Pharmacodynamic Markers of Perifosine Efficacy

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Abstract

Purpose: It is critical to develop methods to quantify the early pharmacodynamic effects of targeted therapeutics in vivo to make drug development more efficient and ensure biologically relevant dosing. Furthermore, an ability to identify patients likely to respond to targeted therapeutics would decrease the size, duration, and cost of clinical trials, resulting in more efficient translation to improved patient outcomes. Recent studies suggest that perifosine inhibits the phosphatidylinositol-3'-kinase (PI3K) pathway by preventing cell membrane recruitment of the AKT pleckstrin homology domain.

Experimental Design: A novel functional proteomics technology, reverse phase protein array, was used to establish and quantify pharmacodynamic markers of perifosine efficacy.

Results: Perifosine selectively prevents AKT recruitment to the membrane and blocks activation of downstream effectors. Perifosine inhibited breast, ovarian, and prostate cancer models. Growth inhibition was associated with apoptosis. Activation of AKT as a consequence of genomic aberrations predicted perifosine efficacy. In cell lines and xenografts, there was a highly statistically significant correlation between the degree of antitumor efficacy of different perifosine doses and quantified down-regulation of phosphorylation of AKT and of its downstream targets, particularly S6.

Conclusions: Because of a strong correlation between proportional modulation of PI3K pathway biomarkers and quantified perifosine efficacy, it is likely that early measurement of such pharmacodynamic biomarkers with reverse phase protein array will optimize selection of responding patients and guide perifosine dosing. Furthermore, PI3K pathway activation status may allow baseline selection of patients most likely to respond to perifosine alone or in combination with other therapies.
to perifosine than lines without such aberrations. Enforced PI3K/AKT pathway activation also renders cells more sensitive to perifosine. Thus, perifosine may be particularly useful in treating tumors with PI3K/AKT-activating genomic anomalies.

Materials and Methods

Antibodies and reagents. Perifosine was provided by Keryx Pharmaceuticals. LY294002 was purchased from Calbiochem. Polyclonal antibodies recognizing total and phosphorylated PI3K/AKT pathway proteins were purchased from Cell Signaling, Inc.: phosphorylated AKT (Ser473 or Thr308), AKT, phosphorylated GSK3α/GSK3β (Ser21/9), total and phosphorylated p70S6K (Thr389), phosphorylated S6 (Ser240/244), phosphorylated MEK (Thr202/T212), phosphorylated c-Jun-NH2-kinase (JNK; Thr183/Tyr185), phosphorylated epidermal growth factor receptor (Tyrc397), Jun, p38, and cleaved caspase-7. Rabbit anti-INK1 and anti-ERK2 antibodies and mouse anti-GSK3 antibody were purchased from Santa Cruz Biotechnology. Rabbit anti–cyclin B1 was obtained from Epitomics. Horseradish peroxidase–conjugated goat anti-rabbit and anti-mouse IgGs were purchased from Bio-Rad. Rabbit antiactive mitogen-activated protein kinase (MAPK) antibody and a nonradioactive cell proliferation assay (Celliter 96 for MTS assay) were purchased from Promega. APO-BRDU reagents for cell cycle and apoptosis assay were purchased from Phoenix Flow Systems, Inc.

Expression vectors. Human AKT was cloned from OVCAR3 cells by reverse transfection—PCR (Titan One Tube RT-PCR System from Roche Applied Science). The myristylation site (ATG-AGC-AGC-AGC-AAG-AGC-AGC-AGC-AGC) and HA-tagged sequence (ATG-TAC-CCA-TAC-GAT-GTT-CCA-TAC-GAT-GCT) were attached to the NH2 terminus of AKT by PCR. Constitutively activated AKT (T308D/S473D) and dominant-negative AKT (K179A/T308A/S473A) were generated by site-directed mutagenesis (Stretagene) on the backbone of human myristylated wild-type AKT. The constructs were entirely sequenced and were without error. Adenovirus-containing kinase inactive (dominant-negative) AKT (K179M) was provided by Dr. Ho-Young Lee (M. D. Anderson Cancer Center). Adenovirus-containing β-gal Lac-Z was prepared in the Core Facility at M. D. Anderson Cancer Center. DNA plasmids containing green fluorescent protein (GFP)-AKT-PH and GFP-PLCγ-PH domain were gifts from Dr. Tobias Meyer (Stanford University).

