become palpable, wortmannin was administered by oral gavage on a daily ×7 schedule at 1 mg kg^{-1} (i.e. on days 4 to 10). Tumor volumes were calculated from tumorimeter measurements on the noted day of the study. Results are expressed as the mean ± SE of eight mice per group (control, treated). **B** At 7 days, when tumor burdens reached approximately 1 g, wortmannin was administered by oral gavage on a daily ×6 schedule at 1 mg kg^{-1} (i.e. on days 7 to 13). Tumor masses were determined at the time of sacrifice. Results are expressed as the mean ± SE of three mice per group. $^*P < 0.05$ *vs* corresponding control (control treated)

Human MCF-7 breast cancer xenograft burdens were decreased by 97% ($P = 0.005$) relative to controls after 14 days of 1 mg kg^{-1} wortmannin administration by oral gavage beginning 1 day after tumor implantation (Fig. 2A). In this study, five of eight mice treated with 1.0 mg kg^{-1} wortmannin had no detectable tumor at the end of the treatment period, although tumors grew in all eight mice after the wortmannin administration period. A second group of mice (the dose-reduced group) were treated with a 1.5 mg kg^{-1} dose of wortmannin for 5 days before initial weight loss necessitated that the dose be decreased to 1.0 mg kg^{-1} for the remainder of the study. The growth rate of treated tumors was significantly slower than that of the controls during wortmannin treatment ($P < 0.0001$ for both the reduced and 1 mg kg^{-1} groups), but was not different from the controls during the postadministration period. The growth rate of the wortmannin-treated tumors was significantly slower during treatment than during the postadministration period ($P = 0.0003$ and 0.002 for the reduced and 1 mg kg^{-1} doses, respectively).

Tumor burdens were decreased by 85% ($P = 0.0004$) when wortmannin administration was begun 7 days after MCF-7 tumor implantation (Fig. 2B). The inhibition of tumor growth rate observed with wortmannin was significant when compared to untreated controls ($P = 0.0001$). During the posttreatment period, all of the wortmannin-treated mice developed tumors which

