Molecular pharmacology and antitumor activity of PX-866, a novel inhibitor of phosphoinositide-3-kinase signaling

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Abstract

We have developed biologically stable semisynthetic viridins as inhibitors of phosphoinositide (PtdIns)-3-kinases. The most active compound was PX-866 (acetic acid (1*S*,4*E*,10*R*,11*R*,13*S*,14*R*)-[4-diallylaminomethylene-6-hydroxy-1-methoxymethyl-10,13-dimethyl-3,7, 17-trioxo-1, 3,4,7,10,11,12,13,14,15,16,17-dodecahydro-2-oxa-cyclopenta[*a*]phenanthren-11-yl ester), which inhibited purified PtdIns-3-kinase with an IC_{50} of 0.1 nmol/L and PtdIns-3-kinase signaling measured by phospho-Ser^{473}-Akt levels in HT-29 colon cancer cells with an IC_{50} of 20 nmol/L. PX-866 administered to mice at 10 mg/kg inhibited phospho-Ser^{473}-Akt in HT-29 colon tumor xenografts up to 80% with recovery taking >48 hours after p.o. administration but more rapidly after i.v. or i.p. administration. PX-866 was eliminated from mouse plasma with a half-life of 18 minutes and a clearance of 360 mL/min/kg following i.v. administration and, when administered i.p. or p.o., showed first-pass metabolism with sequential *N*-deallylation. Synthetic standards of the *N*-deallylated metabolites of PX-866 inhibited PtdIns-3-kinase at low nanomolar per liter concentrations. PX-866 exhibited *in vivo* antitumor activity against s.c. OvCar-3 human ovarian cancer and A-549 human lung cancer xenografts in immunodefficient mice with log cell kills up to 1.2. PX-866 also increased the antitumor activity of cisplatin against A-549 xenografts and radiation treatment against OvCar-3 xenografts. The results show that PX-866 is a biologically stable broad-spectrum PtdIns-3-kinase inhibitor with good pharmacokinetics that causes prolonged inhibition of PtdIns-3-kinase signaling in human tumor xenografts. PX-866 exhibits single agent *in vivo* antitumor activity and increases the antitumor effects of cisplatin and radiation treatment. [Mol Cancer Ther 2004;3(7):763–72]

Introduction

Phosphoinositide (PtdIns)-3-kinases phosphorylate membrane PtdIns on the 3′ position of the *myo*-inositol ring (1). There are eight mammalian PtdIns-3-kinases that are divided into three main classes based on sequence homology and substrate preference (2). Class I enzymes produce PtdIns(3,4,5)P_3 and are subdivided into class Ia, which includes p110α PtdIns-3-kinase and the closely related p110β and p110δ. p110α and p110β are found in all adult human tissues, whereas p110δ is found in leukocytes. All class Ia kinases are associated with a p85 regulatory/adaptor subunit. Three genes that can also generate splice variants encode at least eight p85 adaptor subunits (3). Different p85 subunits show differences in tissue distribution that may be of functional significance, but there does not appear to be a preferential association between the p85 subunits and any of the p110s. Class Ib PtdIns-3-kinase is represented by p110γ (which associates with a p101 adapter subunit) and is confined largely to leukocytes. Class II PtdIns-3-kinases are larger molecules of ~200 kDa without adapter subunits and give rise to PtdIns(3)P and PtdIns(3,4)P_2. Class III PtdIns-3-kinase has one member, Vps34p, which is constitutively active and is involved in lysosomal protein trafficking. The lipid product of Vps34p is PtdIns(3)P.

Likely, all mammalian cells express representatives of each class of PtdIns-3-kinase (3). Class Ia PtdIns-3-kinases are activated by transmembrane receptors and oncogenic tyrosine kinases through the binding to Src homology 2 domains found in all p85 isoforms (4) and by $p21^{ras}$ (5). Class Ib PtdIns-3-kinase is activated by βγ subunits of heterotrimeric G proteins released on the activation of seven-pass transmembrane receptors (6). Class II PtdIns-3-kinases are activated by tyrosine kinase coupled receptors, although their mechanism of activation and function are largely unknown.

The PtdIns-3-kinase superfamily includes protein kinases bearing the conserved PtdIns-3-kinase domain (7), such as DNA-dependent protein kinase, which is involved in the repair of DNA damage; ataxia telangiectasia mutated and ataxia telangiectasia related, which link the DNA repair machinery to control of the cell cycle; and mammalian target of rapamycin, which regulates protein synthesis by phosphorylating substrates controlling mRNA translation.

p110 PtdIns-3-kinase can cause cellular transformation (8). Constitutive activation of PtdIns-3-kinase occurs in

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human small cell lung cancer (9) and in ~40% of human ovarian, head and neck, urinary tract, and cervical cancers (10). The major mechanism for the oncogenic activity of PtdIns-3-kinase is by preventing apoptosis through the downstream activation of Akt (protein kinase B; refs. 11, 12). Akt is a member of the AGC serine-threonine kinase family (13). There are three mammalian genes, *Akt1*, *Akt2*, and *Akt3*, giving proteins that share a high degree of sequence homology in their catalytic and NH_2-terminal pleckstrin homology domains but diverge in other domains (14). All of the Akt isoforms undergo membrane recruitment from the cytoplasm through the binding of their pleckstrin homology domain to membrane PtdIns(3,4,5)P_3 leading Akt activation by phosphorylation on Thr^{308} and Ser^{473} (in *Akt1*) by membrane-associated kinases (14, 15). The kinase responsible for Thr^{308} phosphorylation is PtdIns-dependent kinase-1, whereas the identity of the Ser^{473} kinase is unclear (16, 17). Fully phosphorylated Akt is independent of phospholipid activation and detaches from the plasma membrane moving to the cytoplasm and nucleus (18), where it phosphorylates a variety of target proteins to prevent the expression of death genes or to induce cell survival (19). Promoters of apoptosis that are inhibited by Akt are the forkhead transcription factor family members FKHR, FHHRL1, and AFX (19); the proapoptotic Bcl-2 family member Bad (20); the apoptosis signaling kinase-1, which transduces stress signals to the c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase pathways (21); and procaspase-9, which initiates the caspase cell death cascade (22). Targets that Akt activates to promote cell survival are nuclear factor-κB (23) and cyclic AMP response element binding protein (24). *Akt2* is amplified and overexpressed in 12% of human ovarian cancers, 3% of breast cancers, and 10% of pancreatic cancers (25, 26), and *Akt3* is overexpressed in breast and prostate cancer (27).