Cell lines and transfection. The breast and ovarian cancer cell lines MDA-MB-468 (PTEN-mutant), MDA-MB-231 (RAS/RAF-mutant), SKOV3 (HER2 amplification), OVCAR3 (p85-mutant), and DOV13 (no known PI3K pathway aberrations) were cultured by Keryx Pharmaceuticals. Isogenic pairs of DOV13 cell lines were serum starved for 2 h and incubated with perifosine (2.5, 5, and 10 μM/L) or LY294002 (20 μM/L) for 6 h in serum-free medium. Cells were left unstimulated or stimulated with EGF (20 ng/mL) for 30 min. Cells were washed and fixed with 4% paraformaldehyde at room temperature for 30 min, and the cell nuclei were counterstained by 4′,6-diamidino-2-phenylindole. GFP fusion protein localization was observed using a fluorescence microscope.

Cell localization assay. GFP fusion proteins containing the AKT-PH or PLCγ-PH domain were transfected into the cell line OVCAR3 by Nucleofector (Amaxa Biosystems). Eighteen hours later, cells were serum starved for 2 h and incubated with perifosine (2.5, 5, and 10 μM/L) or LY294002 (20 μM/L) for 6 h in serum-free medium. Cells were left unstimulated or stimulated with EGF (20 ng/mL) for 30 min. Cells were washed and fixed with 4% paraformaldehyde at room temperature for 30 min, and the cell nuclei were counterstained by 4′,6-diamidino-2-phenylindole. GFP fusion protein localization was observed using a fluorescence microscope.

Cell proliferation assay. Cell lines were seeded in 96-well plates (5,000/well) and Z-scores were read at 570 nmol/L (formazan product converted by metabolically active cells was measured at 490 nmol/L absorbance. For the crystal staining assay, absorbance was read at 570 nmol/L. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% bovine serum albumin and incubated at 4°C overnight with anti–phosphorylated p70S6K, anti–phosphorylated GSK3α/β, anti–phosphorylated AKT, anti-AKT, anti–phosphorylated S6 (all 1:1,000 dilution), anti–active MAPK (1:5,000), anti-GSK3, or anti-ERK2 (both 0.2 μg/mL) antibodies. The membranes were washed in TBS-T [10 mL/M/L Triton-HCl (pH 7.4), 1.5% (vol/vol) NaCl, 0.1% Tween 20] and incubated with horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse IgG (1:2,500) at room temperature for 1 h. The membranes were washed, and the proteins were visualized by enhanced chemiluminescence detection (Amersham Pharma Biotech).

RPPA. Lysis buffer was used to lyse cell lines or frozen mouse xenografts by homogenization. Cell/tumor lysates were normalized to 1 μg/mL concentration using bicinecinic acid assay and boiled with 1% SDS, and the supernatants were manually diluted in six or eight 2-fold serial dilutions with lysis buffer. A GeneTAC arrayer (Genomic Solutions, Inc.) created 1,152 spot arrays on nitrocellulose-coated FAST slides (Schleicher & Schuell BioScience, Inc.) from the serial dilutions. Each slide was probed with a validated primary antibody (listed above), and the signal was amplified using a DakoCytomation–catalyzed system. A secondary antibody (antimouse or antirabbit) was used as a starting point for amplification. The slides were scanned, analyzed, and quantitated using Microvigene software (VigeneTech Inc.) to generate serial dilution—signal intensity curves for each sample with the logistic fit model: ln(y) = a + (b − a) / (1 + exp {[c×(d − ln(x))]}). A representative natural logarithmic value of each sample curve on the slide (curve average) was then used as a relative quantification of the amount of each protein in each sample. Protein loading was corrected across samples by correction of the linear expression values using the average expression levels of at least four proteins (e.g., ERK2, GSK3, INK, mTor) to calculate a loading correction factor for each sample. These proteins were found to be stably expressed and not altered on Western blotting and RPPA during perifosine treatment. In the PC3 mouse model, as an example, we observed a 22-fold (2,200%) decrease in AKT phosphorylation at Ser473 with perifosine, and the range of loading correction factors was 0.4 to 2.4. As an alternative, all measured proteins were used to calculate loading correction factors, and results did not differ. To generate heat maps, Treeview (University of Glasgow) and X-cluster softwares were used.

