The phosphatidylinositol 3-kinase inhibitor, PX-866, is a potent inhibitor of cancer cell motility and growth in three-dimensional cultures

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Abstract

The phosphatidylinositol 3-kinase (PI3K) pathway is activated in many human tumors and mediates processes such as cell proliferation, survival, adhesion, and motility. The natural product, wortmannin, has been widely used to study the functional consequences of PI3K inhibition in both normal and transformed cells in culture but is not a suitable cancer chemotherapeutic agent due to stability and toxicity issues. PX-866, an improved wortmannin analogue, displays significant antitumor activity in xenograft models. Here, we directly compare PX-866 and wortmannin in human cancer cell lines cultured in monolayer or as three-dimensional spheroids. Both PI3K inhibitors failed to inhibit monolayer cell growth at concentrations up to 100 nmol/L but strongly suppressed spheroid growth at low nanomolar concentrations, with PX-866 showing greater potency than wortmannin. Relative to wortmannin, PX-866 treatment results in a more sustained loss of Akt phosphorylation, suggesting that the increased potency of PX-866 is related to a more durable inhibition of PI3K signaling. PX-866 and wortmannin both inhibit spheroid growth without causing cytotoxicity, similar to known cytostatic agents, such as rapamycin. PX-866 also inhibits cancer cell motility at subnanomolar concentrations. These findings suggest that the antitumor activities of PX-866 stem from prolonged inhibition of the PI3K pathway and inhibition of cell motility. In addition, we propose that the use of three-dimensional tumor models is more predictive of in vivo growth inhibition by PI3K inhibitors in cancer cell lines lacking phosphatase and tensin homologue activity or expression. [Mol Cancer Ther 2007;6(9):2505–14]

Introduction

Activation of the phosphatidylinositol 3-kinase (PI3K) pathway is a feature common to a variety of human cancers. PI3K gene amplification and protein overexpression are found in lung, breast, and ovarian tumors (1). Activating mutations in the catalytic subunit of PI3K (p110α) are found in colorectal, breast, and brain tumors (2–5). The PI3K pathway is negatively regulated by the tumor suppressor protein phosphatase and tensin homologue (PTEN). In humans, germ-line mutations in PTEN are causally related to Cowden’s disease, which is a multiorgan disease characterized by benign hamartomatous growths that occasionally transition to frank neoplasia. PTEN is also deleted, mutated, or epigenetically repressed in a striking proportion of sporadic human tumor samples (6). These genetic changes result in the stimulation of pathways downstream of PI3K that promote the growth, proliferation, migration, and survival of cancer cells.

The signaling pathways downstream of PI3K were elucidated in part through the use of small-molecule inhibitors, such as wortmannin and LY294002. Although both compounds compete with ATP for binding to the PI3K catalytic domain, LY294002 acts as a reversible inhibitor, whereas wortmannin irreversibly blocks the catalytic site via a covalent interaction with a critical lysine residue (Lys802 in the p110α isoform; ref. 7). These two compounds are best characterized as pan-specific inhibitors of the PI3K superfamily. For example, wortmannin irreversibly inhibits class I PI3Ks in cells treated with nanomolar drug concentrations (30–100 nmol/L), but, when administered at higher concentrations (5–20 μmol/L) that inhibit cell growth, wortmannin irreversibly inhibits several additional kinases that express the PI3K-related catalytic domain, including mammalian target of rapamycin, ATM, and hSMG-1 (8, 9). The class Iα form of PI3K has attracted strong interest as a potential target for anticancer drug discovery. However, neither wortmannin nor LY294002 is considered a viable candidate for clinical development due to poor pharmacokinetics, liver toxicity, and, for wortmannin, chemical instability in aqueous formulations.