The dual-specificity tyrosine-threonine/PtdIns-3-phosphatase tumor suppressor protein phosphatase and tensin homologue detected in chromosome 10 (28, 29) prevents the accumulation of PtdIns(3,4,5)P_3 and thus attenuates PtdIns-3-kinase signaling (30). Phosphatase and tensin homologue is lost in a large number of human cancers including advanced prostate, endometrial, renal, glial, melanoma, and small cell lung cancers (30). Therefore, the PtdIns-3-kinase pathway, through either increased growth factor receptor or oncogenic tyrosine kinase activity, increased PtdIns-3-kinase, increased Akt, and deleted phosphatase and tensin homologue, or a combination of these factors is activated in a large number of human cancers. Thus, inhibiting the PtdIns-3-kinase pathway offers a very attractive target for cancer drug discovery.

Wortmannin is an irreversible inhibitor at nanomolar concentrations of PtdIns-3-kinase (31), binding covalently to Lys^{802} of the catalytic site of p110α (32) and Lys^{883} of p110γ (33). Most PtdIns-3-kinase isoforms are equally inhibited by wortmannin (3). We have reported previously that wortmannin has antitumor activity against tumor xenografts in animals (34); however, this activity is variable and accompanied by liver and hematologic toxicity. Wortmannin is also a biologically unstable molecule. We now report the *in vivo* molecular pharmacology and antitumor activity of novel, biologically stable, and less toxic viridian class inhibitors of PtdIns-3-kinase.

Materials and Methods

Compounds

The viridins shown in Fig. 1 were synthesized from wortmannin (>97% pure) provided by Dr. David Newman (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD), as described elsewhere,[5] and were >95% pure by high-performance liquid chromatography. The *N*-deallyl and *N*-di-deallyl derivatives of PX-866 were synthesized as reference standards for pharmacokinetic studies. For toxicity studies, the compounds were administered by i.p. injection at 2 mg/mL dissolved or suspended in 0.1% Tween 20-0.9% NaCl. For all other studies, PX-866 was dissolved at 1 mg/mL in 5% ethanol in water for i.p. and p.o. administration and in 5% ethanol-0.9% NaCl for i.v. administration. PX-867 and PX-881 were dissolved at 1 mg/mL in pharmaceutical grade 20% hydroxypropyl-β-cyclodextrin (Trappsol) in 5% ethanol-0.9% NaCl for i.v. administration. All solutions were made fresh and administered within 30 minutes. Epidermal growth factor was obtained from R&D Systems (Minneapolis, MN). Rabbit purified anti-phospho-Ser^{473}-Akt and anti-Akt antibodies were from Cell Signaling Technology (Beverly, MA). Trappsol was from Cyclodextrin Technologies Development, Inc. (High Springs, FL).

Cells

HT-29 colon cancer, OvCar-3 ovarian cancer, and A-549 non-small cell lung cancer cells were obtained from American Tissue Type Collection (Rockville, MD). The cells were grown in humidified 95% air-5% CO_2 at 37°C in DMEM supplemented with 10% fetal bovine serum. All cell lines were tested to be mycoplasma free using a PCR ELISA kit (Roche Diagnostics, Inc., Indianapolis, IN).

Toxicity Studies

Viridins were administered i.p. daily for 4 days to male C57BL/6 mice, three per group, with at least four doses between 1 and 30 mg/kg. Wortmannin was administered at doses up to 3 mg/kg. The mice were killed 24 hours after the last dose, and changes in body weight from the start of the study, blood lymphocyte, neutrophil, RBC and platelet counts, and serum glucose, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine were measured. The maximum tolerated dose (MTD) for each compound was determined as the dose that caused ≥4 g body weight loss or death of at least 1 of the 3.8 animals. Liver toxicity was measured as the mean increase in serum alanine aminotransferase and aspartate

[5] Wipf P, Minion DJ, Halter RJ, et al. Synthesis and biological evaluation of synthetic viridins derived from C(20)-hetroalkylation of the steroidal Pl-3-kinase inhibitor wortmannin. Org Biomol Chem. In press 2004.

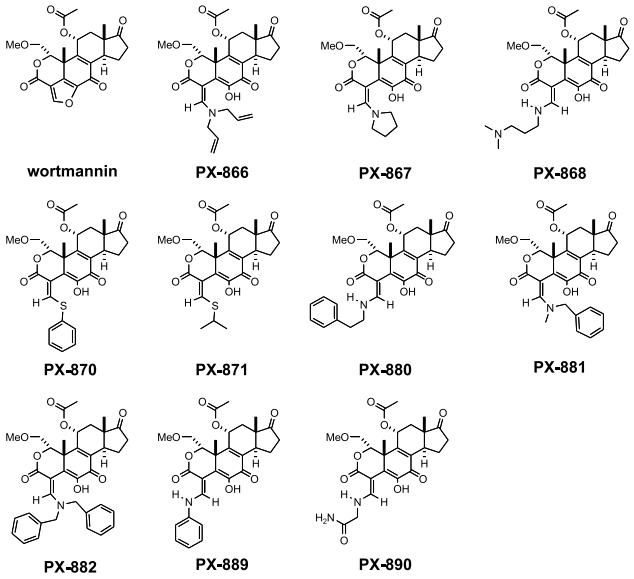

Figure 1. Structures of the semisynthetic viridins and the structure of wortmannin.

aminotransferase and expressed relative to that caused by wortmannin at its MTD, at a dose of the viridin that gave the same decrease in blood lymphocyte count as wortmannin at its MTD.