AKT localization assay. GFP fusion proteins containing the AKT-PH or PLCγ-PH domain were transfected into the cell line OVCAR3 by Nucleofector (Amaxa Biosystems). Eighteen hours later, cells were serum starved for 2 h and incubated with perifosine (2.5, 5, and 10 μM/L) or LY294002 (20 μM/L) for 6 h in serum-free medium. Cells were left unstimulated or stimulated with EGF (20 ng/mL) for 30 min. Cells were washed and fixed with 4% paraformaldehyde at room temperature for 30 min, and the cell nuclei were counterstained by 4′,6-diamidino-2-phenylindole. GFP fusion protein localization was observed using a fluorescence microscope.
Colony formation assay. OVCAR3 cells were incubated with different perifosine concentrations (5 and 10 μmol/L) for 24 h in 0.5% FBS, harvested, and washed. The same number of cells was seeded in culture dishes for an additional 15-day incubation in 10% FBS in the absence of perifosine. The number of colonies was subsequently counted and used as a reference variable for cell growth.

Cell cycle and apoptosis analysis. Cells were cultured in 60-mm dishes, serum starved overnight, and incubated with perifosine in the presence of 0.5% FBS for the time period indicated. Both floating and attached cells were collected and fixed using 4% paraformaldehyde. Cells were labeled by APO-BRDU reagents (Phoenix Flow Systems, Inc.), a two-color TUNEL assay for labeling DNA breaks and total DNA. Cell cycle change and apoptosis were measured by flow cytometry and analyzed by CellQuest (Becton Dickinson).

Tumor growth in vivo. BALB-c nude mice (female, 3 weeks old) were purchased from Harlan and adapted in the M.D. Anderson Cancer Center animal facility for a week. SKOV3 cells were injected orthotopically into the peritoneal cavity at 8 to 10 × 10^6 cells/0.2 mL medium. Each mouse received one injection. Each treatment group included five mice. Seven days later, perifosine (in vehicle PBS) was administered orally as 200 mg/kg daily or 70 mg/kg thrice weekly. Vehicle only was given to control mice. Mouse abdominal circumference was measured every 2 days. Mice were euthanized on the 24th day; tumors were excised and measured by weight, herein represented using the mean weight from each group. In a separate study, for pharmacodynamic measurements, mice were inoculated with PC3 or DU145 prostatic cells. Docetaxel was given i.v. at 12.5 mg/kg every other day for three treatments, or perifosine was administrated orally by gavage beginning when the tumor grew to 5 mm^3 in volume (day 1). In the control group, vehicle (sterile water) was given daily. The tumor was monitored until day 24 (PC3) or day 106 (DU145). Tumor weight was calculated by weight (mg) = [width^2 (mm^2) × length (mm)] / 2. Mice were then euthanized, and the tumors were excised and homogenized in lysis buffer for RPPA. In a third study, mice were inoculated with PC3, A431, or BT474 cells and treated with vehicle or perifosine. The tumor was monitored until day 28 (PC3 and A431) or day 20 (BT474), and the animals were sacrificed. Additional mice were sacrificed at days 0, 1, 2, 7, 14 (BT474 only), and 21 (PC3/A431 only), and tumor was extracted for RPPA.

Mouse magnetic resonance imaging. Initial magnetic resonance imaging of six mice bearing i.p. tumors was done 7 days after injection of SKOV3 cells. On the same day, three animals were treated with perifosine (200 mg/kg orally weekly) and three with vehicle. Imaging was repeated on day 14. For magnetic resonance, animals were anesthetized with 2% isoflurane. Magnetic resonance was done using a 4.7 T Biospec small animal imager (Bruker Biospin USA). Images from a coronal T2-weighted fast-spin echo sequence (echo time, 70 ms; repetition time, 4,000 ms; nax 4; slice thickness, 1.3 mm; field of view, 3.7 × 5 cm; matrix, 256 × 256; spatial resolution, 195 μm) were used for tumor measurements with Image J program (Version 1.33 u, NIH, 2004; ref. 17). To assess therapeutic effect, tumor weight from the early time point was subtracted from that at the later time point.