PX-866 is a recently developed wortmannin analogue that displays increased potency and favorable pharmacokinetics relative to the parent compound (10). PX-866 inhibited purified PI3K (p85–p110α heterodimer) at a 10-fold lower IC₅₀ than wortmannin in immune complex

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kinase assays (0.1 versus 1.2 nmol/L). The maximum tolerated dose of PX-866 in mice was 19.5 mg/kg, which was over six times higher than wortmannin, and liver toxicity, as measured by mean increases in serum alanine and aspartate aminotransferases, was reduced by 65%. In human tumor xenograft models, PX-866 has shown promising single-agent antitumor activity, and, in combination therapy, PX-866 potentiated tumor growth inhibition induced by radiation, cisplatin, or gefitinib (10, 11).

To further probe the mechanisms underlying the anti-tumor activity of PX-866, we did direct comparisons of PX-866 and wortmannin in human cancer cell lines cultured either as plastic-attached monolayers or as three-dimensional, extracellular matrix-embedded, spheroids. As in vitro models for studies of tumor biology and cancer drug pharmacology, three-dimensional cell cultures offer several advantages over monolayer culture, such as the presence of cell-cell and cell-matrix interactions as well as nutrient, oxygen, and growth factor gradients (12).

The present study shows that three-dimensional cultures involving PTEN-deficient human cancer cell lines as well as cell lines that contain mutations in the catalytic subunit of PI3K exhibit dramatically higher sensitivities to PX-866 or wortmannin, relative to the same cells cultured as monolayers. Furthermore, PX-866 was considerably more potent than wortmannin as a suppressor of the growth of U87 glioblastoma, PC3 prostate, T47D breast, and HCT116 colon cancer cells in three-dimensional cultures. Both compounds suppressed spheroid growth primarily through the induction of cytostasis, rather than cytotoxic activity. Finally, PX-866, like wortmannin, was a powerful inhibitor of cancer cell migration. The results obtained with three-dimensional cultures are correlated with the relative antitumor activities of these compounds against human tumor xenografts in immunodeficient mice (11), which suggests that the three-dimensional culture system may be more predictive of the therapeutic activities of PI3K pathway inhibitors currently under development.

Materials and Methods

Compounds and Reagents

PX-866 (13) was generously provided by Dr. D. Lynn Kirkpatrick (ProX Pharmaceuticals, Tucson, AZ), dissolved in DMSO, and stored at −80°C until use. Wortmannin was purchased from Sigma, dissolved in DMSO, and stored at −80°C until use. Matrigel was purchased from BD Biosciences. The Cell Motility HitKit was purchased from Cellomics. The CellTiter 96 AQueous One Solution Immunoprecipitation Assay and the CytoTox 96 Nonradioactive Cytotoxicity Assay were purchased from Promega.

Cells and Antibodies

U87 glioblastoma, PC3 prostate cancer, T47D breast cancer, HCT116 colon cancer, and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection. U87 cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS). PC3 and T47D cells were cultured in RPMI 1640 plus 10% FBS. HCT116 cells were cultured in McCoy’s 5A medium supplemented with 10% FBS. MDA-MB-231 cells were cultured in 50:50 F12/DMEM supplemented with 10% FBS. The polyclonal antibodies against phospho-Akt (Ser473) and total Akt were purchased from Cell Signaling Technologies.

Spheroid Culture Conditions

PC3 spheroids were grown in 35-mm dishes containing 125 μL Matrigel (BD Biosciences). Five thousand cells were seeded onto each 35-mm dish in RPMI 1640 plus 10% FBS supplemented with 2% Matrigel. Spheroids grew to ~50 microns in diameter after 3 days in culture under these conditions.

U87 cells were induced to form spheroids in a spinner flask or via a hanging drop method (14), whereas T47D and HCT116 spheroids were formed solely by the hanging drop method. For spinner cultures, 1 × 10^7 cells were added to 125 mL DMEM plus 10% FBS in a 250-mL spinner flask. After 3 days of gentle agitation, 15 mL of the spheroid suspension were collected and centrifuged at 200 × g, and the spheroids were resuspended in 0.25% low melting point agarose in DMEM for plating onto 35-mm dishes. After the agarose had solidified, 2 mL of DMEM plus 10% FBS were added to each dish. For the hanging drop method, U87, T47D, or HCT116 cells were seeded at a density of 250 cells per 20 μL of the appropriate growth medium in a Nunc 60-well MiniTray. The tray was then covered, inverted, and incubated for 5 days. On day 5, the U87 spheroids measured ~250 microns in diameter and were transferred to a 48-well plate coated with 1% agarose to prevent them from adhering.