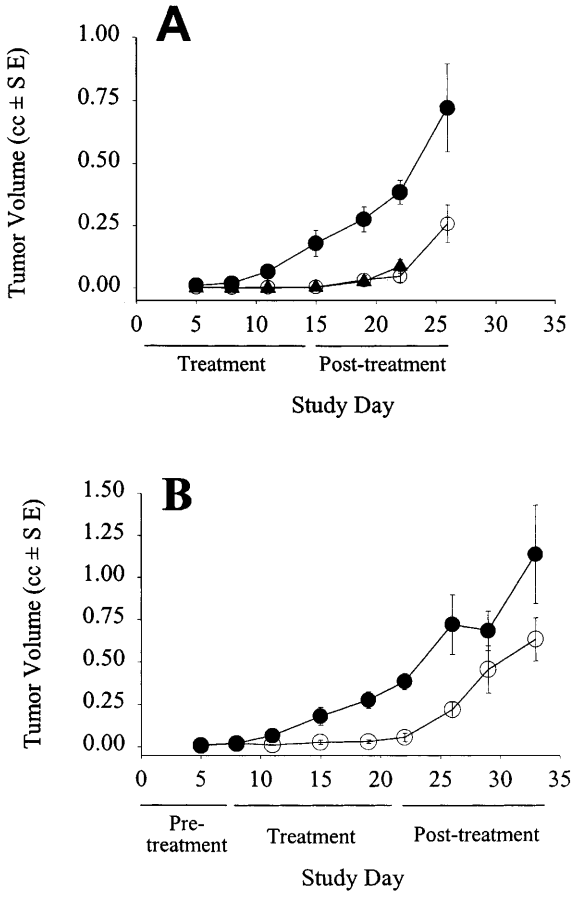

Fig. 2A, B Effect of wortmannin on MCF-7 tumors in *scid* mice. Mice were innoculated s.c. with 10^7 MCF-7 cells. **A** One day after tumor inoculation wortmannin was administered by oral gavage on a daily ×14 day schedule (i.e. on days 1 to 14). One group of eight mice was dosed with 1 mg kg^{-1} wortmannin for all 14 days. The second group of eight mice was dosed with wortmannin 1.5 mg kg^{-1} for the first 5 days and the dose was decreased to 1 mg kg^{-1} for the remaining treatment period. Tumor volumes were calculated from caliper measurements and are expressed as the mean ± SE of eight mice per group (control, 1 mg kg^{-1} wortmannin for 14 days, 1.5 mg kg^{-1} wortmannin for 5 days then 1.0 mg kg^{-1} wortmannin for 9 days). **B** Tumors were allowed to grow for 7 days, by which time the mean tumor volume was 0.02 ml, and wortmannin was then administered by oral gavage at 1 mg kg^{-1} daily ×14 days (i.e. on days 8 to 21). Tumor volumes were calculated from caliper measurements and are expressed as the mean ± SE of eight mice per group. (control, 1 mg kg^{-1} wortmannin for 14 days)

grew at a rate which was not significantly different from that of controls, but was significantly different than their own growth rate during wortmannin administration ($P = 0.0001$).

Inhibition of tumor PtdIns 3-kinase

We investigated whether the PtdIns 3-kinase activity of tumor and normal mouse tissues was altered by in vivo wortmannin treatment (Table 1). There were no significant differences in total PtdIns 3-kinase activity in tumors or in spleen, liver and brain of nontreated and wortmannin-treated mice. No differences in in vitro wortmannin sensitivity were detected between tumor homogenates from control and wortmannin treated animals (Fig. 3).

Table 1 PtdIns 3-kinase activity in tissues harvested from C3H mice. Tissues from mice treated with wortmannin daily for 6 days were homogenized, diluted to 0.01 to 0.1 mg ml^{-1} protein and assayed for total PtdIns 3-kinase activity. Values are means ± SE from duplicate determinations for tissue from each of the three mice in the group

Tissue	PtdIns 3-kinase activity (pmol/mg protein per 45 min)	
	Control	Treated
Tumor	161.4 ± 24.5	147.0 ± 14.9
Brain	142.6 ± 9.5	116.8 ± 14.5
Spleen	8.4 ± 0.7	6.2 ± 0.9
Liver	31.5 ± 3.7	43.0 ± 9.1

Cellular PtdIns 3-kinase

Because tissue PtdIns 3-kinase appeared to be resistant to inhibition by wortmannin, we investigated the effects of wortmannin on PtdIns 3-kinase activity in breast cancer cell lysates. MCF-7 and C3H cell lysate PtdIns 3-kinase activity was inhibited in vitro by 49% and 44%, respectively, at 5 n*M* wortmannin (Fig. 4). Wortmannin at 1 μ*M* inhibited PtdIns 3-kinase in MCF-7 and C3H lysates by 83.0% and 69.6%, respectively. Significantly different PtdIns 3-kinase inhibition slopes existed for the 0 to 10 and 50 to 1000 n*M* wortmannin concentrations in each cell line ($P = 0.0001$). It is noteworthy that while 60% of C3H cell lysate PtdIns 3-kinase was inhibited by 50 n*M* wortmannin (Fig. 4), even at 1 μ*M*

