

Research Article

Molecular Pharmacology and Antitumor Activity of PHT-427, a Novel Akt/Phosphatidylinositide-Dependent Protein Kinase 1 Pleckstrin Homology Domain Inhibitor

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Abstract

Phosphatidylinositol 3-kinase/phosphatidylinositide-dependent protein kinase 1 (PDK1)/Akt signaling plays a critical role in activating proliferation and survival pathways within cancer cells. We report the molecular pharmacology and antitumor activity of PHT-427, a compound designed to bind to the pleckstrin homology (PH) binding domain of signaling molecules important in cancer. Although originally designed to bind the PH domain of Akt, we now report that PHT-427 also binds to the PH domain of PDK1. A series of PHT-427 analogues with variable C-4 to C-16 alkyl chain length were synthesized and tested. PHT-427 itself (C-12 chain) bound with the highest affinity to the PH domains of both PDK1 and Akt. PHT-427 inhibited Akt and PDK1 signaling and their downstream targets in sensitive but not resistant cells and tumor xenografts. When given orally, PHT-427 inhibited the growth of human tumor xenografts in immunodeficient mice, with up to 80% inhibition in the most sensitive tumors, and showed greater activity than analogues with C4, C6, or C8 alkyl chains. Inhibition of PDK1 was more closely correlated to antitumor activity than Akt inhibition. Tumors with PIK3CA mutation were the most sensitive, and K-Ras mutant tumors were the least sensitive. Combination studies showed that PHT-427 has greater than additive antitumor activity with paclitaxel in breast cancer and with erlotinib in non-small cell lung cancer. When given >5 days, PHT-427 caused no weight loss or change in blood chemistry. Thus, we report a novel PH domain binding inhibitor of PDK1/Akt signaling with significant *in vivo* antitumor activity and minimal toxicity. *Mol Cancer Ther*; 9(3); 706–17. ©2010 AACR.

Introduction

The pleckstrin homology (PH) domain is a region of 100 to 120 amino acids found in >250 human proteins (1). Although the amino acid sequence of PH domains is not universally conserved, the tertiary structure is remarkably conserved. Although PH domains bind to a variety of different targets, a unique property of ~40 PH domains is the specificity with which they bind phosphorylated phosphatidylinositide (PtdIns) lipids within the biological cell membrane. PtdIns phosphorylation and the subsequent binding of PH domain-containing proteins are vital components of signal transduction

pathways that regulate cell growth and survival and, thus, are opportunistic targets for upregulation and oncogenic determinism (2).

The PtdIns-3-kinase/PtdIns-dependent protein kinase 1 (PDK1)/Akt (protein kinase B) signaling pathway is critical for the proliferation and survival of many types of cancer cells (3). Its activation is found in a variety of cancers, associated with mutation or loss of the mixed function lipid phosphatase PTEN (4) or mutations of PtdIns-3-kinase (5). PtdIns-3-kinase phosphorylates PtdIns(4,5)P₂ to give PtdIns(3,4,5)P₃, which binds to the PH domain of Akt, allowing Akt to translocate from the cytoplasm to the inner leaflet of the plasma membrane (6) where it is phosphorylated on Thr³⁰⁸ by PDK1, another PH domain-containing protein (7). Subsequent phosphorylation of Akt on the Ser⁴⁷³ residue occurs either by integrin-linked kinase, by the kinase activity of Akt itself, or by mTORC2 (8), causing Akt to translocate back to the cytoplasm and to the nucleus where it phosphorylates a variety of downstream targets. These include promoters of apoptosis, such as forkhead transcription factors and AFX, and the Bcl-2 family member Bad (3). Akt also promotes cell survival by activating CREB (9) and promotes proliferation by activating mammalian

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doi: 10.1158/1535-7163.MCT-09-0985

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target of rapamycin and, indirectly, p70 ribosomal S6 kinase (RSK; ref. 10) and glycogen synthase kinase-3 β (GSK-3 β), which contributes to cyclin D accumulation of cell cycle entry (11). Furthermore, Akt acts as a mediator for both vascular endothelial growth factor production and angiogenesis by phosphorylation of mammalian target of rapamycin (12).

Aberrant PtdIns-3-kinase signaling has been found to play an important role in multiple aspects of tumorigenesis, including uncontrolled proliferation, resistance to apoptosis, angiogenesis, and metastatic capability. This aberrant signaling may occur through dysfunction of pathways upstream of PtdIns-3-kinase, such as mutationally activated growth factor receptors, and Ras, or activation of the pathway itself. The first mechanism discovered by which the phosphatidylinositol 3-kinase/Akt pathway is directly activated was the loss or inactivation of the PTEN tumor suppressor (13). *PIK3CA*, the gene encoding PtdIns-3-kinase p110 α , is frequently mutated in several common cancers (5). Eighty percent of these mutations occur in one of three hotspots, leading to gain-of-function amino acid substitutions in the helical or kinase domain of the enzyme. There seem to be at least two molecular mechanisms for the gain of function in PtdIns-3-kinase p110 α , with helical domain mutations abolishing the binding of the p85 inhibitory subunit and kinase domain mutations mimicking the conformational change caused by the interaction with Ras (5). The other *PIK3CA* mutations are widely distributed and far less potent in activating PtdIns-3-kinase enzyme activity than the hotspot mutations and are only marginally oncogenic. Other PtdIns-3-kinase p110 isoforms do not show cancer-specific mutations. However, they are often differentially expressed in cancer and are oncogenic when overexpressed in cell culture. There is no indication that either class II PtdIns-3-kinase or class III PtdIns-3-kinase is linked to cancer. Most recently, mutations in the PH domain of Akt1, which causes electrostatic alterations leading to increased binding of the Akt PH domain with PtdIns(3,4,5)P₃ and increased phosphorylation, have been found to aberrantly activate the pathway (14). Thus far, the initial mutation found at amino acid 17 of the Akt PH domain has been identified in 8% of the breast tumors studied, 6% of colorectal tumors, and 2% of ovarian cancers. Most attempts to develop inhibitors of Akt have focused on compounds that bind to the kinase ATP-binding pocket. Due to the similarity of the ATP pocket among serine/threonine kinases, particularly AGC family kinases to which Akt belongs, achieving target specificity has been extremely difficult (15). All the reported Akt ATP pocket inhibitors also inhibit protein kinase A (PKA), which may account for the relatively high toxicity of this type of inhibitor observed in animals and in patients.

PDPK1 phosphorylates and activates many members of the AGC subfamily of serine/threonine kinases through phosphorylation of their autoinhibitory activation loops (16). Most PDPK1 substrates do not have a

PH domain and are phosphorylated by PDPK1 in the cytosol (17). Akt is the only known PH domain-containing PDPK1 target and PDPK1 has to be localized to the plasma membrane to activate Akt through binding of its PH domain to PtdIns(3,4,5)P₃ (18). Substrates phosphorylated by PDPK1, in addition to Akt, include p70 RSK (19), p90 RSK (20), protein kinase C (PKC) isoforms (21), and serum/glucocorticoid-regulated kinases (SGK; ref. 22). PDPK1 copy numbers and levels are increased in human breast cancer, leading to the increased ability of upstream lesions in the PtdIns-3-kinase pathway to signal to Akt (23). Tumors with *PIK3CA* mutations seem to preferentially signal through PDPK1 and its downstream target SGK3 (24). Thus, PDPK1 is also an attractive target for cancer drug discovery. However, as with Akt, it has proven difficult to achieve catalytic ATP pocket inhibitor selectivity for PDPK1 compared with other serine/threonine kinases such as PKA (15). Thus, the PH domain of PDPK1 presents an alternate target for the development of inhibitors with the potential for antitumor activity.