Measurement of PtdIns-3-Kinase

The ability of the semisynthetic viridins to directly inhibit immunoprecipitated PtdIns-3-kinases was measured using a five-point concentration response curve between 0.05 and 10 nmol/L (31) and confirmed the results of the screening studies.[5] Inhibition of cellular PtdIns-3-kinase was measured indirectly by the effect on Akt activation. Briefly, HT-29 cells were grown in 35 mm well plates to 75% confluency in DMEM supplemented with 10% heat-inactivated fetal bovine serum-4.5 g/L glucose in a 5% CO_2 atmosphere. The medium was replaced by DMEM without fetal bovine serum for 16 hours, and the cells were incubated with wortmannin or the viridins for 2 hours at 37°C before the addition of epidermal growth factor (50 ng/mL). After 20 minutes, the culture medium was aspirated and the cells were lysed in 50 mmol/L HEPES buffer (pH 7.5), 50 mmol/L NaCl, 0.2 mmol/L NaF, 0.2 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1% NP40, and 0.25% sodium deoxycholate (lysis buffer). Total cell lysate protein (50 μg) was boiled for 5 minutes, loaded on a 10% acrylamide/bisacrylamide gel, and separated by electrophoresis at 150 V for 40 minutes. Proteins were electrophoretically transferred to a polyvinylidene fluoride membrane, preincubated with a blocking buffer of 137 mmol/L NaCl, 2.7 mmol/L KCl, 897 mmol/L $CaCl_2$, 491 mmol/L $MgCl_2$, 3.4 mmol/L Na_2HPO_4, 593 mmol/L KH_2PO_4, and 5% bovine serum albumin, and incubated overnight with anti-phospho-Ser^{473}-Akt or anti-Akt polyclonal antibody. Detection used donkey anti-rabbit IgG peroxidase-coupled secondary antibody and the Renaissance chemiluminescence system on Kodak X-Omat Blue ML films (Eastman Kodak, New Haven, CT). Bands were quantified using Eagle Eye software (Stratagene Corp., La Jolla, CA).

Pharmacodynamic Studies

HT-29 colon cancer cells (10^7) were injected s.c. into the flanks of male severe combined immunodefcient (*scid*) mice and allowed to grow to ~300 mm^3. Mice were administered an i.p. dose of viridins at 5, 10, and 20 mg/kg in 0.9% NaCl, 0.1% Tween 20, or vehicle alone. Mice were killed at various times, and the tumors were removed and immediately frozen in liquid N_2. For the assay, the tumors were homogenized in lysis buffer and Western blotting was performed using anti-phospho-Ser^{473}-Akt and anti-Akt antibodies. Tumor Akt activity was expressed as the ratio of phospho-Ser^{473}-Akt to total Akt.

Pharmacokinetic Studies

Male C57BL/6 mice were administered PX-866, PX-867, or PX-881 i.p. at doses of 5, 10, or 15 mg/kg or PX-866 i.p., i.v., or p.o. at a dose of 10 mg/kg,. The mice were killed at different times, blood was collected into heparinized tubes, and plasma was prepared. When bile was collected, the entire gall bladder of the mouse was removed. Plasma (0.2 mL) and gall bladders were immediately mixed with 0.2 mL of 0.25 mol/L sodium phosphate buffer (pH 10) and extracted for 1 hour by inversion with 4 mL ethyl acetate. After centrifugation, organic layer (3.8 mL) was removed and evaporated under N_2 and the residue was dried on a lyophilizer. LC/mass spectrometry analysis was performed on a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer in tandem with a Surveyor LC system (San Jose, CA). Chromatographic separation was achieved with a Waters Symmetry C-18 3.9 × 150 mm column (Waters, Milford, PA) with a mobile phase of 0.1% trifluoroactetic acid in 60% methanol at a flow rate of 0.4 mL/min. For the assay, the sample residue was dissolved in 100 μL mobile phase and centrifuged at 15,000 × *g* for 5 minutes at 4°C. The autosampler sample tray was maintained at 4°C, and the injection volume was 50 μL. Analytes were ionized by positive electrospray ionization with a spray voltage of 5,000 V, a sheath gas pressure of 30 psi, and a capillary temperature of 280°C. N_2 was used as the sheath gas and argon as the collision gas at a pressure of 1.5 mTorr. Detection of analytes employed selective reaction monitoring that only allows ions of a selected mass to pass through the first quadropole into the collision cell. After fragmentation in the collision cell, the third quadropole allows only fragments of a selected mass to pass on to the detector. The following reactions were monitored using the selective reaction monitoring scan mode for compounds for which authentic standards were available [i.e., (with parent mass/fragment mass) PX-866 (526.2/336.1) at a collision energy of 36 eV, *N*-mono-deallyl PX-866 (486.0/294.1) at a collision energy of 24 eV, and *N*-di-deallyl PX-866 (446.0/254.023) at a collision energy of 28 eV]. The limit of detection of the assay for all the compounds from 0.2 mL mouse plasma was 0.1 ng/mL.

Antitumor Studies

HT-29 colon cancer, OvCar-3 ovarian cancer, or A-549 non-small cell lung cancer cells (10^7) in log cell growth suspended in 0.2 mL Matrigel (Becton Dickinson Biosciences, Palo Alto, CA) were injected s.c. into the flanks of *scid* mice. The animals were weighed weekly, and tumor diameters were measured twice weekly at right angles (d_{short} and d_{long}) with electronic calipers and converted to volume by the formula volume = $(d_{short})^2 \times (d_{long}) \div 2$ (35). When the tumors reached volumes between 150 and 300 mm^3, the mice were stratified into groups of eight animals having approximately equal mean tumor volumes, and administration of the viridins was started. When the tumor volume reached ≥2,000 mm^3 or became necrotic, the animals were euthanized. Tumor growth rate was calculated from the linear portion of the least squares regression of the cube root of the tumor volume. Log_{10} cell kill was calculated by the formula log_{10} cell kill = [tumor growth delay (day)] × [tumor doubling time (day) × 3.32] (36). One-way ANOVA using the general linear model (37) was used to test for the effect of treatment on tumor growth rate and growth delay.

Results

Viridins

Ten semisynthetic viridins (with structures in Fig. 1) were chosen for scaleup and *in vivo* testing from a compound library of >100 viridins and for their ability to inhibit PtdIns-3-kinase at or above the level of wortmannin and to inhibit the growth of cancer cells in short-term culture (Table 1).