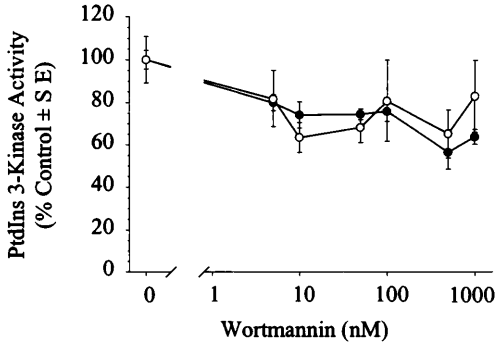

Fig. 3 In vitro wortmannin sensitivity of C3H tumor PtdIns 3-kinase. Homogenates of tumors from mice treated in vivo with wortmannin daily for 6 days and control mouse tumor were diluted 0.01 to 0.1 μg μl^{-1} protein. PtdIns 3-kinase reactions were performed on diluted homogenate in the presence of various concentrations of wortmannin. Results are expressed as mean ± SE of triplicate determinations for one mouse in the control and treated groups, respectively. Values from one of two representative experiments are expressed as percent of control (6 day control, 6 day treated)

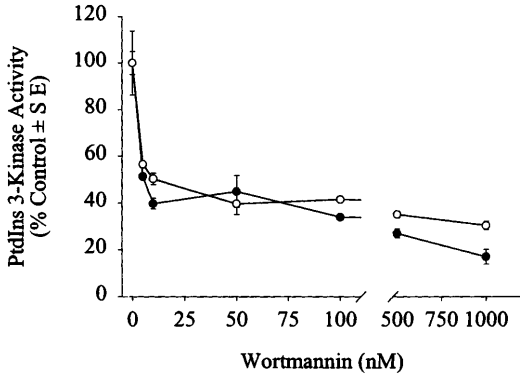

Fig. 4 Inhibition of PtdIns 3-kinase in breast cancer cell lysate. Cell lysates of monolayer cultures of MCF-7 and C3H cells were prepared, diluted to 0.01 to 0.1 μg μl^{-1} protein and used as the enzyme source in PtdIns 3-kinase assays. The MCF-7 cell line tested was that used in our in vivo studies. The C3H line was a derivative of the trocar-passaged tumor which had been adapted for monolayer culture. Reactions were performed in the presence of various concentrations of wortmannin. Data from one representative experiment are included expressed as the means and SE of triplicate determinations at each wortmannin concentration ($n = 2$ experiments). Activity is expressed as percent of control, (MCF-7, C3H)

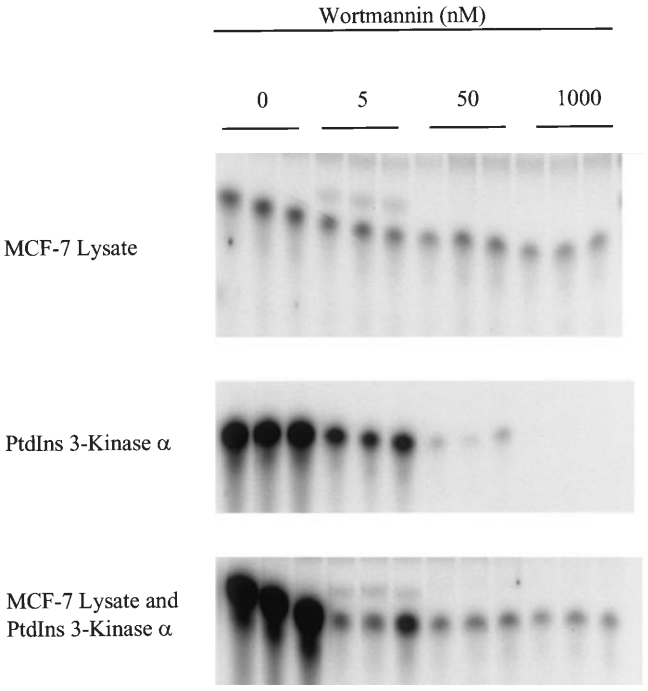

Fig. 5 Incorporation of [^{32}P] into PtdIns-3-phosphate by MCF-7 cell lysate and human recombinant PtdIns 3-kinase. Aliquots of the chloroform layer from PtdIns 3-kinase reaction extracts were applied to Silica gel 60 plates. Samples were developed in a mobile phase of 30% 2 *M* acetic acid 70% *n*-propanol until the solvent front had migrated two-thirds of the distance to the top of the plate. After drying, the TLC plates were cut to remove the sample loading zone and the undeveloped portion of the plate. The plates were exposed to a phosphorescent screen for 24 h before being scanned on a Molecular Dynamics Phosphorimager using ImageQuant analysis software

wortmannin ($200 \times IC_{50}$ for PtdIns 3-kinase) 50% inhibition of C3H tumor PtdIns 3-kinase was not observed (Fig. 3).