The approach we have adopted to inhibit Akt and PDPK1 uses structure-based design of small molecules that bind to the PH domains of the proteins, thus inhibiting their activity. We have identified a novel chemical scaffold in several compounds that binds selectively to the PH domain of Akt, inducing a decrease in Akt activation and causing apoptosis at low micromolar concentrations (25). One compound, PHT-427 (4-dodecyl-N-(5-(5-(methyl(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)pentyl-1,3,4-thiadiazol-2-yl)benzenesulfonamide), inhibited Akt and its downstream targets in cells and showed *in vivo* antitumor activity when administered by the i.p. route in a pancreatic cancer xenograft (26). We now report the cellular and *in vivo* pharmacology of PHT-427 and some analogues with different alkyl chain lengths and show that PHT-427 binds to the PH domains of both Akt and PDPK1 and inhibits the activity of both proteins in cells and tumor xenografts. We have developed an oral formulation for PHT-427 and show its antitumor activity against several human tumor xenografts, particularly those with *PIK3CA* mutations.

Materials and Methods

Cells and Reagents

BxPC-3, Panc-1, and MiaPaCa-2 pancreatic cancer cells; PC-3 prostate cancer cells; SKOV-3 ovarian cancer cells; MCF-7 breast cancer cells; and A-549 and NCI-H441 non-small cell lung cancer (NSCLC) cells were obtained from the American Tissue Type Collection. All cell lines were characterized by short tandem repeat profiling before use by the M.D. Anderson Cell Characterization Service. The cells were grown in humidified 95% air and 5% CO₂ at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS). All cell lines were tested to be *Mycoplasma*-free using a PCR ELISA kit (Roche Diagnostics, Inc.). PHT-427 (Fig. 1A) and its C2 to C14 carbon

chain length analogues (Fig. 1B) were synthesized as previously described (26, 27). The Akt inhibitors triciribine (NSC 154020), perifosine, and edelfosine were purchased from Cayman Chemical, and DPIEL (D1-3,4-dideoxyphosphatidylinositol ether lipid) and MK-2206 were synthesized by the M.D. Anderson Translational Chemistry Service. PHT-427 was formulated for oral administration suspended at 40 to 50 mg/mL in sesame seed oil. Expression and purification of recombinant human Akt2 and PDPK1 PH domains was done as previously described (26).

Surface Plasmon Resonance Spectroscopy Binding Assays

All interaction analyses were done with a Biacore 2000 using SA chips, Biacore 2000 Control Software v3.2, and the BIAevaluation v4.1 analysis software (GE Healthcare) as previously described (25). Competitive binding assays and K_i determination used PtdIns(3,4,5)P₃ phosphate biotin-labeled liposomes (Echelon Biosciences) and increasing concentrations of the compounds being tested.

Signaling Pathway Inhibition

Reverse-phase protein array (RPPA) analysis was carried out as previously described (28). Inhibition of the phosphorylation of Akt, PDPK1, and their downstream targets was measured by Western blotting using rabbit

polyclonal antibodies to phospho-Ser⁴⁷³-Akt, phospho-Thr³⁰⁸-Akt, total Akt, phospho-Ser²⁴¹-PDPK1, phospho-Ser⁹-GSK-3 β , phospho-Ser²¹-GSK-3 β , phospho-Ser⁶⁵⁷-PKC, and phospho-Ser²⁴⁰-p70S6 kinase (Cell Signaling Technology, Inc.) as described previously (26). PDPK1-specific inhibition was measured by phospho-Ser²²¹-RSK (R&D Systems). β -Actin (Santa Cruz Biotechnology) was used as a loading control and blots were quantified using an ImageQuant image analyzer (Molecular Dynamics).

Cell Imaging

Panc-1 cells stably transfected with green fluorescent protein (GFP)-tagged Akt or PDPK1 PH domains were serum starved in phenol red-free growth medium on glass-bottomed 96-well imaging plates (Matrical) for 16 h. They were then treated with PHT-427 at 1, 5, and 10 μ mol/L or PI-103 (Cayman Chemical) for 4 h and stimulated with 50 ng/mL insulin-like growth factor-I (IGF-I; R&D Systems) for 10 min. Images were taken before and after IGF-I treatment using an IN Cell Analyzer 1000 (GE Healthcare Life Sciences) instrument with a Nikon Plan Fluor ELWD 20 \times /0.45 objective loaded and using a 300-ms exposure time.

Antitumor Studies

Approximately 10⁷ BxPC-3, Panc-1, MiaPaCa-2, PC-3, SKOV-3, A-549, or MCF-7 cells in log cell growth were suspended in 0.2 mL PBS and injected s.c. into the flanks of female *scid* mice. The MCF-7 cells were suspended in Matrigel (Becton Dickinson Biosciences), and the mice were implanted 1 d previously with a 60-d 17- β -estradiol release pellet (Innovative Research of America). 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 following formula: $v = (d_{short})^2 \times (d_{long}) / 2$ (29). When the tumors reached volumes between 150 and 300 mm³, the mice were stratified into groups of eight animals having approximately equal mean tumor volumes, and administration of PHT-427 or its analogues, dissolved on 0.1 mL sesame seed oil, was begun. Control animals received vehicle alone. When the tumor volume reached $\geq 1,500$ mm³ or became necrotic, the animals were euthanized. The growth rate of individual tumors was measured over the 10-d period of dosing, and the mean growth rate for each treatment group was compared with the control using Student's *t* test. NCI-H441 NSCLCs were implanted orthotopically in the left lung of *nu/nu* nude mice as previously described (30), and 20 d later, oral PHT-427 administration started at 200 mg/kg twice daily for 10 d. Erlotinib was administered orally, also beginning at 20 d, at 50 mg/kg daily until the end of the study. All mice were killed at day 63 when the control animals became moribund, and tumor volumes were measured as above.