Toxicity of Wortmannin and the Semisynthetic Viridins

Previous studies have shown that administering multiple daily doses of wortmannin to rats causes lymphocytopenia and increases blood liver enzymes (RAID Report: Wortmannin NSC-221019. National Cancer Institute, 2003). This was confirmed in the present study in which i.p. wortmannin at its MTD of 3 mg/kg/d for 4 days caused a 6.2-fold increase in blood alanine aminotransferase and a 1.6-fold increase in blood aspartate aminotransferase compared with pretreatment values (both $P < 0.05$). Wortmannin at its MTD also caused a 60% increase in blood glucose ($P < 0.05$) and a 68% decrease in the lymphocyte count ($P < 0.05$). There was no significant change in neutrophil, RBC, or platelets counts and no change in blood urea nitrogen or creatinine, indicating no renal toxicity. The viridins had MTDs when administered i.p. to mice 3- to 10-fold higher than wortmannin. Liver toxicity for each of the viridins relative to wortmannin was measured as the mean increase in blood alanine aminotransferase and aspartate aminotransferase at equivalent lymphocyte lowering doses to wortmannin at its MTD (Table 1). Based on the results, the viridins PX-866, PX-867, and PX-881 were taken for further evaluation as antitumor agents. All three viridins were stable in 20 mmol/L sodium phosphate buffer at pH 7.0 with $t_{1/2}$ values of >15 hours.

Table 1. Relative toxicities of the compounds administered to mice

Compound	PtdIns-3-K Inhibition IC_{50} (nmol/L)	Cytotoxicity NCI Cell Panel IC_{50} (μmol/L)	MTD (mg/kg)	Liver Toxicity
Wortmannin	1.2	8.9	3.0	1.00
PX-866	0.1	2.2	19.5	0.35
PX-867	1.1	0.5	18.0	0.23
PX-868	1.0	11.9	>30	ND
PX-870	1.0	7.1	6.9	0.94
PX-871	0.1	10.2	9.0	0.48
PX-880	2.0	8.1	>30	ND
PX-881	0.8	0.7	29.5	0.37
PX-882	0.3	10.2	18.0	0.92
PX-889	>10	21.0	>30	ND
PX-890	10	33.0	30	0.91

NOTE: Wortmannin and 10 semisynthetic viridins were chosen for study based on their IC_{50} for PtdIns-3-kinase and in the National Cancer Institute 60 human tumor cell line 3-day cytotoxicity assay. Groups of three C57BL6 mice were administered wortmannin or the semisynthetic viridins i.p. at least four doses of up to 30 mg/kg/d for 4 days. The animals were killed 24 hours after the last dose, and differential blood counts and serum chemistry were determined. The MTD is the dose that gave ≥4 g body weight loss or death of at least one of the three animals. Liver toxicity is expressed relative to wortmannin at its MTD at a dose of the viridin that gave the same myelosuppression as wortmannin at its MTD. PtdIns-3-K, PtdIns-3-kinase; NCI, National Cancer Institute; ND, could not be determined.

Inhibition of Cellular Akt

The ability of the semisynthetic viridins to inhibit the epidermal growth factor–dependent activation of Akt was measured in HT-29 colon cancer cells by Western hybridization using anti-phospho-Ser^{473}-Akt antibody (Fig. 2A). Quantitation of the blots to give the ratio of phospho-Ser^{473}-Akt to total Akt (Fig. 2B) gave IC_{50} values (±SE) for PX-866 of 16.8 ± 2.2 nmol/L, PX-867 of 26.7 ± 3.5 nmol/L, and PX-881 of 13.7 ± 0.6 nmol/L.

Inhibition of Tumor Akt Activation

Administration of PX-866, PX-867, and PX-881 as a single i.p. dose to *scid* mice bearing HT-29 human colon cancer xenografts significantly decreased the ratio of tumor phospho-Ser^{473}-Akt to total Akt after 4 hours with effective doses giving 50% inhibition (ED_{50}) for PX-866 of 7.5 mg/kg, PX-867 of >20 mg/kg, and PX-881 of 14.5 mg/kg (Fig. 3A). Wortmannin under the same conditions had an ED_{50} of 0.45 mg/kg (data not shown). PX-866 was the most active synthetic viridin and was used for time course and scheduling studies. A single i.p. dose of PX-866 of 10 mg/kg inhibited tumor phospho-Ser^{473}-Akt to total Akt by 58% at 2 hours compared with pretreatment values ($P < 0.01$) but had returned to pretreatment levels by 24 hours (Fig. 3B). Both i.v. and p.o. administration of PX-866 at 10 mg/kg gave prolonged inhibition of phospho-Ser^{473}-Akt to total Akt with 64% inhibition by 24 hours ($P < 0.1$) following i.v. administration and a return to pretreatment values by 48 hours and 78% inhibition by 24 hours ($P < 0.01$) following p.o. administration, and inhibition was still 69% at 48 hours ($P < 0.05$).

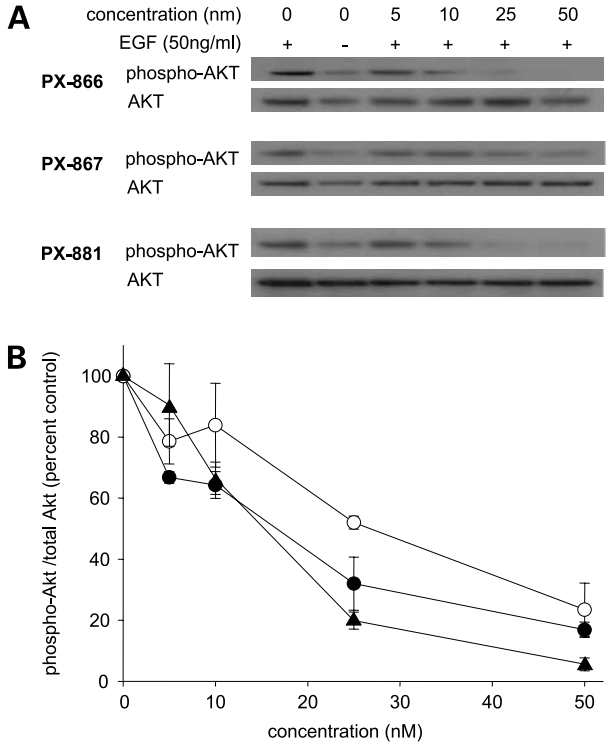

Figure 2. Inhibition of cellular Akt in HT-29 cells. **A,** cells were treated with the semisynthetic viridins at the indicated concentrations for 2 hours and stimulated with epidermal growth factor (50 ng/mL) for 20 minutes. The cells were lysed and the proteins were separated on SDS-PAGE. Typical Western blots for PX-866, PX-867, or PX-881 showing phospho-Ser^{473}-Akt and total Akt. **B,** plots of densitometry scans of the Western blots for PX-866 (●), PX-867 (○), and PX-881 (▲) expressed as the ratio of phospho-Ser^{473}-Akt to total Akt as a percentage of untreated control. *Points,* mean of three separate experiments; *bars,* SE.