A panel of PtdIns 3-kinase inhibitors were assessed for their ability to inhibit MCF-7 cell lysate PtdIns 3-kinase at ten times their IC_{50} for bovine brain PtdIns 3-kinase. Of the compounds tested (Table 2), only quinone XVIII was able to inhibit more than 50% of the PtdIns 3-kinase in MCF-7 lysate.

Inhibition of human recombinant PtdIns 3-kinase was used to confirm that the wortmannin-insensitivity of breast cancer cells was not an artifact of the assay system. As shown in Fig. 5, the incorporation of [^{32}P]-ATP into PtdIns 3-phosphate by recombinant PtdIns 3-kinase was inhibited by 84% at 5 n*M* wortmannin and completely blocked (>99%) by 50 n*M* wortmannin. Combining MCF-7 cell lysate and recombinant PtdIns 3-kinase in the kinase reaction appeared to potentiate the activity of the recombinant enzyme while maintaining its sensitivity to wortmannin.

Table 2 Inhibition of MCF-7 cell lysate PtdIns 3-kinase. Compounds known to inhibit bovine brain PtdIns 3-kinase were tested at 10× their respective IC_{50} [6, 7] for inhibition of MCF-7 cell lysate PtdIns 3-kinase activity in the in vitro assay. Values listed were from one representative experiment ($n = 2$) and the SE of triplicate determinations did not exceed 10% of the mean

Compound	Dose (μ*M*)	Inhibition (%)
Pentagalloylglucose	5	10
Hypericin	20	1
Quercitin	50	9
LY294002	50	38
Suramin	100	39
Quinone XVIII	1000	54

Partial purification of wortmannin-insensitive PtdIns 3-kinase

Fractionation of MCF-7 breast cancer cell lysate demonstrated that PtdIns 3-kinase accounted for most of the PtdIns kinase activity in these cells (Fig. 6). As expected the majority of this PtdIns 3-kinase activity was correlated to p85 subunit expression. PtdIns 3-kinase activity detected in fractions 12 and 13 (Fig. 6) was not associated with p85 subunit expression and was not wortmannin-sensitive (data not shown). The anti-p85 antibody does not detect the p110 subunit of PtdIns 3-kinase, so the identity of the 110-kDa cross-reacting band was unknown.

Discussion

Wortmannin has previously been reported to be an effective antitumor agent in prepalpable murine C3H mammary and human BxPC-3 pancreatic tumor models [16]. The results of the current study demonstrate the effectiveness of wortmannin as an inhibitor of the growth of palpable murine C3H mammary tumors and both prepalpable and palpable human MCF-7 mam-