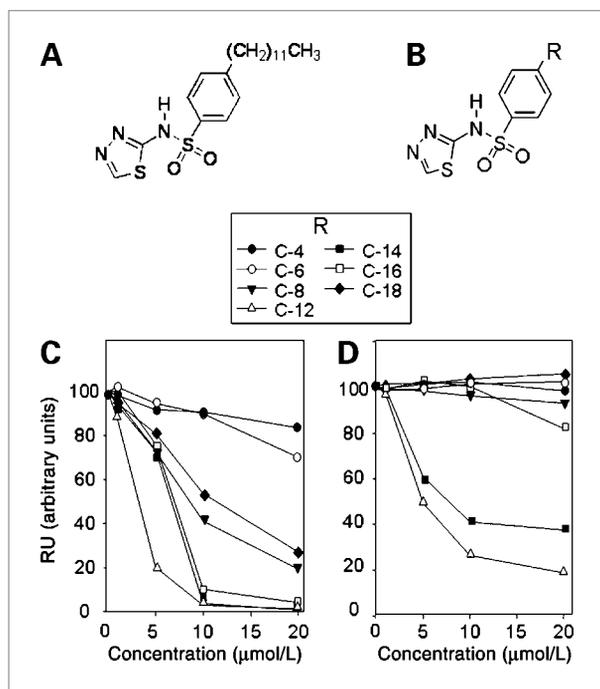


Figure 1. Relative binding of PHT-427 analogues with different carbon chain lengths to the expressed PH domains of Akt and PDK. A, structure of PHT-427. B, structure of analogues where R is a C-4 to C-14 carbon chain. SPR spectroscopy was used to measure the binding affinity (K_i) for the expressed PH domain of Akt2 (C) and PDPK1 (D) by competitive binding of the compounds for the natural ligand PtdIns(3,4,5)P₃.

Pharmacokinetic Studies

Female C57BL/6 mice were administered PHT-427 as a single oral dose of 200 mg/kg. The mice were killed at different times (three mice at each time point), blood was collected into heparinized tubes, and plasma was prepared and stored frozen at -80°C . For assay, 0.2 mL plasma was mixed with 0.2 mL of 0.1 mol/L sodium phosphate buffer (pH 4.0) and extracted for 1 h by inversion with 1 mL ethyl acetate. After centrifugation, 0.8 mL of the organic layer was removed, evaporated under N_2 , and redissolved in 0.2 mL ethanol, and 10 μL were injected onto a Waters Quattro Ultima tandem mass spectrometer using a Phenomenex Luna 3.0 μm , 2.0×50 mm C8 analytic column (Phenomenex), with detection and quantification by multiple reaction monitoring with the mass spectrometer operating in electrospray positive ionization mode. The mass transition m/z 410.2 \rightarrow 91.3 was used to detect and quantify PHT-427 with quantification by external standardization, with a peak retention time for PHT-427 of 6.1 min. The lowest level of detection for plasma PHT-427 was <1 ng/mL.

Pharmacodynamic Studies

BxPC-3 or MiaPaCa-2 pancreatic cancer cells (1×10^7) were injected s.c. into the flanks of female *scid* mice and allowed to grow to ~ 300 mm³. Mice received a single oral dose of PHT-427 of 200 mg/kg in 0.1 mL vehicle. Mice were killed after various times, and the tumors were removed and immediately frozen in liquid N_2 . The tumors were homogenized in 50 mmol/L HEPES buffer (pH 7.5), 50 mmol/L NaCl, 1% NP40, and 0.25% sodium deoxycholate. Western blotting was done as described above.

Toxicity Studies

PHT-427 in vehicle or vehicle alone was administered orally twice a day for 5 d to female C57BL/6 mice, five per group. The mice were killed 16 h after the last dose, and changes in body weight from the start of the study, spleen weight, WBCs, RBCs, hemoglobin, platelet or differential count blood, and serum glucose, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine were measured.

Results

PH Domain Binding of PHT-427 Analogues

The binding affinities of PHT-427 and its derivatives (Fig. 1A and B) to the PH domains of Akt (Fig. 1C) and PDPK1 (Fig. 1D) were measured by a surface plasmon resonance (SPR) displacement assay using PtdIns(3,4,5)P₃-rich liposomes with increasing concentrations of the compounds under investigation. Maximum displacement of the PH domain of both Akt and PDPK1 bound to PtdIns(3,4,5)P₃ phosphate-rich liposomes was obtained with the compound bearing a 12-carbon alkyl group C-12 (PHT-427, hereafter), with K_i values of 2.7 ± 0.4 and 5.2 ± 0.4 $\mu\text{mol/L}$, respectively (Table 1). Of note is

Table 1. Binding of compounds to PH domains of Akt and PDPK1

Compounds	Akt (K_i $\mu\text{mol/L}$)	PDPK1 (K_i $\mu\text{mol/L}$)
PtdIns(3,4,5)P ₃	0.52 ± 0.11	1.85 ± 0.20
DPIEL	1.59 ± 0.17	13.35 ± 1.05
Perifosine	>50.0	>50.0
Edelfosine	>50.0	>50.0
Triciribine	>50.0	ND
MK-2206	48.35 ± 1.45	>50.0
C4	>50.0	>50.0
C6	>50.0	>50.0
C8	>50.0	>50.0
C12 (PHT-427)	2.67 ± 0.37	5.20 ± 0.45
C14	5.56 ± 0.54	11.3 ± 3.35
C16	7.57 ± 0.34	39.5 ± 2.75
C18	12.15 ± 0.85	>50.0

NOTE: K_i s for reported Akt PH domain inhibitors, and PHT-427 and its C-4–C-14 carbon chain analogues. K_i s were measured as the concentration of compound that displaces 50% of the PH domain bound to lipid vesicles enriched in PtdIns(3,4,5)P₃ in the absence of drug. Values are the mean of four determinations \pm SE.

Abbreviation: ND, not determined.

that whereas compounds C-14, C-16, and C-18 showed lower, although still appreciable, binding to the PH domain of Akt, only C-12 and C-14 showed binding to the PH domain of PDPK1. We have not investigated the binding of PHT-427 to other PH domains of other proteins, except for Tiam1 to which it does not bind (data not shown). DPIEL was used as a reference compound and bound with high affinity to the PH domain of Akt but with lower affinity to the PH domain of PDPK1, as previously reported (31). We also studied other compounds that have been suggested to bind to the PH domain of Akt and to inhibit its activity, including the alkyl-lysophospholipid compounds perifosine (32), edelfosine (33), and triciribine (34). None of these compounds bound to the PH domain of Akt or PDPK1 at concentrations up to 50 $\mu\text{mol/L}$. MK-2206 is a recently reported allosteric inhibitor of Akt that binds to a site outside the PH domain (35). We found that MK-2206 bound very weakly to the PH domain of Akt and not at all to the PH domain of PDPK1.

RPPA Studies

The effects of PHT-427 on cell signaling were investigated by RPPA using a panel of 86 antibodies to phosphorylated and nonphosphorylated signaling protein related to PtdIns-3-kinase/PDPK1/Akt signaling in PC-3 prostate cells, where PtdIns-3-kinase/PDPK1/Akt signaling is activated because of homozygous PTEN

mutation. Wortmannin, a PtdIns-3-kinase inhibitor, was used as a positive control. A heat map of the results is shown in Fig. 2A, and quantitations of relevant proteins are illustrated in Fig. 2B. After 16 hours, a reduction was observed in phospho-Ser²⁴¹-PDPK1 and phospho-Thr³⁰⁸-Akt by both 10 μ mol/L PHT-427 and 0.1 μ mol/L wortmannin. In contrast, the PDPK1-inde-

pendent phospho-Ser⁴⁷³-Akt was slightly increased by PHT-427 but completely inhibited by wortmannin. Finally, phospho-Ser⁶⁵⁷-PKC and total SGK1, both previously shown to be dependent on the activation of PDPK1 (36, 37), were decreased by treatment with both PHT-427 and wortmannin. These results suggest that, at 10 μ mol/L, PHT-427 inhibits both Akt and PDPK1.