Pharmacokinetics

The highest parent compound plasma concentration at 3 minutes following a single i.v. dose of 10 mg/kg was achieved by PX-866 (Fig. 4A). The total area under the plasma concentration time curve (AUC) was much greater for PX-866 (27.7 min μg/mL) than for PX-867 (5.2 min μg/mL) or PX-881 (8.3 min μg/mL; Table 2). PX-866 showed a parent compound AUC following i.p. administration of 0.89 min μg/mL and following p.o. administration of 0.29 min μg/mL (Fig. 4B). Thus, the bioavailability compared with i.v. administration for parent PX-866 was 0.03 following i.p. administration and 0.01 following p.o. administration. These differences suggest first-pass metabolism by the liver, and we therefore studied the metabolism of PX-866. The proposed metabolic pathway for PX-866 is shown in Fig. 5. PX-866 and its *N*-deallylated and *N*-di-deallylated metabolites (compounds **1** and **2**) were identified by their chromatographic retention, mass, and fragmentation patterns compared with authentic reference compound. Other metabolites provisionally identified by their mass and fragmentation patterns were the PX-866 epoxide (3) and its dihydroxy-metabolite after ring opening (4) and the deacylated form of PX-866. Mouse plasma deacetylated PX-866 to give compound **5** with a half-life of 28 minutes at 37°C. Compounds **6** and **7** were unidentified metabolites of compounds **1** and **2**, respectively. Metabolites of PX-866 detected in the mouse plasma were compounds **1**, **4**, **6**, and **7** (Table 3), with levels that peaked and declined at the same rate as PX-866. PX-866 itself was the largest peak in bile, and digestion of bile with β-glucuronidase increased the size of this peak by 3-fold (data not shown). Digestion of bile with sulfatase had no effect on the size of the PX-866 peak. Metabolites of PX-866 present in mouse bile were compounds **1**, **2**, **6**, and **7** and small amounts of compounds **3** and **4**. The total amount of PX-866 and metabolites recovered from mouse bile >2 hours was ~10% of the total PX-866 administered.

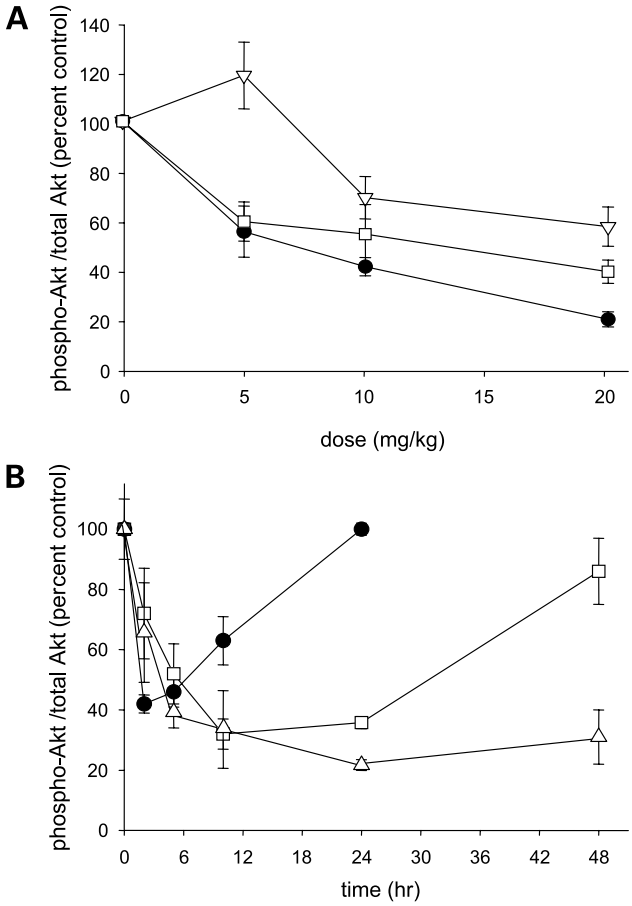

Figure 3. Inhibition of PtdIns-3-kinase signaling in HT-29 colon cancer xenografts. Male *scid* mice with 300 mm^3 HT-29 colon cancer xenografts were administered the semisynthetic viridins, and the xenografts were removed after various times. Akt activity was measured by Western blotting and expressed as the ratio of phospho-Ser^{473}-Akt to total Akt. **A,** dose dependency of the inhibition of tumor Akt at 4 hours after a single i.p. dose of PX-866 (●), PX-867 (▽), or PX-881 (□). **B,** time course of Akt inhibition following the administration of PX-866 at a dose of 10 mg/kg by either i.p. (●), p.o. (△), or i.v. (□) route. *Points,* mean of three mice; *bars,* SE.