Biochemical Analysis of Spheroids and Monolayer Cells

Immunoblot analysis was done on soluble proteins from U87 and T47D spheroids after lysis in ice-cold radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.5% SDS, 0.5% sodium deoxycholate, 1 mmol/L EDTA plus protease and phosphatase inhibitors]. The lysates were passed thrice through a 27-gauge needle and incubated on ice for 30 min. Monolayer cells were washed with cold PBS and lysed with radioimmunoprecipitation assay buffer in the dish for 15 min. Lowry protein assays were done on all cell extracts, and equal amounts of protein (30–50 μg) were resolved on 8% SDS-PAGE gels followed by immunoblot analysis.

Bruton’s Tyrosine Kinase-Pleckstrin Homology-Green Fluorescent Protein Translocation Assay

Visualization of PIP3-induced Bruton’s tyrosine kinase-pleckstrin homology-green fluorescent protein (Btk-PH-GFP) was done essentially as described by Balla and Varnai (15). Briefly, the Btk-PH-GFP plasmid was transfected into 5 × 10^5 U87 cells seeded onto a 6-cm dish using Fugene reagent (Roche) according to the manufacturer’s protocol. Twenty-four hours later, the U87 cells were trypsinized and 10,000 cells per well were seeded onto four-well chamber slides in DMEM plus 10% FBS. After allowing the cells to adhere overnight, the wells were washed twice with...
serum-free DMEM and then incubated with the same serum-free medium for 5 h. During the last 30 min of serum starvation, the appropriate concentrations of PX-866 or wortmannin were added. The cells were then switched to Modified Krebs-Ringers medium (15) and photographed with a SPOT digital camera on a Zeiss fluorescent microscope. ImagePro software (MediaCybernetics) was used to analyze the intensity of fluorescence at the cell borders before and after serum stimulation (final serum concentration of 10%) on five to six cells per condition.

**Cytotoxicity Assay**

Cell death was measured in U87 glioblastoma cells treated with 1 nmol/L rapamycin, 100 nmol/L PX-866, 100 nmol/L wortmannin, or 750 nmol/L doxorubicin (Adriamycin) according to the CytoTox 96 Nonradioactive Cytotoxicity Assay manufacturer’s protocol. Briefly, 4 days after treatment, 200 µL of medium were aspirated from each well, transferred to a microcentrifuge tube, and centrifuged at 250 × g for 4 min at 4°C. Fifty microliters of this medium were then added to a 96-well microwell plate in duplicate, to which 50 µL of the coupled enzymatic substrate buffer were added.

After 30 min at room temperature, 50 µL of stop solution were added to each well and the resulting color change was quantitated on a Biotek EL800 absorbance plate reader at 490 nm. The released lactate dehydrogenase (LDH) was normalized to the total LDH in each well following spheroid lysis with cell lysis buffer (supplied by the

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**Figure 1.** U87 cell growth in the presence of PX-866 or wortmannin. A, cells grown as a monolayer were treated with PX-866 (Px) or wortmannin (Wm) at the indicated concentrations. The CellTiter 96 proliferation assay was done 18, 40, and 68 h later. For the 68-h time point, PX-866 or wortmannin was readded at the appropriate concentration again after 48 h in culture (arrow). Representative of three independent experiments, which were done in triplicate. Points, growth; bars, SD. B, U87 cells grown as spheroids in a spinner flask were plated onto agarose and treated with the indicated concentration of PX-866 or wortmannin every other day, for a total of 6 d. Phase-contrast photos were taken of representative fields. C, spheroid length and width were measured with an optical micrometer. These values were used to calculate spheroid volume at days 0, 2, and 6. Representative of three independent experiments.
manufacturer) for 1 h at 37°C. The LDH released into the medium during lysis was quantitated in the same manner as described above.