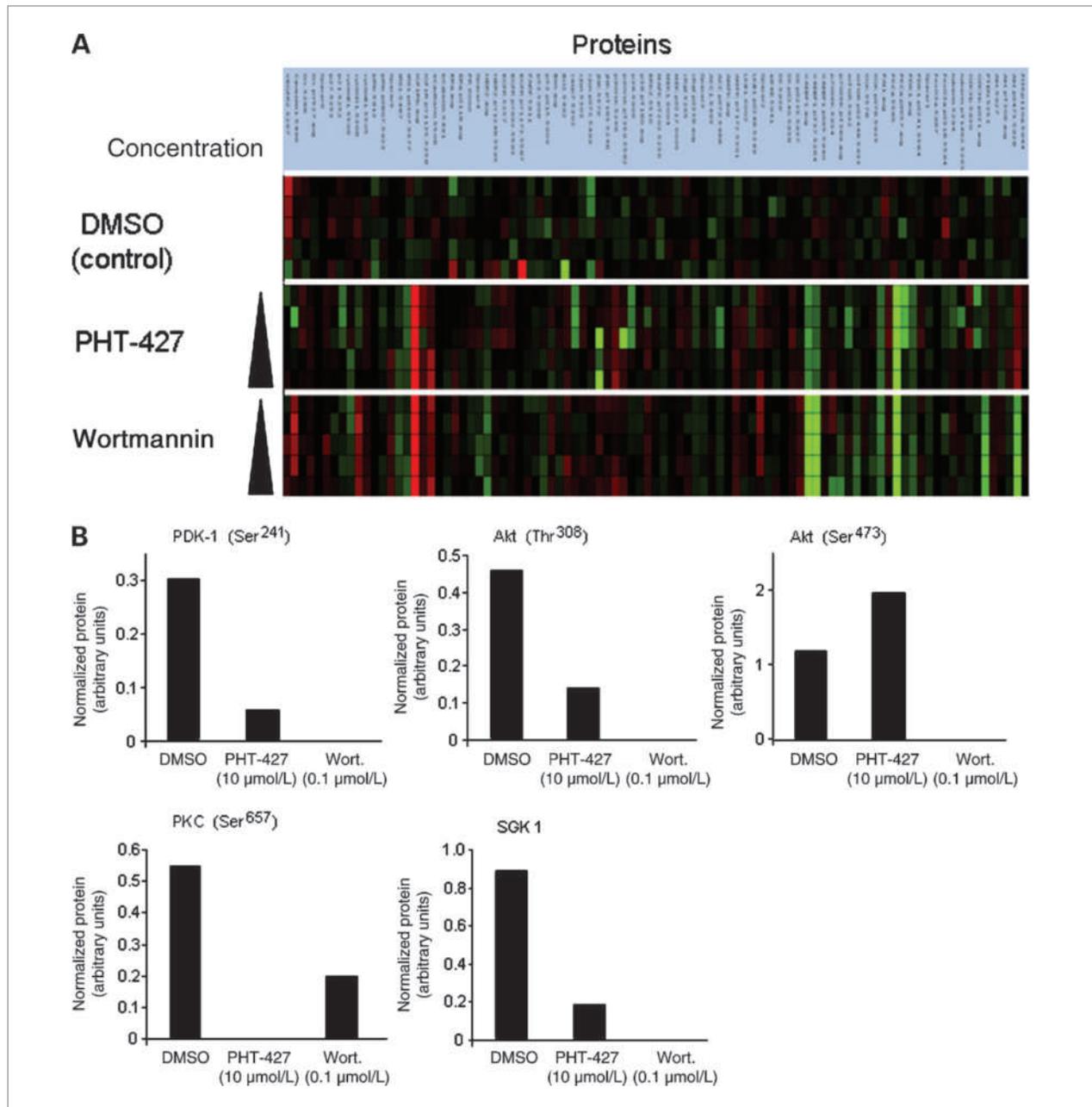
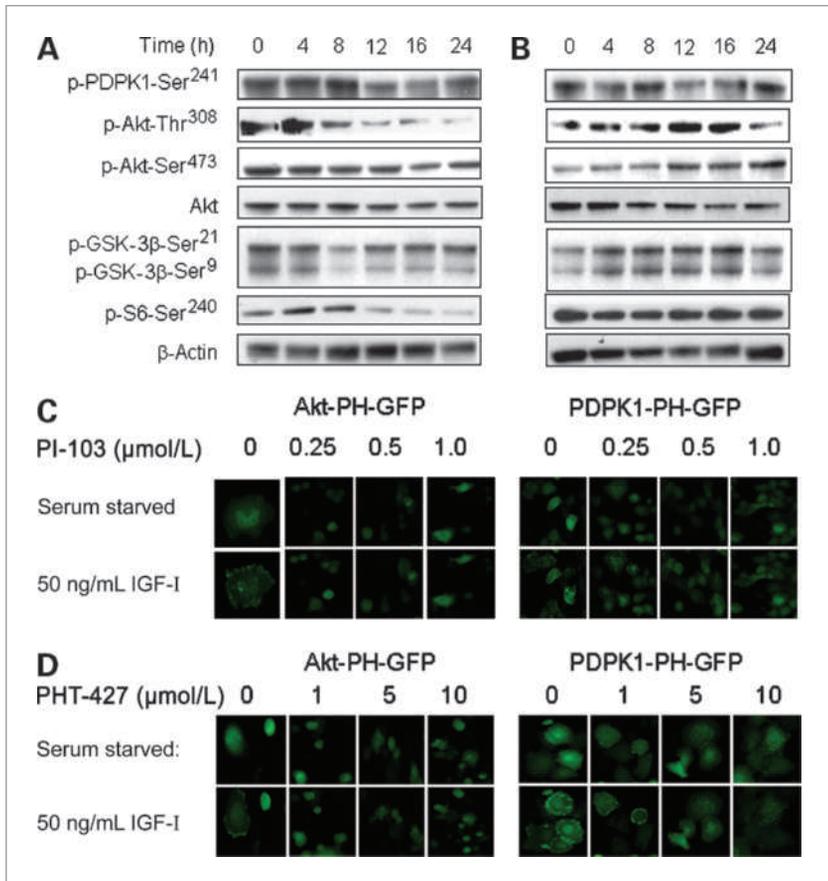


Figure 2. RPPA of PHT-427 effects in PC-3 prostate cancer cells. A, heat map of PC-3 cells was exposed to serum-free medium for 8 h and wortmannin or PHT-427 at 0.1, 0.5, 1, 5, and 10 μ mol/L (shown by increasing arrow) or DMSO vehicle for 16 h, and protein was isolated and subjected to quantitative RPPA with a panel of 74 validated antibodies related to PtdIns-3-kinase/PDPK1/Akt signaling. Values are expressed as the mean of three determinations of expression relative to DMSO control. Red, high; green, inhibition; black, no change. B, histograms of phospho-Ser²⁴¹-PDPK1, phospho-Thr³⁰⁸-Akt, phospho-Ser⁴⁷³-Akt, phospho-Ser⁶⁵⁷-PKC, and total SGK1 levels with DMSO vehicle control, 10 μ mol/L PHT-427, and 0.1 μ mol/L wortmannin (wort).