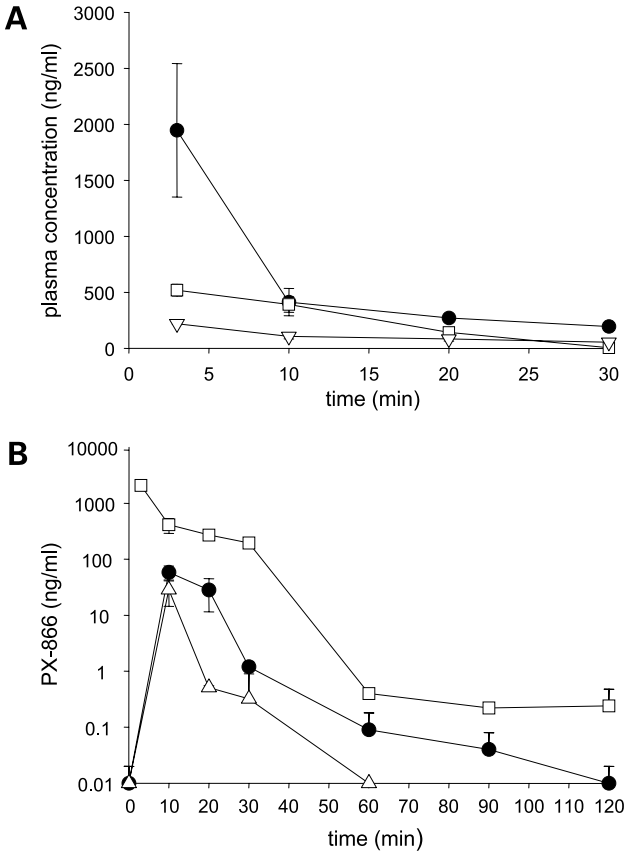

Figure 4. Plasma level of wortmannin analogues in mice. **A,** plasma levels of PX-866 (●), PX-867 (▽), and PX-881 (□) following administration of the compounds as a single i.p. dose of 10 mg/kg to male C57BL/6 mice. **B,** plasma levels of PX-866 administered to male C57BL/6 mice at a dose of 10 mg/kg by either i.v. (□), i.p. (●), or p.o. (△) routes. *Points,* mean of three mice per time point; *bars,* SE.

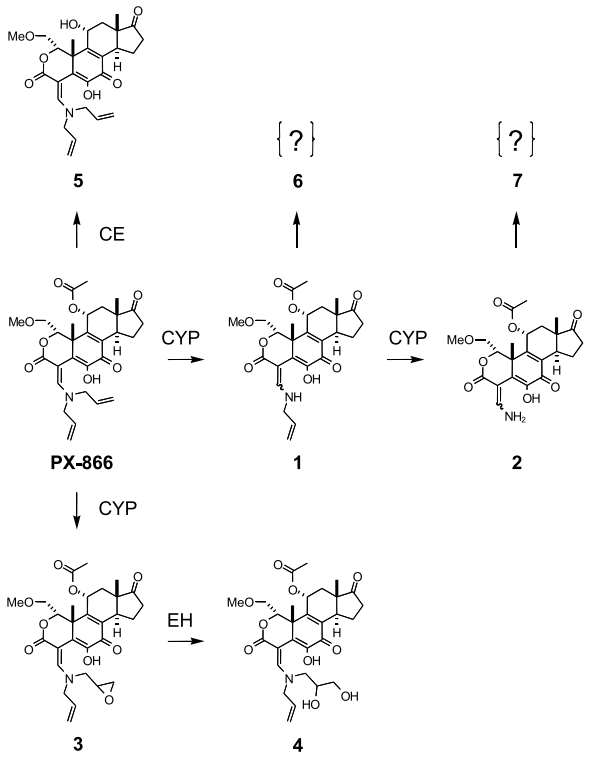

Figure 5. Metabolism of PX-866 in the mouse. PX-866 undergoes sequential *N*-deallylation probably catalyzed by cytochrome *P*450 (*CYP*) to give the *N*-mono- and *N*-di-deallylated metabolites **1** and **2**. *CYP*-dependent metabolism may also give the epoxide **3**, which undergoes epoxide ring opening by epoxide hydrolase (*EH*) to give **4**. Deacetylation of PX-866 by plasma carboxylesterases (*CE*) gives compound **5**. Compounds **6** and **7** are unidentified metabolites of **1** and **2**, respectively.

PtdIns-3-Kinase Inhibition by the Metabolites

The ability of the reference compounds to inhibit PtdIns-3-kinase was studied. PX-866 inhibited immunoprecipitated PtdIns-3-kinase with an IC_{50} of 0.1 nmol/L, the *N*-deallylated metabolite **1** with an IC_{50} of 7.5 nmol/L, and the *N*-di-deallylated metabolite **2** with an IC_{50} of 2.0 nmol/L.

Antitumor Activity

The antitumor activity of the viridins was investigated using s.c. implanted human tumor xenografts in *scid* mice. The compounds were administered at ~75% of their MTD at the schedule and route employed (Table 4). Treatment of mice with established 120 mm^3 OvCar-3 ovarian cancer tumor xenografts with PX-866 i.p. daily for 9 days gave the best antitumor response with an optimal treated versus control (T/C) of 30% and a calculated log_{10} cell kill of 1.2. All semisynthetic viridins were more active than wortmannin (T/C 47%; log_{10} cell kill 0.5), and PX-866 was more active than PX-867 (T/C 41%; log_{10} cell kill 0.6) or PX-881 (T/C 52%; log_{10} cell kill 0.8). In the rapidly growing HT-29 colon cancer xenograft, PX-866 administered i.p. showed the same activity as wortmannin with T/C of 39% and a

Table 2. Pharmacokinetic parameters of PX-866, PX-867, and PX-881

Compound	C_{max} (μg/mL)	$t_{1/2}$ (min)	AUC (0→∞) (min μg/mL)	Cl (mL/min/kg)	V_d (L/kg)
PX-866	1.95	18.4	27.70	360	9.61
PX-867	0.22	20.3	5.19	1,925	56.3
PX-881	0.60	3.32	8.31	56	5.8

NOTE: Mice were administered PX-866, PX-867, and PX-881 i.v. at 10 mg/kg. The pharmacokinetic parameters are C_{max} (maximum plasma concentration), $t_{1/2}$ (half-life), AUC from time 0 minute to infinity, Cl (plasma clearance), and V_d (volume of distribution).

Table 3. Metabolites of PX-866 in plasma and bile

Route	PX-866 (Rt = 10.1 min)	**1** (Rt = 11.5 min)	**2** (Rt = 5.5 min)	**4** (Rt = 4.9 min)	**6** (Rt = 7.4 min)	**7** (Rt = 7.7 min)
i.v. plasma	23.70	1.62	ND	ND	3.11	0.24
p.o. plasma	0.30	0.06	ND	ND	0.28	0.55
Bile	15.6	0.20	0.23	0.65	0.71	1.68

NOTE: PX-866 was administered at 10 mg/kg by either i.v. or p.o. route to male C57BL/6 mice. Plasma and bile were collected at up to 120 minutes, and metabolites were measured by LC/mass spectrometry/mass spectrometry. Values are the AUC for plasma in min μg/mL and bile in min μg.

calculated $\log_{10}$ cell kill of 0.4 and was more active than the other analogues. In A-549 non-small cell lung cancer xenografts, PX-866 administered i.v. at 9 mg/kg/d for 9 days gave a T/C of 26%, and when given i.v. at 12 mg/kg/d every other day for 5 days, the T/C was 45% and the $\log_{10}$ cell kill was 0.7. PX-866 administered p.o. at 4 mg/kg/d every other day for 5 days, which was 75% of the MTD by this route and schedule, gave a T/C of 47% and a $\log_{10}$ cell kill of 0.9.