**Cell Motility Assay**

Motility was measured in MDA-MB-231 breast cancer cells treated with PX-866 or wortmannin according to the Cellomics Cell Motility HitKit manufacturer’s protocol. Briefly, fluorescent microspheres were coated onto collagen-covered 96-well plates (BD Biosciences). The micro-spheres were overlaid with MDA-MB-231 cells (400 cells per well), in a total volume of 100 μL F12/DMEM 50:50 medium containing either serum (5%) or serum plus PX-866 or wortmannin. Triplicate wells were seeded without serum to serve as negative controls. Following 18 h of incubation, the wells were fixed, the cells were stained with phalloidin, and plate was subjected to high-throughput fluorescence microscopy on an EDAQ 100 (Beckman Coulter) to visualize the paths cleared.

**Figure 2.** Inhibition of PC3 spheroid growth by PX-866 and wortmannin. PC3 spheroids were treated with PX-866 or wortmannin at the indicated concentrations every other day. A, phase-contrast photographs of PC3 spheroids on day 7 following three rounds of treatment. B, spheroid volume measurements were calculated from micrometer length and width measurements on days 0, 2, 4, 6, and 8. Representative of three independent experiments.
path areas cleared by 20 cells per treatment group were analyzed with ImagePro software to yield the average migration.

**Results**

**Effect of PX-866 on the Growth of Tumor Cell Spheroids**

In this study, we examined the antiproliferative properties of the wortmannin analogue PX-866 by examining the growth of tumor cells in monolayer versus three-dimensional cultures. Interestingly, neither of these PI3K inhibitors, at concentrations up to 100 nmol/L, suppressed the proliferation of U87 glioblastoma cells cultured as plastic-attached cell monolayers (Fig. 1A).

In contrast, treatment of U87 spheroids with nanomolar concentrations (1–100 nmol/L) of PX-866 or wortmannin every other day for a total of 6 days strongly suppressed cell growth/proliferation, as measured by changes in spheroid volume. The wortmannin analogue, PX-866, was a more potent inhibitor in this spheroid growth assay than was the parent compound, wortmannin. Treatment with PX-866 at a concentration of 1 nmol/L caused a dramatic reduction in spheroid growth, whereas approximately a 10-fold higher concentration of wortmannin was required to achieve a similar effect in this three-dimensional culture system (Fig. 1B and C). At concentrations of 10 to 100 nmol/L, both drugs blocked the growth of U87 spheroids. Qualitatively similar results were obtained with spheroids derived from PC3 prostate cancer cells, although neither compound induced a complete cessation of PC3 spheroid growth at the maximal concentration (100 nmol/L) used in these experiments (Fig. 2A and B). We also tested two other cell lines, T47D and HCT116, in both monolayer and spheroid growth assays. As observed with the U87 cells, neither inhibitor suppressed the growth of T47D or HCT116 cells grown as monolayer (Fig. 3A; data not shown). Similar to the PC3 cells, both compounds were able to reduce spheroid growth, with PX-866 showing greater potency than wortmannin (Fig. 3B; data not shown). These results indicate that growth of U87, PC3, T47D, and HCT116 cells in three-dimensional cultures is considerably more sensitive to PI3K inhibition than is growth of the same cell lines as plastic-attached monolayers.