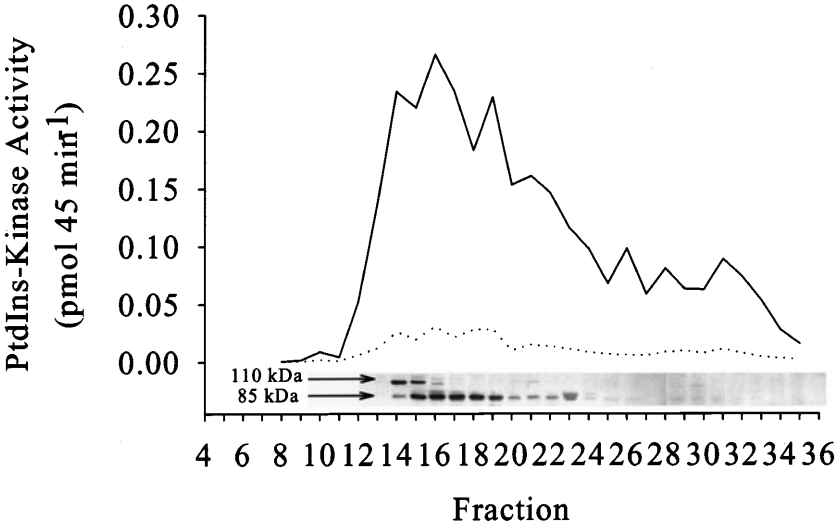

Fig. 6 Fractionation of MCF-7 cell lysate PtdIns 3-kinase activity by Mono Q anion exchange chromatography. MCF-7 cell lysate was prepared and fractionated using a Mono Q anion exchange FPLC column at 4 °C. PtdIns 3-kinase activity and non-PtdIns 3-kinase activity was determined with PtdIns and 3′-deoxy-PtdIns as substrates, respectively. PtdIns 3-kinase activity was calculated from the difference between these two substrates. Data from one representative experiment ($n = 3$) are expressed as picomoles product produced per reaction to allow tracking of those fractions with the highest total activity (*solid line* PtdIns substrate, *dashed line* 3′-deoxy PtdIns substrate, *inset* Western blot of fractions probed with antirat PtdIns 3-kinase p85 antibody, species cross-reactivity: human, monkey, rat and mouse)

mary tumor xenografts. Significant inhibition of palpable C3H tumor growth rates were observed both during and after drug treatment. The growth rates of both prepalpable and palpable MCF-7/*scid* xenografts were significantly inhibited during wortmannin treatment and growth rates paralleled those of the control after treatment was discontinued. The ability of MCF-7 tumors to resume a growth rate similar to that of the controls after treatment was discontinued suggests that wortmannin was inhibiting the growth of these human tumors by a cytostatic mechanism.

Previous studies of the antitumor activity of wortmannin did not determine if tumor PtdIns 3-kinase was being altered by in vivo drug treatment [16]. In the current study, no differences in PtdIns 3-kinase activity or wortmannin sensitivity were detected between control and in vivo wortmannin-treated tumor, brain, spleen or liver homogenate from C3H mice.

Further investigation yielded the unexpected finding that C3H tumor PtdIns 3-kinase activity for both control and wortmannin-treated animals was wortmannin-insensitive. However, human MCF-7 and murine C3H mammary tumor cell lines appeared to contain two types of PtdIns 3-kinase activity as demonstrated by biphasic wortmannin inhibition curves: one population of PtdIns 3-kinase which was wortmannin-sensitive with an IC_{50} of approximately 5 n*M* and another population which was wortmannin-insensitive with an IC_{50} ranging from 200 to 500 n*M*, respectively.

The wortmannin sensitivity profile observed in the C3H cell lysate (Fig. 4) was different to that observed in the primary C3H tumor homogenate (Fig. 3). Primary C3H tumors appeared to lack wortmannin-sensitive PtdIns 3-kinase activity compared to lysates of monolayer C3H cultures. The presence of wortmannin-sensitive PtdIns 3-kinase activity in lysates of monolayer cultures may indicate that it is more important for anchorage-dependent growth than the wortmannin-insensitive PtdIns 3-kinase. Another possible explanation for the difference between cell and tumor lysates could be that factors to which the tumors are exposed in vivo could stimulate the tumor cells to express a different profile of PtdIns 3-kinase isoforms than expressed in vitro. The conclusion that different forms of PtdIns 3-kinase are necessary for anchorage-dependent and -independent growth is supported by studies in which differential display was used to demonstrate that PtdIns 3-kinase $p55^{PIK}$ mRNA is downregulated in HT-29 colon carcinoma cells grown as monolayer cultures compared to HT-29 colonies in soft agar (Dr. L.C. Yeates, personal communication). Many other differences also exist between transformed cell lines and the tumors from which they were originally derived. Any of these differences could be responsible for altering the profile of PtdIns 3-kinase enzyme expressed in the samples and, for this reason the difference observed between the C3H cell lysate and tumor homogenate are not considered surprising.