PX-866 was combined with cisplatin or radiation to explore potential additive or synergistic effects on antitumor activity. When combined with cisplatin, 1 mg/kg/d i.p. for 5 days against A-549 lung cancer xenografts, the T/C at 37 days for cisplatin was 80% ($P > 0.05$), PX-866 alone was 74% ($P > 0.05$), and PX-866 given 2 hours before the cisplatin was 44.8% ($P < 0.05$), suggesting that there was more than an additive effect of the two treatments (Fig. 6). When combined with 1 Gy radiation daily for 5 days against OvCar-3 ovarian cancer xenografts, the T/C at 84 days for radiation was 59% ($P > 0.05$), for PX-866 at 8 mg/kg 112% and 12.5 mg/kg 52% ($P < 0.01$), and for radiation 2 hours after PX-866 at 8 mg/kg 35.3% ($P < 0.01$) and 12.5 mg/kg 12% ($P < 0.01$; Fig. 7). Thus, PX-866 activity is more than additive when combined with radiation and most clearly at the lower dose of PX-866.

Discussion

The PtdIns-3-kinases are a diverse group of enzymes with a multiplicity of cellular functions. It has been suggested that selective inhibitors of PtdIns-3-kinase might have an improved therapeutic index as anticancer agents compared with broad-spectrum inhibitors (3). However, attempts to develop isoform-selective inhibitors of PtdIns-3-kinases are hampered by a lack of detailed knowledge of the functions of the different PtdIns-3-kinases (3, 38, 39). Furthermore, the high degree of functional redundancy among PtdIns-3-kinases might make the selective inhibition of cellular function, such as inhibition of cancer cell growth, an elusive goal. To date, there are no reports that selective PtdIns-3-kinase inhibitors, although they exist (reviewed in ref. 39), have significant antitumor activity. Antitumor activity has, however, been reported for the broad-spectrum PtdIns-3-kinase inhibitors wortmannin (34) and LY294002 (40, 41). In fact, developing highly selective inhibitors of PtdIns-3-kinase may not be desirable. It is increasingly apparent that many presumed selective tyrosine kinase inhibitors inhibit multiple kinases, which is responsible for their antitumor activity (42, 43). There are now several multitargeted tyrosine kinase inhibitors in clinical development.

We chose to develop broad-spectrum inhibitors of PtdIns-3-kinase by building on our earlier studies showing

Table 4. Antitumor activity of wortmannin analogues against human tumor xenografts in *scid* mice

Tumor (Doubling Time)	Initial Tumor Volume (mm^3)	Compound	mg/kg/d	Route	Schedule	T/C% (d)	Growth Delay (d)	Log_{10} Cell Kill	*P*
OvCar-3 ovarian (6.0 d)	120	Wortmannin	0.75	i.p.	Q1D × 9	47 (40)	5	0.5	*
	120	PX-866	8	i.p.	Q1D × 9	30 (40)	12	1.2	*
	120	PX-867	13	i.p.	Q1D × 9	41 (40)	6	0.6	*
	120	PX-881	12	i.p.	Q1D × 9	52 (40)	8	0.8	*
HT-29 colon (5.2 d)	180	Wortmannin	0.75	i.p.	Q1D × 9	36 (16)	5	0.3	*
	170	PX-866	12	i.p.	Q1D × 9	39 (16)	6	0.4	*
	170	PX-867	13	i.p.	Q1D × 9	80 (16)	0	0	
	170	PX-881	12	i.p.	Q1D × 9	62 (16)	2	0.2	
A-549 lung (5.8 d)	65	PX-866	6	i.p.	Q1D × 9	62 (32)	4	0.2	
	65	PX-866	9	i.p.	Q1D × 9	26 (32)	8	0.4	*
	60	PX-866	12	i.v.	Q2D × 5	45 (21)	5	0.7	*
	60	PX-866	4	p.o.	Q2D × 5	47 (21)	6	0.9	*

NOTE: Mice were implanted s.c. with 10^7 tumor cells. When the tumor volume reached the value shown, the mice were randomized into groups of eight and drug treatment started at the indicated doses. The doubling time for each tumor during its log phase of growth is shown in parentheses.
*$P < 0.05$ compared with non-drug-treated control tumor growth rate.

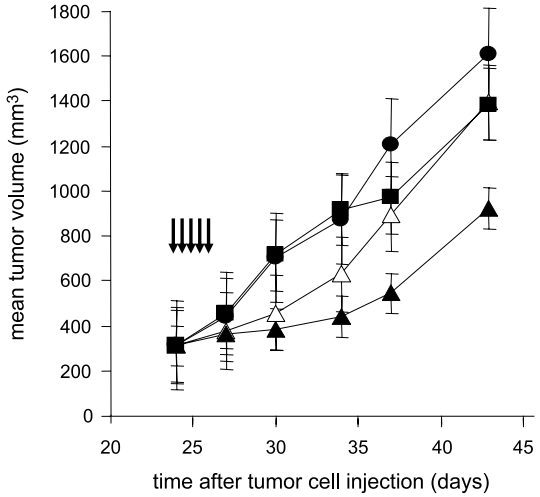

Figure 6. Antitumor activity of PX-866 with cisplatin against A-549 lung cancer xenografts. Female *scid* mice were implanted s.c. with 10^7 A-549 human non-small cell lung cancer cells. Drug injection was begun on day 24, when the tumors were 300 mm^3 daily i.p. for 5 days (*arrows*) with vehicle alone (●), cisplatin 1 mg/kg (■), PX-866 6 mg/kg (△), or PX-866 6 mg/kg administered 2 hours before cisplatin (▲, 6 mg/kg). *Points,* mean of eight mice in each group; *bars,* SE.

that wortmannin has antitumor activity against a variety of tumor xenografts in animal models (34). The limitations to the use of wortmannin as an antitumor agent are its acute toxicity, including hepatotoxicity, and its biological instability. For example, wortmannin cannot be detected in blood after administration to mice (RAID Report: Wortmannin NSC-221019. National Cancer Institute, 2003).[6] From a series of 10 semisynthetic viridins chosen for their ability to inhibit PtdIns-3-kinase and for their growth inhibition of cancer cells in short-term culture, three were chosen for *in vivo* investigation. The compounds PX-866, PX-867, and PX-881 were stable in solution at physiologic pH, and they achieved measurable concentrations in plasma following i.v. administration with plasma half-lives up to 20 minutes. All three semisynthetic viridins inhibited cell and tumor xenograft phospho-Ser^{473}-Akt used as an integrated measure of the activity of the PtdIns-3-kinase signaling pathway.