**Visualization of PI3K Inhibition by PX-866**

To examine the effects of PX-866 on levels of the bioactive PI3K metabolite PIP3 in the plasma membrane, we used the...
PH domain of Btk. A fusion protein comprising the PH domain of Btk fused to GFP has been used to visualize the formation of PIP3 stimulated by epidermal growth factor or platelet-derived growth factor stimulation (15). The PH domain of Btk mediates localization of the fusion protein to PIP3-containing lipid bilayers. To determine whether PX-866 inhibited PIP3 formation at the same concentrations that inhibited spheroid growth, we transfected U87 glioblastoma cells with Btk-PH-GFP–encoding plasmid DNA. We observed that the serum-stimulated localization of this fluorescent probe to focal regions of the plasma membrane was inhibited by either PX-866 or wortmannin pretreatment (Fig. 4A). In addition, we quantitated the fluorescent pixel intensity at the cell edge following serum stimulation in the absence or presence of PX-866 or wortmannin (Fig. 4B). The average change in pixel intensity at the edge of the cell was increased by 15% in serum-stimulated cells. PX-866 pretreatment inhibited Btk-PH-GFP translocation only moderately at 10 nmol/L, but this response was nearly abolished in cells treated with 100 nmol/L PX-866. In contrast, wortmannin significantly prevented Btk-PH-GFP translocation at both the 10 and 100 nmol/L concentrations. Thus, in short-term assays, wortmannin was a more potent suppressor of the steady-state levels of PIP3 in PTEN-deficient U87 glioblastoma cells.

Biochemical Examination of PI3K Pathway Components in the Presence of PX-866

Previous studies showed that PX-866 blocks epidermal growth factor–stimulated Akt phosphorylation in colon cancer cells grown as a monolayer (10). We examined Akt phosphorylation in U87 and T47D cell monolayer versus spheroid cultures in the presence of PX-866 or wortmannin (Figs. 5 and 6). A direct comparison of wortmannin and PX-866 revealed differences in the kinetics of PI3K inhibition, as measured by Akt phosphorylation. In U87 cells, we...
observed that treatment of the cells with 10 nmol/L wortmannin strongly diminished Akt phosphorylation at the early time point (2 h) in both monolayer and three-dimensional culture. Similar results were obtained with a 10-fold higher concentration (100 nmol/L) of PX-866. Under both culture conditions, however, the inhibitory effect of PX-866 on Akt phosphorylation was more durable than that induced by wortmannin. Indeed, after 16 h of treatment with a lower concentration (10 nmol/L) of PX-866, the phosphorylation of Akt remained lower than that observed in control cultures. The latter finding is consistent with a more persistent inhibitory effect of PX-866 on PI3K signaling. The T47D cells were slightly less sensitive to both wortmannin and PX-866 with inhibition of Akt phosphorylation observed in monolayers treated with 100 nmol/L. However, a reproducible observation in both cell lines was that cellular exposure to either wortmannin or PX-866 caused a more dramatic inhibition of Akt phosphorylation in three-dimensional cultures than in monolayer culture. This finding may be explained in part by the fact that three-dimensional cultures, unlike

Figure 5. Inhibition of Akt phosphorylation in U87 cells. Monolayer cells (A) or spheroids (B) were treated with PX-866 or wortmannin at the indicated concentrations. Two or 16 h later, cell extracts were subjected to immunoblot analysis for phospho-Akt (P-Akt) and total Akt levels. Representative blot and quantitative analysis of the same experiment.

Figure 6. Inhibition of Akt phosphorylation in T47D cells. Monolayer cells (A) or spheroids (B) were treated with PX-866 or wortmannin at the indicated concentrations. Two or 16 h later, cell lysates were prepared and subjected to immunoblot analysis for phospho-Akt and total Akt levels. Representative blot and quantitative analysis of the same experiment.
monolayer cultures, exhibit outside-to-inside gradients of proliferating cells, due to the more limited availability of soluble growth factors, oxygen, and nutrients in the interior regions of the spheroid. Both Akt phosphorylation and cell cycle progression may be more sensitive to PI3K inhibition by these agents compared with exponentially growing cells exposed to optimal mitogen and nutrient concentrations (16).