The ability of wortmannin to inhibit PtdIns 3-kinase in the presence of a threefold excess of MCF-7 cell lysate protein demonstrated that there were no factors in the lysate samples that blocked the ability of wortmannin to inhibit PtdIns 3-kinase activity and showed that the wortmannin-insensitive PtdIns 3-kinase in breast cancer cells was not an artifact of the assay conditions.

Attempts to detect known catalytic forms of PtdIns 3-kinase with commercially available antibodies were not successful. An alternative approach was used to confirm that wortmannin-insensitive activity was not due to previously characterized PtdIns 3-kinase isoforms by partially purifying MCF-7 cell lysate and testing fractions for PtdIns 3-kinase activity and p85 regulatory subunit expression. PtdIns 3-kinase is the only classic PtdIns 3-kinase isoform that is not associated with p85 regulatory subunit expression and this isoform is wortmannin-sensitive [20]. Thus, the wortmannin-insensitive peak of activity which was independent of p85 in MCF-7 cells was believed to be the novel isoform.

The majority of the human PtdIns 3-kinases identified to date have been isolated from cell culture lysates or expressed from cloned cDNA and characterized as at least partially wortmannin-sensitive (wortmannin IC_{50} values in the low nanomolar range) [14, 20]. Recently, a human PtdIns 3-kinase with a C-terminal C2 domain, PtdIns 3-kinase-C2, has been cloned and characterized as wortmannin- and LY294002-insensitive (IC_{50} 420 n*M* and 19 μ*M*, respectively) [5]. A bovine PtdIns specific 3-kinase has also been characterized as insensitive to wortmannin (IC_{50} 350 n*M*) [19]. The wortmannin-insensitive PtdIns 3-kinase of human MCF-7 mammary tumors cells (IC_{50} 200 n*M* and >50 μ*M* for wortmannin and LY294002, respectively) will have to be purified and characterized before it will be possible to determine if this enzyme is related to the human PtdIns 3-kinase-C2 or bovine PtdIns 3-kinase.

The lack of correlation between inhibition of mammary tumor growth by wortmannin and in vitro PtdIns 3-kinase wortmannin sensitivity indicates that the antitumor activity of wortmannin is not exclusively due to inhibition of classic forms of PtdIns 3-kinase. The ability of wortmannin to inhibit the growth of even established mammary tumors, despite the presence of wortmannin-insensitive PtdIns 3-kinase in these tumors, demonstrates the potential of wortmannin as a chemotherapeutic agent and suggests that other cellular targets are responsible for this activity.