Measurement of vascular endothelial growth factor production. Supernatants of cultured cells were quantified for vascular endothelial growth factor by ELISA using the human vascular endothelial growth factor–specific QuantiGlo vascular endothelial growth factor kit (R&D Systems). Vascular endothelial growth factor concentration was calculated by comparing sample absorbance to standard curves.

Results

Perifosine selectively inhibits AKT membrane translocation. Perifosine has been proposed to inhibit PI3K/AKT signaling by blocking membrane AKT translocation (2); however, selectivity for the AKT-PH domain has not been established. We, therefore, assessed the ability of perifosine to alter localization of the PH domain of AKT1, which preferentially binds
phosphatidylinositol (PI)(3,4,5)P3, and of PLCγ, which preferentially binds P(4,5)P2 (18, 19). In OVCAR3 cells expressing GFP-PH domain fusion constructs, perifosine induced a concentration-dependent inhibition of EGF-induced AKT-PH domain membrane translocation without altering PLCγ-PH domain localization (Fig. 1). Thus, perifosine has selectivity for the AKT-PH domain, potentially by preventing interaction with PI(3,4,5)P3.

Perifosine inhibits PI3K/AKT signaling. Because perifosine inhibits AKT translocation, we determined its effect on intracellular PI3K/AKT pathway activation in two ovarian cancer cell lines incubated for 4 h with 5 and 10 μmol/L perifosine using immunoblotting. Perifosine decreased basal and EGF-induced phosphorylation of multiple PI3K/AKT pathway components in SKOV3 (Fig. 2A) and OVCAR3 (not presented). Equimolar amounts of the PI3K inhibitor LY294002 were not as efficient at inhibiting signaling, particularly with EGF, possibly because LY294002 is a competitive inhibitor.

We also explored the ability of perifosine to alter signaling through several pathways using RPPA to simultaneously analyze multiple components of specific pathways in three breast and ovarian cancer cell lines (Fig. 2B). Perifosine decreased phosphorylation of AKT at Ser473 (AKT(p473)) and Thr308 (AKT(p308)) and of downstream AKT effectors (e.g., S6) in each cell line but did not alter phosphorylation of MAPK/ERK1/2 or MEK (not presented) in two of the three lines, demonstrating PI3K/AKT pathway selectivity. Perifosine induced an increase in epidermal growth factor receptor phosphorylation, potentially due to feedback loop activation, and consistently increased Jun levels through as yet undetermined mechanisms. Thus, monitoring of the PI3K/AKT pathway or Jun levels could provide pharmacodynamic markers. The ability of RPPA to simultaneously monitor multiple pathways allows for rapid screening of potential targets and markers.

RPPA also has the potential to provide quantitative analysis. Perifosine at 5 μmol/L reduced AKT(p473) by 65% (±27%) in resting MDAMB468 cells and by 77% (±6%) in EGF-stimulated cells. We previously found, by comparing lysates derived from resting and EGF-stimulated MDAMB468 cells to phosphopeptide controls using RPPA, that these cells have 0.2 and 0.87 amol AKT(p473) per cell, respectively (21). Thus, 5 μmol/L perifosine reduces AKT(p473) in each resting MDAMB468 cell by 0.13 amol (±0.05 amol) and in each EGF-stimulated cell by 0.67 amol (±0.05 amol).

Perifosine-induced growth inhibition correlates with PI3K/AKT pathway genomic status. To determine if the PI3K/AKT pathway mutational status alters perifosine sensitivity, we used RPPA to assess four ovarian/breast cancer cell lines that have previously been comprehensively characterized for PI3K/AKT genomic aberrations, three with activating anomalies (OVCAR3-p85α mutation, PIK3CA amplification; SKOV3-HER2/neu amplification; MDAMB468-PTEN mutation, epidermal growth factor receptor amplification), and one without known aberrations in the core PI3K/AKT pathway (MDAMB231-RAS/RAF mutations).6 Baseline AKT(p473) levels correlated with growth inhibition induced by a 72-h treatment with 2.5 μmol/L (Fig. 3A) and 5 μmol/L perifosine in 0.5% FBS. Higher AKT(p473) levels were associated with increased sensitivity to perifosine-induced growth inhibition in cells possessing PI3K/AKT-activating genomic abnormalities versus MDAMB231, the latter having low basal AKT(p473) levels despite possessing RAS/RAF mutations. Perifosine concentrations of ≥10 μmol/L induced marked growth inhibition of all four cell lines in 0.5% FBS, possibly reflecting “off-target” effects or an AKT inhibition threshold, even in cells without PI3K/AKT pathway aberrations. Thus, genomic PI3K/AKT pathway activation seems to predict the likelihood of response to perifosine. In support of the effects of perifosine being dependent on PI3K/AKT inhibition and of genomic pathway activation being an indicator of perifosine sensitivity, a dominant-negative AKT (K179M) transgene significantly reduced growth of MDAMB468 cells with little effect on MDAMB231, although both expressed equivalent transgene levels (not shown). Thus, genomic PI3K/AKT pathway activation sensitizes tumor cells to pathway inhibition and could potentially be used to select patients for clinical trials with perifosine.