Figure 3. Effects of PHT-427 in cells. Western blots of Akt activity measured by phospho-Ser⁴⁷³-Akt and PDPK1 activity by phospho-Ser²⁴¹-PDPK1 and activation of selected downstream targets. β -Actin was used as a loading control. A, BxPC-3 pancreatic cancer cells exposed to 10 mmol/L PHT-427 in medium with 10% fetal bovine serum for various times. B, MiaPaCa-2 pancreatic cancer cells exposed to 10 mmol/L PHT-427 in medium with 10% fetal bovine serum for various times. C, Panc-1 pancreatic cancer cells stably transfected with Akt-PH domain-GFP or PDPK1-PH domain-GFP were cultured in serum-free medium for 16 h, exposed to the PtdIns-3-kinase inhibitor PI-103 at different concentrations for 4 h, and then stimulated with 50 ng/mL IGF-I for 20 min or no stimulation. Cellular fluorescence was measured with an IN Cell Analyzer 1000. D, similar studies with Akt-PH domain-GFP or PDPK1-PH domain-GFP Panc-1 cells exposed to PHT-427 at different concentrations.



Western Blotting

The BxPC-3 and MiaPaCa-2 pancreatic cancer cell lines were probed by Western blotting following up to 24-hour exposure to 10 μ mol/L PHT-427, which is below the IC₅₀ for cell growth inhibition of \sim 30 μ mol/L (26), to determine the effects of PHT-427 on the PtdIns-3-kinase/PDPK1/Akt signaling pathway components. We have previously reported that the BxPC-3 cells, which give xenografts that are sensitive to the antitumor activity of PHT-427 (see below), showed a decrease of both phospho-Ser⁴⁷³-Akt and phospho-Thr³⁰⁸-Akt at 12 and 16 hours (Fig. 3A), whereas both were increased at these time points in MiaPaCa-2 cells, which give resistant xenografts (Fig. 3B). Total Akt was decreased at 16 hours in both cell lines. Phospho-Ser²⁴¹-PDPK1 was inhibited in both lines at 12 to 16 hours, whereas phospho-Ser²⁴⁰-RSK was decreased in the sensitive BxPC-3 cells but not in the resistant MiaPaCa-2 line.

Subcellular Translocation

GFP-tagged PH domains were used to follow the subcellular localization of Akt and PDPK1 on IGF-I stimulation in Panc-1 cells. There was diffuse cytoplasmic fluorescence in unstimulated cells with movement of the fluorescence to the plasma membrane on stimulation

with IGF-I (Fig. 3C and D). The plasma membrane translocation of both the Akt and PDPK1 PH domains was inhibited by PHT-427 at between 5 and 10 μ mol/L. This inhibition was comparable with that obtained when cells are treated with the PtdIns-3-kinase inhibitor PI-103.

Antitumor Activity

Effect of the Carbon Chain Length. Mice with BxPC-3 pancreatic, MCF-7 breast, or A-549 NSCLC xenografts were administered PHT-427 or its analogues with a C-4, C-6, or C-8 alkyl chain by oral gavage twice a day for 10 days. The results show that PHT-427 had the greatest antitumor activity, with the C-8 chain analogue having less activity and analogues with a C-4 or C-6 chain very little activity (Fig. 4A). All further antitumor studies were conducted using the compound PHT-427.

Activity of PHT-427 in Different Tumors. The antitumor activity of PHT-427 at doses of 125 to 250 mg/kg in different tumors is shown in Table 2 and graphically in Fig. 4B. PHT-427 gave up to an 80% inhibition of tumor growth in the most sensitive tumors. Tumors with PIK3CA mutation were among the most sensitive. Tumors with a K-Ras mutation were less sensitive irrespective of the dose of PHT-427. The pattern of inhibition in different tumors is similar to that we have seen using the