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References

1. Abraham RT (1996) Phosphatidylinositol 3-kinase related kinases. Curr Opin Immunol 8: 412
2. Boulton S, Kyle S, Yalcintepe L, Durkacz BW (1996) Wortmannin is a potent inhibitor of DNA double strand break but not single strand break repair in Chinese hamster ovary cells. Carcinogenesis 17: 2285
3. Brunn GJ, Williams J, Sabers C, Wiederrecht G, Lawrence JC Jr., Abraham RT (1996) Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J 15: 5256
4. Carpenter CL, Cantley LC (1990) Phosphoinositide kinases. Biochemistry 29: 11147
5. Domin J, Pages F, Volinia S, Rittenhouse SE, Zvelebil MJ, Stein RC (1997) Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. Biochem J 326: 139
6. Frew T, Powis G, Berggren M, Abraham RT, Ashendel CL, Zalkow LH, Hudson C, Qazia S, Gruszecka-Kowalik E, Merriman R, Bonjouklian R (1994) A multiwell assay for inhibitors of phosphatidylinositol-3-kinase and the identification of natural product inhibitors. Anticancer Res 14: 2425
7. Frew T, Powis G, Berggren M, Gellegos A, Abraham RT, Ashendel CL, Zalkow LH, Hudson C, Gruszecka-Kowalik E, Burgess EM, Benedetti-Doctorovich V, Kerrigan JE, Lambropoulos J, Merriman R, Bonjouklian R (1995) Novel quinone antiproliferative inhibitors of phosphatidylinositol-3-kinase. Anticancer Drug Design 10: 347
8. Hazeki O, Hazeki K, Katada T, Ui M (1996) Inhibitory effect of wortmannin on phosphatidylinositol 3-kinase-mediated cellular events. J Lipid Mediat Cell Signal 14: 259
9. Hunter T (1995) When is a lipid kinase not a lipid kinase? When it is a protein kinase. Cell 83: 1
10. Kaplan DR, Whitman M, Schaffhausen B, Pallas DC, White M, Cantley L, Roberts TM (1987) Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell 50: 1021
11. Keith CT, Schreiber SL (1995) PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. Science 270: 50
12. Kozikowski AP, Kiddle JJ, Frew T, Berggren M, Powis G (1995) Synthesis and biology of 1D-3-deoxyphosphatidylinositol: a putative antimetabolite of phosphatidylinositol-3-phosphate and an inhibitor of cancer cell colony formation. J Med Chem 38: 1053
13. Powis G, Kozikowski A (1994) Inhibitors of *myo*-inositol signalling. In: Kerr DJ, Workman P (eds) New targets for cancer chemotherapy. CRC Press, Baton Rouge, p 81
14. Powis G, Bonjouklian R, Berggren MM, Gallegos A, Abraham R, Ashendel C, Zalkow L, Matter WF, Dodge J, Grindey G, Vlahos CJ (1994) Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. Cancer Res 54: 2419
15. Ryu SH, Kim U-H, Wahl MI, Brown AB, Carpenter G, Huang K-P, Rhee SG (1990) Feedback regulation of phospholipase C by protein kinase C. J Biol Chem 265: 17941
16. Schultz RM, Merriman RL, Andis SL, Bonjouklian R, Grindey GB, Rutherford PG, Gallegos A, Massey K, Powis G (1995) In vitro and in vivo antitumor activity of the phosphatidylinositol-3-kinase inhibitor, wortmannin. Anticancer Res 15: 1135
17. Serunian LA, Auger KR, Roberts T, Cantley LC (1989) Production of novel polyphosphoinositides in vivo is linked to cell transformation by polyomavirus middle T antigen. J Virol 64: 4718
18. Sjölander A, Yamamoto K, Huber BE, Lapetina EG (1991) Association of p21*ras* with phosphatidylinositol 3-kinase. Proc Natl Acad Sci USA 88: 7908
19. Stephens L, Cooke FT, Walters R, Jackson T, Volinia S, Gout I, Waterfield MD, Hawkins PT (1994) Characterization of a phosphatidylinositol-specific phosphoinositide 3-kinase from mammalian cells. Curr Biol 4: 203
20. Thomason PA, James SR, Casey PJ, Downes CP (1994) A G-protein beta-subunit-responsive phosphoinositide 3-kinase activity in human platelet cytosol. J Biol Chem 269: 16525
21. Toker A, Cantley LC (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature 387: 673
22. Wymann MP, Bulgarelli-leva G, Zvelebil MJ, Pirola L, Vanhaesebroeck B, Waterfield MD, Panayotou G (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. Mol Cell Biol 16: 1722
23. Yao R, Cooper GM (1995) Requirement for phosphatidylinositol-kinase in the prevention of apoptosis by nerve growth factor. Science 267: 2003