We have reported that stable introduction of myristylated-activated PI3K subunit p110α (DOV13mp110) sensitizes PI3K/AKT wild-type DOV13 cells to PI3K inhibition by LY294002 while rendering them taxane resistant (21). The effects of LY294002 are mimicked by introduction of a dominant-negative AKT (K179M) transgene significantly reduced growth of MDAMB468 cells with little effect on MDAMB231, although both expressed equivalent transgene levels (not shown). Thus, genomic PI3K/AKT pathway activation sensitzes tumor cells to pathway inhibition and could potentially be used to select patients for clinical trials with perifosine.

Because perifosine selectively inhibits AKT membrane translocation (Fig. 1), membrane-targeted AKT should bypass growth inhibitory effects if the latter are mainly due to PI3K/AKT signaling.
AKT inhibition. Indeed, a membrane-targeted (myristylated) activated AKT (DOV13AKT2dd) almost completely bypassed the effects of perifosine on DOV13 proliferation (Fig. 3B). Furthermore, perifosine failed to sensitize DOV13AKT2dd cells to paclitaxel.

In these assays, perifosine was present throughout incubation. To determine the effects of brief exposure, which may occur during patient treatment, OVCAR3 cells were incubated with perifosine for 24 h, washed, and replated in the absence of perifosine. Perifosine induced a dose-dependent irreversible inhibition of proliferative competence (Supplementary Fig. S1). In contrast, LY294002 did not significantly alter colony formation. This suggests that perifosine induces irreversible growth inhibition, potentially through induction of apoptosis.

Fig. 2 Continued. B, perifosine consistently decreased RPPA-quantified phosphorylation of AKT at Ser\(^{473}\) (AKT\(p_\text{S}473\)) and Thr\(^{308}\) (AKT\(p_\text{T}308\)) but not of mitogen-activated protein kinase (MAPKp) pathway components in three cell lines incubated for 4 h with 1.25, 5, and 20 μmol/L perifosine followed by EGF stimulation and cell lysis. In each case, the Y axis represents quantified phosphorylation relative to control. S6p\(240-244\) phosphorylation of S6 ribosomal protein at Ser\(^{240}\) and Ser\(^{244}\). EGFRp992 phosphorylation of EGFR at amino acid 992. Jun, a transcription factor.
The differing effects of perifosine and LY294002 are possibly due to selective inhibition of AKT by perifosine while leaving other parts of the PI3K pathway (e.g., PDK1) intact or, alternatively, to AKT-independent effects of perifosine. In contrast, LY294002, by inhibiting PI3K, blocks the complete pathway, resulting in reversible growth arrest.

**Perifosine induces apoptosis.** Based on the ability of a brief perifosine incubation to block colony formation, we characterized its effects on cell cycle arrest and apoptosis in OVCAR3. After incubation with perifosine for 72 h in medium containing 0.5% FBS, cells were harvested and apoptosis was determined by propidium iodide and BrdUrd staining. Both perifosine and LY294002 induced G1 arrest, more marked with equimolar LY294002 concentrations (Supplementary Table S1). Unlike LY294002, perifosine, particularly at 10 μmol/L, dramatically increased apoptosis. Similar effects occurred in SKOV3 (not shown). Thus, the selective ability of a brief perifosine incubation to block colony formation is likely due to apoptosis rather than replicative arrest.

**Perifosine inhibits xenograft growth.** To explore the effects of perifosine on orthotopic tumor growth, SKOV3 cells were inoculated i.p. in athymic nude mice. Seven days later, perifosine was administered orally (70 mg/kg thrice or 200 mg/kg once weekly). Perifosine at 200 mg/kg weekly significantly inhibited tumor growth (Supplementary Fig. S2A). This was also shown using T2-weighted magnetic resonance images at baseline and 14 days into study treatment (Supplementary Fig. S2B).