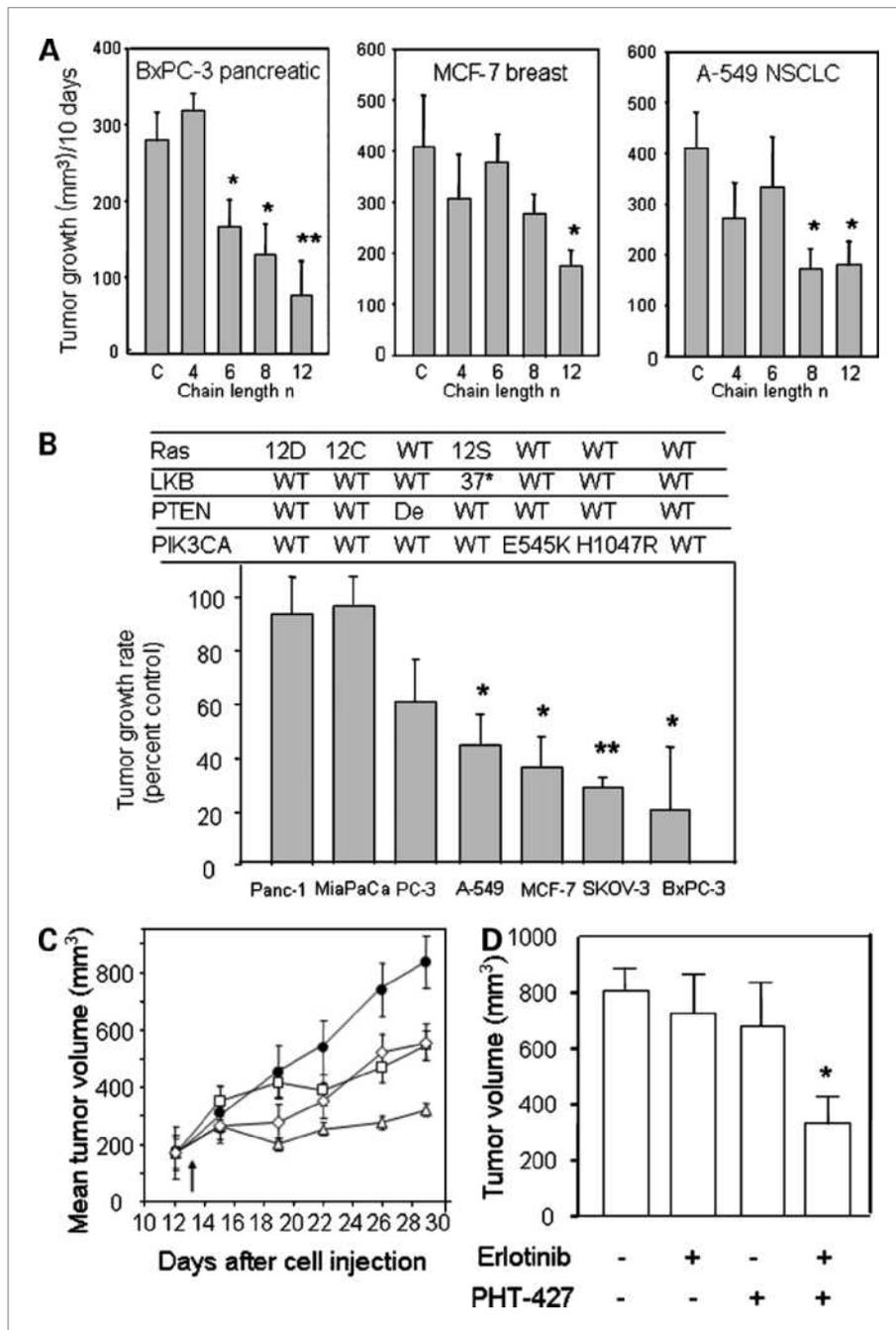


Figure 4. Antitumor activity of PHT-427 analogues. **A**, effect of carbon chain length. Mice with BxPC-3 pancreatic, MCF-7 breast, and A-549 NSCLC s.c. xenografts were treated with vehicle alone (C) or PHT-427 and its analogues with different C chain lengths at 200 mg/kg twice a day for 10 d and tumor growth rate was measured. Columns, mean ($n = 8$ mice per group); bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with control. **B**, tumor growth inhibition by PHT-427 in different tumors. Doses and schedules: PC-3 prostate cancer, 125 mg/kg twice a day \times 5 d; A-549 NSCLC, 200 mg/kg twice a day \times 10 d; MCF-7 breast cancer, 200 mg/kg twice a day \times 10 d; SKOV-3 ovarian cancer, 250 mg/kg twice a day \times 10 d; BxPC-3 pancreatic cancer, 250 mg/kg twice a day \times 5 d. Results are expressed as the growth rate of the PHT-427-treated tumors relative to the control tumors. Columns, mean ($n = 8$ mice per group); bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with control. **C**, combination of PHT-427 with paclitaxel in MCF-7 human breast cancer xenografts. Female *scid* mice with a s.c. implanted 60-d estradiol release pellet were injected s.c. with 10^7 MCF-7 human breast cancer cell. When the tumors reached ~ 180 mm³, dosing was started on day 13 (shown by \uparrow). ●, vehicle control orally twice a day for 10 d; ◇, 200 mg/kg PHT-427 orally twice a day for 10 d; □, 10 mg/kg paclitaxel i.p. every other day for five doses; Δ, 200 mg/kg PHT-427 orally twice a day for 10 d and 10 mg/kg paclitaxel i.p. every other day for 5 doses. **D**, mice were implanted orthotopically with NCI-H441 NSCLC cells into their lungs, and 20 d later, treatment was begun with daily oral 50 mg/kg erlotinib until the end of the experiment or daily orally of 200 mg/kg PHT-427 for 10 d. The mice were killed and autopsied at day 63 when control animals became moribund, and primary left lung tumor volume was measured. Columns, mean of 10 animals per group; bars, SE. *, $P < 0.05$.

PtdIns-3-kinase inhibitor PX-866 (38), suggesting that the antitumor activity of PHT-427 is due to inhibition of the PtdIns-3K/PDPK1/Akt signaling pathway.

Antitumor Activity in Combination with Chemotherapy. PHT-427 administered orally to mice with s.c. MCF-7 human breast cancer xenografts showed antitumor that was additive with that of paclitaxel (Fig. 4C). PHT-427 administered orally for 10 days to mice with orthotopic NCI-H441 NSCLC xenografts increased the antitumor activity of continuous daily erlotinib, which by itself had no antitumor activity in this model (Fig. 4D). NCI-H441 has a mutant K-Ras, which is a negative predictor of erlotinib activity in NSCLC.

Pharmacokinetics. Plasma levels of PHT-427 following oral administration to mice of a dose of 200 mg/kg showed rapid absorption, without a lag phase, C_{max} was 8.2 $\mu\text{g/mL}$ 1 hour following dosing, and the elimination half-life was 1.4 hours with a terminal PHT-427 concentration of 0.1 $\mu\text{g/mL}$ 10 hours after dosing (Fig. 5A). The plasma concentration of PHT-427 was above the level that gave inhibition of Akt and PDPK1 signaling in cells of 10 $\mu\text{mol/L}$ (4 $\mu\text{g/mL}$) for at least 3 hours.

Pharmacodynamics. Xenografts derived from both the MiaPaCa-2 and BxPC-3 lines were excised and subjected to Western blotting for markers of PtdIns-3-kinase/

PDPK1/Akt signaling after a single 200 mg/kg dose at times up to 12 hours (Fig. 5B and C). Quantitation of the blots for MiaPaCa-2 and BxPC-3 xenografts showed maximal decreases at 8 hours compared with nontreated control, for phospho-Ser⁴⁷³-Akt of 68.7% and 10%, phospho-Thr³⁰⁸-Akt of 74.1% and 100%, and phospho-Ser²⁴¹-PDPK1 of 69.6% and 14.8%, respectively. Total Akt itself showed a small decrease of ~25% at 8 hours. Phospho-Ser²²¹-RSK, which is phosphorylated independently of Akt activation (24), was decreased by 85.5% in MiaPaCa-2 and 37.2% in BxPC-3 xenografts. Phospho-Ser²⁴⁰-RSK levels showed a decrease at 0.5 hour and again at 4 to 6 hours in BxPC-3 xenografts but not in MiaPaCa-2 xenografts. Thus, the xenograft studies confirm the previous cell line studies with inhibition of phospho-Ser²⁴¹-PDPK1, phospho-Ser⁴⁷³-Akt, and phospho-Thr³⁰⁸-Akt in both BxPC-3 and MiaPaCa-2, whereas inhibition of phospho-Ser²⁴⁰-RSK was only seen in the sensitive BxPC-3 but not in the resistant MiaPaCa-2.

Toxicity. Groups of four female C57BL/6 mice were administered 0.1 mL vehicle alone or PHT-427 in 0.1 mL vehicle at 200 mg/kg twice a day for 5 days, and blood was collected 16 hours after the last dose. PHT-427 administration gave no significant change in blood chemistry (WBCs, RBCs, hemoglobin, platelet, or differential

Table 2. Antitumor activity of PHT-427

Tumor	Volume at start (mm ³)	Dose (mg/kg)	Schedule	Tumor growth rate (mm ³)/10 d	T/C %	P
BxPC-3 pancreatic	156	Control	BID \times 5 d	228 \pm 46		
		125	BID \times 5 d	67 \pm 35	29.4	0.030
		250	BID \times 5 d	46 \pm 53	20.1	0.027
	97	Control	BID \times 10 d	279 \pm 37		
		100	BID \times 10 d	181 \pm 52	64.8	NS
		200	BID \times 10 d	77 \pm 44	27.6	0.004
Panc-1 pancreatic	200	Control	BID \times 10 d	318 \pm 53		
		200	BID \times 10 d	308 \pm 59	97.1	NS
MiaPaCa-2 pancreatic	200	Control	BID \times 10 d	318 \pm 53		
		200	BID \times 10 d	308 \pm 59	97.1	NS
PC-3 prostate	229	Control	BID \times 5 d	780 \pm 161		
		125	BID \times 5 d	470 \pm 121	60.3	NS
SKOV-3 ovarian	192	Control	BID \times 10 d	432 \pm 59		
		250	BID \times 10 d	122 \pm 16	28.3	0.001
A-549 NSCLC	157	Control	BID \times 10 d	413 \pm 37		
		200	BID \times 10 d	182 \pm 47	44.1	0.016
MCF-7 breast	142	Control	BID \times 10 d	410 \pm 101		
		100	BID \times 10 d	383 \pm 139	93.4	NS
		200	BID \times 10 d	156 \pm 30	38.0	0.042

NOTE: There were eight female mice per group. Control mice received vehicle only (0.1 mL sesame seed oil). The growth rate of individual tumors was measured over the period of dosing, and the mean for each treatment group was compared with control using Student's *t* test to obtain *P* values. Values are the mean \pm SE.