PX-866 is a Cytostatic Agent

To determine if the spheroid growth inhibition observed in PX-866-treated spheroids was due to the inhibition of cell growth or to increased cell death, we assessed cell viability in the spheroid cultures with a LDH release assay. After 4 days, LDH release from cells treated with 100 nmol/L PX-866 or wortmannin was similar to control wells or cells treated with the cytostatic agent, rapamycin (Fig. 7). In contrast, cultures treated with doxorubicin, a well-established cytotoxic agent, showed a significant increase in LDH release. Thus, we attribute the profound inhibition of U87 spheroid growth induced by the PI3K inhibitors to cytostatic rather than cytotoxic effects of these compounds.

PX-866 Inhibits the Motility of Cancer Cells

PI3K signaling plays a critical role in cell migration and motility (17, 18). The effect of PX-866 on cancer cell migratory activity has not been assessed previously. Consequently, we comparatively evaluated the effects of PX-866 and wortmannin on breast cancer cell motility using a fluorescence-based cell migration assay. MDA-MB-231 breast carcinoma cells were plated on a bed of fluorescent microspheres, which are pushed aside to create a microscopically detectable path as cells migrate on the plastic surface. We observed significant suppression of the average migration path length in cell cultures treated with nanomolar concentrations of PX-866 or wortmannin (Fig. 8).

Discussion

As a family of kinases that control cell proliferation, survival, and migration, the PI3Ks are attractive antitumor targets and are currently the focus of intensive drug discovery efforts (19, 20). The broad-spectrum PI3K inhibitor wortmannin exhibits antitumor activity in xenograft models (21) but is not considered a viable drug candidate due to its chemical instability and toxic side effects. PX-866 is a wortmannin-like PI3K inhibitor with improved stability and reduced hepatotoxicity and has been shown to exert antitumor activities in human ovarian and colon cancer xenograft models (10). In this study, we observed that PX-866 inhibited the growth of human glioblastoma, prostate, breast, and colon cancer cell lines grown as three-dimensional spheroids. Moreover, PX-866...
seemed to be a considerably more potent inhibitor of spheroid growth than the parent compound, wortmannin. In biochemical studies, PX-866 also caused a more persistent inhibition of Akt phosphorylation than wortmannin, suggesting that its increased potency as a spheroid growth inhibitor was due to a more durable inhibition of PI3K signaling than that imposed by the chemically unstable wortmannin.

A major focus of this study was to define the mechanistic basis of the tumor growth inhibition by PX-866. The first article to describe PX-866 reported an in vitro basis of the tumor growth inhibition by PX-866. The first unstable wortmannin.

PI3K signaling than that imposed by the chemically persistent inhibition of Akt phosphorylation than wortmannin. In biochemical studies, PX-866 also caused a more profound inhibition of U87 cell spheroid growth induced by PX-866 or wortmannin reflects a cytostatic rather than cytotoxic effect of PI3K inhibition. Therefore, the current results suggest that cancer therapy with PI3K inhibitors may induce primarily stable disease when administered as monotherapy. Hence, these agents may be most effective when given in combination with conventional cytotoxic agents or radiotherapy. Indeed, PI3K inhibition may increase the sensitivity of cancer cells to killing by DNA-damaging chemotherapy or ionizing radiation. In xenograft models, PX-866 combined with cisplatin or single-dose irradiation resulted in a more than additive suppression of tumor growth compared with either agent alone (10). Inhibition of downstream effectors of the PI3K pathway, such as mammalian target of rapamycin by rapamycin, is also largely cytostatic in cells cultured in monolayer but can cause cell death in tumors in vivo perhaps through additional effects on the tumor microenvironment, including the tumor-associated vascular supply (30).

The present findings show that PX-866 inhibits PI3K activity with a resulting decrease in Akt phosphorylation, decreased cell motility, and profound growth inhibition of human cancer cell lines grown as multicellular spheroids. Furthermore, this study emphasizes the importance of duration of action against a molecular target as a determinant of anticancer drug efficacy in cancer cells and underscores the predictive power of three-dimensional cultures to assess the efficacy of small-molecule PI3K pathway inhibitors.

References

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