**Biologically relevant dosing.** It is critical to develop methods that assess the pharmacodynamic effects of targeted therapeutics to improve drug development and ensure that patients receive a biologically active dose. Because the antitumor efficacy of perifosine seems dependent on PI3K/AKT inhibition, we used RPPA to quantify effects of perifosine on activation of components of this pathway to identify biomarkers of efficacy that may facilitate early identification of responding patients. The studies described with the two breast and two ovarian cancer cell lines above suggested that PI3K/AKT pathway aberrations, activation status, and inhibition may be accurate predictive and response markers. We sought to test these contentions in a series of additional cell lines across multiple tumor lineages. Thus, IC25, IC50, and IC75 values were determined for six human cancer cell lines representing a range of epithelial tumors, some possessing genomic PI3K/AKT pathway aberrations [BT474 (HER2-amplified PIK3CA-mutant), MCF7 (PIK3CA-mutant), PC3 (PTEN-mutant), DU145 (no detectable aberrations), A431, and HeLa (not fully characterized)]. This approach allows correction for individual variations in perifosine uptake, transport, and metabolism, allowing correlation of changes in proliferation with degree of pathway activation. Each cell line was treated with vehicle or perifosine (IC25, IC50, and IC75; Supplementary Table S2) for 0.5, 1, 4, and 8 h in serum-replete conditions, and phosphoprotein levels were determined by RPPA (4 h is presented in Fig. 4; similar results were observed at each time point). Despite the wide range of concentrations required to achieve IC25, IC50, and IC75, AKTp473 inhibition induced by IC25, IC50, and IC75 doses averaged across all six lines approximated growth inhibition (Fig. 4A). The signaling effects of perifosine IC25, IC50, and IC75 were remarkably
consistent with higher doses blocking PI3K/AKT pathway activity as measured by phosphorylation of AKT, GSK3, and S6, particularly at later time points (Fig. 4B). However, there were subtle differences at midrange perifosine concentrations, with the IC\textsubscript{50} decreasing AKTp473 but not AKTp308 in MCF7/A431 and inhibiting both sites in PC3/BT474. Perifosine did not consistently alter MAPK signaling. The correlation between growth and PI3K/AKT inhibition suggests that phosphorylation

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**Fig. 4.** Perifosine effects on AKT activation are dose dependent. A, after 4 h of control or perifosine treatment of six human cancer cell lines at the individual cell line IC\textsubscript{50}s followed by lysis, RPPA, quantitation, and load controlling of AKT phosphorylation (Ser\textsuperscript{473}), the percentage inhibition of AKT phosphorylation at Ser\textsuperscript{473} (AKTp473) closely approximated the percentage growth inhibition at each perifosine concentration. B, heat maps showing relative changes in phosphorylation of four components of the PI3K signaling pathway (AKTp473, AKTp308, S6p240-4, and GSK3p21-9) in the four indicated cell lines after treatment with increasing IC dose levels of perifosine. To generate heat maps, phosphoprotein levels were quantified with RPPA, and the quantification data of each phosphoprotein were then mean centered. Red, high phosphoprotein levels relative to mean or average (increasing brightness of red indicates increasing phosphoprotein levels); black, mean; green, low phosphoprotein levels. Phosphorylation of MAPK was relatively unchanged in comparison with AKT phosphorylation.
of pathway components may be useful early biomarkers of perifosine efficacy.