Abbreviations: T, mean growth rate of test (drug treated); C, mean growth rate of control tumor expressed as a percent; NS, not significantly different (*P* > 0.05). BID, twice a day.

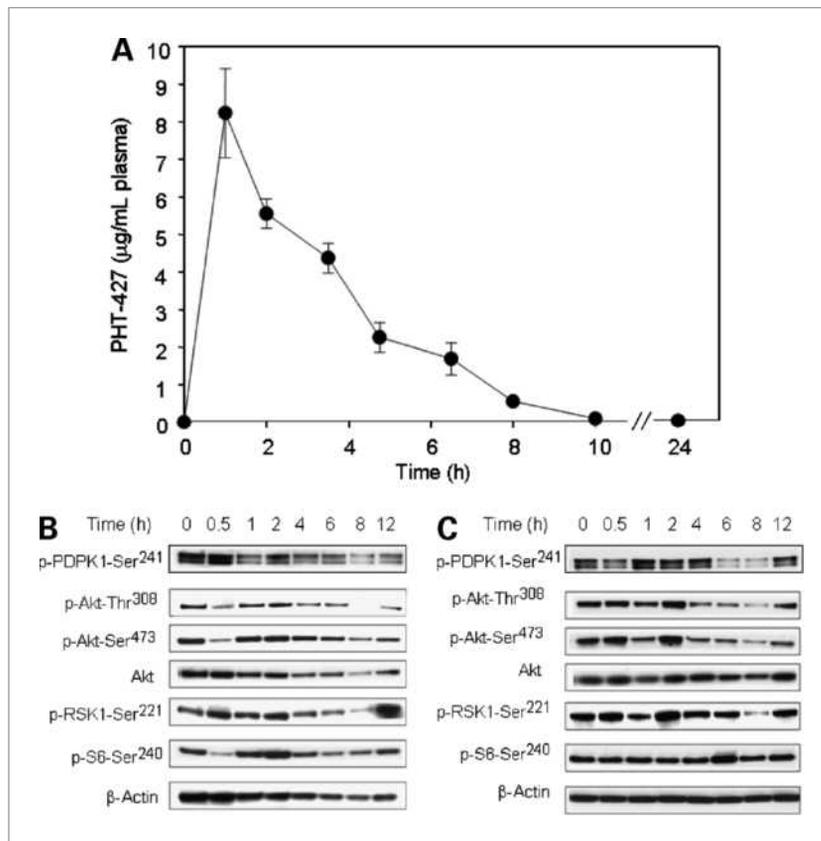


Figure 5. *In vivo* effects of PHT-427. A, C57BL/6 female mice were administered PHT-427 at 200 mg/kg orally in 0.1 mL sesame oil and plasma concentrations of parent compound were measured by high-performance liquid chromatography–mass spectrometry. Points, mean of three mice per group; bars, SE. Western blots of BxPC-3 pancreatic cell xenografts (B) and MiaPaCa-2 pancreatic cell xenografts (C) in *scid* mice administered a single oral dose of 200 mg/kg PHT-427 in 0.1 mL vehicle. Mice were killed after various times, and the tumors were removed and immediately frozen in liquid N₂.

count), serum glucose, creatinine, or blood urea nitrogen, but there was a significant decrease in serum aspartate aminotransferase from 167.2 ± 29.4 to 86.4 ± 13.3 units/L ($P < 0.05$) and in serum alanine aminotransferase from 28.2 ± 2.3 to 20.8 ± 0.9 units/L ($P < 0.05$), possibly suggestive of mild liver dysfunction. There was no significant change in body weight or the weight of the spleens.

Discussion

Because of their roles in cellular apoptosis and survival pathways, the AGC family serine/threonine kinases Akt and PDPK1 have emerged as attractive therapeutic targets for cancer (3, 15). Attempts to develop Akt and PDPK1 inhibitors targeting the ATP binding pocket have generally produced compounds that also inhibit other ACG family kinases, such as PKA, which may account for the toxicity of this type of inhibitor seen in animals and in patients (15). Recently, an allosteric Akt inhibitor MK-2206 that binds to a region outside the ATP catalytic site has been described and is in early clinical trial (35). We have developed an alternative approach to inhibiting Akt and other signaling proteins through compounds that bind to the PH domain (25, 26, 31, 39). The PH domain is essential for the binding of several cytosolic sig-

naling proteins to plasma membrane PtdIns(3,4,5)P₃ formed by the activity of PtdIns-3-kinase, thus causing allosteric activation or bringing the proteins into proximity with their effectors and substrates, leading to the activation of signaling cascades (1).

Although PHT-427 was originally developed as an inhibitor of the PH domain of Akt (26), new results suggest that it is also an inhibitor of PDPK1. We first showed by SPR studies that PHT-427 binds to the expressed PH domain of PDPK1 with an affinity similar to that of binding to the PH domain of Akt (K_i for PDPK1, 5.2 $\mu\text{mol/L}$; K_i for Akt, 2.7 $\mu\text{mol/L}$). We also investigated the effect of varying the length of the alkyl chain attached to the benzene ring, which our modeling studies (data not shown) suggested fits into a shallow channel in the PH domain. For both Akt and PDPK1, the optimum chain length giving maximum binding was C-12 to C-14. Whereas the C-14, C-16, and C-18 analogues also showed some binding to the Akt PH domain, only the C-12 and C-14 analogues showed appreciable binding to the PH domain of PDPK1. Other compounds that have been suggested to bind to the PH domain of Akt include perifosine (32) and triciribine (34), and both compounds are currently in clinical trial as Akt inhibitors. However, in our hands, neither of them bound to the PH domains of Akt or PDPK1.

MK-2206 is an allosteric inhibitor of Akt that was reported to bind to a region outside the ATP site of Akt (35). Our SPR studies confirmed that it does not bind significantly to the PH domain of Akt or PDPK1. Thus, PHT-427 distinguishes itself from other inhibitors by showing high affinity binding for the PH domains of Akt and PDPK1.