PX-866 was chosen for further evaluation being the best inhibitor of tumor phospho-Ser^{473}-Akt, being water soluble for i.v. administration, and giving the largest plasma AUC for parent compound in mice. PX-866 showed marked differences in the time course of its inhibition of tumor phospho-Ser^{473}-Akt depending on its route of administration, with inhibition lasting <24 hours following i.p. administration, 36 hours following i.v. administration, and >48 hours following p.o. administration. There was no correlation between tumor phospho-Ser^{473}-Akt inhibition and plasma AUC for parent drug following administration by the different routes. PX-866 was metabolized to give the *N*-deallylated and *N*-di-deallylated metabolites, both of which retained the ability to inhibit PtdIns-3-kinase at low nanomolar per liter concentrations, as well as other unidentified metabolites. There is clearly a discrepancy between the levels of PX-866 and the metabolites that we have been able to identify in the plasma and the antitumor activity of PX-866 particularly comparing i.p. and p.o. administration. PX-866 metabolites were secreted in the bile so that enterohepatic cycling might contribute to the long-term inhibition of tumor PtdIns-3-kinase signaling particularly following p.o. administration. Another possibility is that there are as yet unidentified metabolites of PX-866 that are responsible for the inhibition of PtdIns-3-kinase signaling. This will require further study. Finally, PX-866 and its metabolites are irreversible inhibitors of PtdIns-3-kinase so that the time course of inhibition of PtdIns-3-kinase may bear little direct correlation to plasma concentrations.

In vivo antitumor activity was seen with PX-866, PX-867, and PX-881, but PX-866 was the most active compound with i.v. and p.o. administration showing greater antitumor activity than i.p. administration. Inhibition of the PtdIns-3-kinase/Akt cell survival pathway with wortmannin has been reported to enhance the activity of other anticancer drugs, including gemcitabine against pancreatic cell lines *in vitro* (44), Gleevec against Philadelphia chromosome positive chronic myelogenous leukemia *in vitro* (45), and gemcitabine against orthotopic pancreatic tumor xenografts *in vivo* (46). In our study, PX-866 increased the antitumor activity of cisplatin against A-549 non-small cell lung cancer xenografts. We also found that PX-866 potentiated *in vivo* growth inhibition by radiation in OvCar-3 human tumor xenografts. The potentiation by wortmannin of radiation cell killing has been ascribed in cell lines to inhibition of tumor cell DNA-dependent kinase (47) and in tumors to inhibition of PtdIns-3-kinase in vascular endothelium (45).

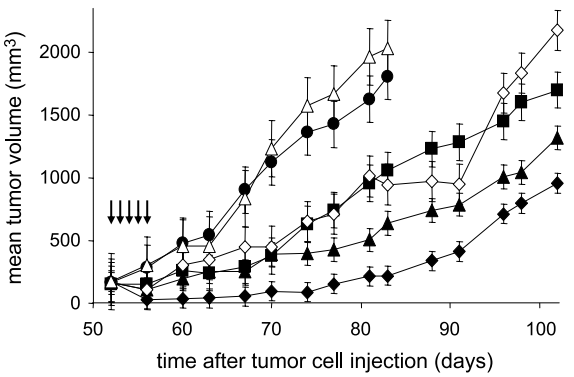

Figure 7. Antitumor activity of PX-866 with radiation against OvCar-3 ovarian cancer xenografts. Female *scid* mice were implanted s.c. with 10^7 OvCar-3 human ovarian cancer cells. Treatment was begun on day 52, when the tumors were 150 mm^3 daily for 5 days (*arrows*) with vehicle alone (●), 1 Gy radiation (■), PX-866 8 mg/kg i.p. (△), PX-866 12.5 mg/kg i.p. (◇), PX-866 8 mg/kg i.p. administered 2 hours before 1 Gy radiation (▲), and PX-866 12.5 mg/kg i.p. administered 2 hours before 1 Gy radiation (◆). *Points,* mean of eight mice in each group; *bars,* SE.

[6] G. Powis, unpublished results.

PX-866 and wortmannin inhibit PtdIns-3-kinases at 0.1 and 1.2 nmol/L, respectively. At concentrations >50 nmol/L, wortmannin inhibits other PtdIns-3-kinase family members such as DNA-dependent protein kinase, ataxia telangiectasia mutated, ataxia telangiectasia related, mammalian target of rapamycin, and PtdIns-4-kinase. We have found that PX-866 does not inhibit mammalian target of rapamycin kinase activity at concentrations up to 10 μmol/L.[7] Unrelated proteins such as phospholipase C, phospholipase D, and myosin light chain kinase are also inhibited by wortmannin (3, 38, 48). Compared with purified enzyme, cells require at least 10-fold higher concentrations of PX-866 and wortmannin to inhibit PtdIns-3-kinase signaling, and higher concentrations are also required to inhibit the other enzymes. We cannot address directly whether the PtdIns-3-kinases or other targets are responsible for the *in vivo* antitumor activity of PX-866. However, the low plasma concentrations of PX-866 and its metabolites, which after p.o. administration do not rise >40 nmol/L, make it likely that the primary target is indeed PtdIns-3-kinase.

In summary, we have shown that biologically stable semisynthetic viridins inhibit cell and tumor xenograft PtdIns-3-kinase signaling and have *in vivo* antitumor activity with PX-866 being the most active compound. Prolonged inhibition of tumor PtdIns-3-kinase signaling is seen following p.o. and i.v. administration of PX-866. There is first-pass metabolism of PX-866, and synthetic standards of several metabolites were shown to also inhibit PtdIns-3-kinase. PX-866 has single agent *in vivo* antitumor activity and enhances the antitumor activity of other chemotherapeutic drugs and radiation.

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