RPPA studies in PC-3 prostate cancer cells showed that PHT-427 causes a reduction in phospho-Thr³⁰⁸-Akt and also in phospho-Ser²⁴¹-PDPK1 and its downstream targets, phospho-Ser⁶⁵⁷-PKC and total SGK1 (36, 37). Western blotting using BxPC-3 pancreatic cancer cells showed that PHT-427 causes a decrease at both phospho-Thr³⁰⁸-Akt and phospho-Ser⁴⁷³-Akt and also in phospho-Ser²⁴¹-PDPK1 and phospho-Ser⁹-GSK-3 β . In contrast, MiaPaCa-2 pancreatic cancer cells showed an increase in phospho-Thr³⁰⁸-Akt and phospho-Ser⁴⁷³-Akt with no decrease in phospho-Ser⁹-GSK-3 β on treatment with PHT-427. *In vivo* studies in mice with BxPC-3 and MiaPaCa-2 xenografts treated with a 200 mg/kg dose of PHT-427 showed a decrease in phospho-Ser⁴⁷³-Akt, phospho-Thr³⁰⁸-Akt, phospho-Ser²⁴¹-PDPK1, and in the PDPK1-specific downstream target phospho-Ser²²¹-RSK (24). Of note was that phospho-Ser²⁴⁰-RSK was decreased by PHT-427 in BxPC-3 cells and xenografts but not in MiaPaCa-2 cells or xenografts that were resistant to the antitumor effects of PHT-427. Phospho-Ser²⁴⁰-RSK has been previously reported to be a biomarker for Akt pathway inhibition in sensitive tumors (40). Thus, our studies show that PHT-427 inhibits both Akt and PDPK1 signaling in sensitive cancer cells but that resistant cancer cells have a rebound increase in Akt activity. Consequently, PDPK1 inhibition may be more important for the antitumor activity of PHT-427 than inhibition of Akt.

Two mechanisms have been suggested for the inhibition of PH domain proteins by small-molecule inhibitors. A study of the binding of 2-hydroxymethyl-carbonylether-lipid, a compound related to DPIEL identified by our group as a PH domain inhibitor of Akt (31), suggested that the binding to the PtdIns pocket of the PH domain causes an open interdomain conformation, where the NH₂-terminal PH domain and COOH-terminal regulatory domains move away from the kinase domain, preventing translocation of Akt to the plasma membrane, thus blocking its activation (41). Another study using an allosteric inhibitor of Akt, which did not bind directly the PtdIns pocket of the PH domain of Akt but instead interacted with Trp⁸⁰ outside of the pocket, found that the PH domain was folded back on the kinase domain, thus blocking its activity (42). We have found that PHT-427 displaces PtdIns(3,4,5)P₃ from the Akt PH domain in a manner similar to DPIEL, whereas the allosteric inhibitor MK-2206 did not displace PtdIns(3,4,5)P₃, suggesting that the binding of PHT-427 causes an open conformation preventing the translocation of Akt to the plasma membrane. This is consistent with our observation of the inhibition of the cellular translo-

cation of both an Akt and a PDPK1 PH domain GFP construct by PHT-427. The inhibition of translocation occurs even if there is no antitumor response to PHT-427 because of the presence of a K-Ras mutation.

Activating mutations in the p110 α catalytic subunit (PIK3CA) are present in several human tumors (5), with the most common sites clustering around the amino acid 1047 site in the kinase domain and amino acid 545 in the helical domain (43). Recent data suggest that cells with PIK3CA mutation may be divided into two classes. The first are cells that show dependency on Akt signaling, an effect that is augmented by additional activation of the pathway, for example, and loss of the tumor suppressor PTEN. The second class of cells has decreased Akt signaling and an increased dependence on PDPK1 signaling, and the effects it has on targets were independent of Akt signaling, most notably the AGC kinase SGK (22). It has recently been shown that in PIK3CA mutant breast cancer cells, PDPK1 can signal independently of Akt by phosphorylating and activating SGK due to altered PtdIns(3,4,5)P₃ levels caused by mutant PIK3CA (22). Mutant PIK3CA cells are therefore dependent on PDPK1 and SGK for viability. We observed good antitumor activity of PHT-427 in one tumor xenograft with a PIK3CA mutation, SKOV-3 ovarian cancer, which was better than with a PC-3 prostate cancer xenograft, which has loss of PTEN, although this was with a lower dose of PHT-427. Thus, although not conclusive, the weight of evidence suggests that it is the PDPK1 inhibitory activity of PHT-427, a consequence of its binding to the PH domain of PDPK1, which could be primarily responsible for the antitumor activity of PHT-427. Inhibition of Akt through binding to the PH domain of Akt cannot, however, be discounted as a contributing to the antitumor activity of PHT-427 and may vary depending on the cell type.

We found that the presence of mutant K-Ras predicts for resistance. This is similar to the pattern of sensitivity to the antitumor activity of a PtdIns-3-kinase inhibitor, with K-Ras bypassing Akt signaling through activation of the mitogen-activated protein/extracellular signal-regulated kinase pathway (38). However, the bypass is not complete because A-549 NSCLC, which has mutant K-Ras, shows some sensitivity to the antitumor activity of PHT-427, as it does to PtdIns-3-kinase inhibition (38). Orthotopic NCI-H441 NSCLC, which also has mutant K-Ras, was resistant to the antitumor activity of PHT-427 alone but showed synergy when PHT-427 was combined with the epidermal growth factor receptor inhibitor erlotinib.

In summary, a series of PHT-427 analogues with C-4 to C-16 alkyl chain length were synthesized and tested. PHT-427 (C-12 chain) and the C-14 analogue bound with the highest affinity to the PH domains of PDPK1 and Akt. PHT-427 gives a transient inhibition of Akt signaling in cells and tumor xenografts but a longer-lasting inhibition of PDPK1 signaling that correlated with decreased activation of downstream signaling tar-

gets for both proteins. PHT-427 given orally on a twice daily schedule inhibited the growth rate of human tumor xenografts in immunodeficient mice as long as it was given, with up to an 80% inhibition in the most sensitive tumors. The pharmacokinetics of PHT-427 showed a relatively short plasma half-life of 1.4 hours and levels that corresponded to the time course of the inhibition of signaling pathways in tumor xenografts. There was no weight loss or change in blood chemistry associated with the administration of PHT-427. Tumors with a K-Ras mutation were less sensitive to PHT-427 as has been observed for other inhibitors of the PtdIns-3-kinase signaling pathway. Combination antitumor studies showed that PHT-427 has greater than additive activity with paclitaxel in breast cancer and with erlotinib in NSCLC.

Disclosure of Potential Conflicts of Interest

G. Powis, E.J. Meuillet, and S. Zhang: cofounders and shareholders, PHusis Therapeutics, Inc.; D.L. Kirkpatrick: employee and shareholder, PHusis Therapeutics, Inc. No other potential conflicts of interest were disclosed.

Grant Support

National Cancer Institute grants RO1 CA 061015 and P30 CA 23074 (G. Powis), MGE@MSA Fellowship and a minority supplement for CA 061015 (S.A. Moses), and Lung Cancer Research Foundation (J.J. Jacoby).

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Received 10/23/2009; revised 12/29/2009; accepted 01/14/2010; published OnlineFirst 03/02/2010.

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Molecular Pharmacology and Antitumor Activity of PHT-427, a Novel Akt/Phosphatidylinositide-Dependent Protein Kinase 1 Pleckstrin Homology Domain Inhibitor

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Mol Cancer Ther 2010;9:706-717. Published OnlineFirst March 2, 2010.

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