To investigate the utility of phosphorylation of PI3K/AKT pathway components as potential biomarkers of perifosine efficacy in vivo, we used RPPA to quantify phosphoproteins in PC3 and DU145 xenografts from perifosine-treated and vehicle-treated nude mice. In the PC3 model, there was a striking correlation between perifosine cumulative dose and antitumor efficacy (Fig. 5A). Four dosing schedules that delivered the maximum tolerated dose (315 mg/kg weekly) resulted in marked equivalent growth inhibition. Strikingly, the maximum tolerated dose also induced marked down-regulation of AKTp473 and of phosphorylation of S6 (S6p240-244), with somewhat less significant down-regulation of AKTp308 and of phosphorylation of GSK3 (GSK3p21/9). AKTp473 and S6p240-244 are robust antibodies with strong signals, potentially contributing to their utility. There were significant correlations between antitumor efficacy of different perifosine schedules (Table 1) and changes in phosphorylation of PI3K/AKT pathway components (Fig. 5A and B; phosphoprotein levels were corrected for protein loading; see Materials and Methods). In the DU145 model, significant down-regulation of AKTp473 and S6p240-244 were also generally observed at perifosine doses that were associated with significant growth inhibition (Supplementary Fig. S3). Docetaxel inhibited xenograft growth in the PC3 model but not phosphorylation of PI3K/AKT pathway components, suggesting that phosphoprotein decreases in perifosine-treated animals were due to an effect of perifosine rather than decreased tumor growth per se. Perifosine toxicity has previously been attributed to JNK phosphorylation (22). JNK phosphorylation increases were observed in several perifosine-treated in vitro models but not in the xenograft models (Fig. 5B). Thus, perifosine antitumor responses in vivo correlated

**Table 1. Treatment groups**

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<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1</td>
<td>Control</td>
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<tr>
<td>2</td>
<td>78.75 mg/kg weekly orally × 4</td>
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<tr>
<td>3</td>
<td>157.5 mg/kg weekly orally × 4</td>
</tr>
<tr>
<td>4</td>
<td>315 mg/kg weekly orally × 4</td>
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<tr>
<td>5</td>
<td>39.375 mg/kg twice weekly orally × 4</td>
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</tr>
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<td>8</td>
<td>19.6875 mg/kg four times weekly orally × 4</td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>10</td>
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<tr>
<td>11</td>
<td>45 mg/kg daily orally × 28</td>
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<tr>
<td>12</td>
<td>Docetaxel</td>
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NOTE: Groups 2 to 11 indicates perifosine doses/schedules.
with inhibition of PI3K/AKT pathway markers previously identified in vitro (AKTp473, S6p240-244). Quantitation of PI3K/AKT pathway activity may provide an early human biological surrogate indicating optimal perifosine dosing and likely antitumor activity. Furthermore, the correlation between inhibition of growth and PI3K/AKT signaling suggests that AKT inhibition contributes significantly to perifosine effects in vivo.

In an attempt to determine the ideal timing of biopsies in human perifosine trials that aim to facilitate optimal biological dosing, mice possessing PC3, A431, or BT474 xenografts were sacrificed at days 0, 1, 2, 7, 14 (BT474 only), and 21 (PC3/A431 only) of perifosine or vehicle treatment for tumor RPPA. PC3 was chosen as a sensitive model, and both A431 and BT474 were chosen as resistant models (Supplementary Table S2). Figure 6 shows that significant AKTp473 inhibition occurred at day 7 and day 21 in the PC3 model, where these decreases correlated with marked xenograft growth inhibition, whereas perifosine did not inhibit AKTp473, other downstream PI3K/AKT pathway components, or xenograft growth in the A431 model (Fig. 6) or the BT474 model (not shown). In the PC3 experiment, AKTp308 and AKTp473 were significantly decreased at 7 days, whereas activation of PI3K/AKT pathway components downstream from AKT (e.g., S6p235-236, S6p240-244) were significantly inhibited only at 21 days (data not shown). Furthermore, at 21 days, down-regulation of cyclin B1 and up-regulation of caspase-7 cleavage were marked most (not shown). We conclude that biopsies to facilitate perifosine optimal biological dosing in human trials should be conducted after 7 days of therapy. These three xenograft models also support the conclusion that PI3K/AKT signaling inhibition correlates with antitumor efficacy of perifosine.

**AKT-independent effects of perifosine.** Perifosine-induced cell proliferation inhibition and taxane sensitization correlated with...
PI3K/AKT activation and were reversed by a membrane-targeted AKT, implicating PI3K/AKT inhibition in these effects. We thus used the same approach to determine whether two additional effects of perifosine, inhibition of motility/invasion and production of neovascular factors, could be attributed to PI3K/AKT inhibition. Strikingly, although perifosine inhibited cell motility, invasion, and vascular endothelial growth factor production by cell lines, these effects did not correlate with PI3K/AKT pathway status and were not reversed by AKT2dd (not shown). Thus, perifosine likely exhibits PI3K/AKT pathway-dependent and pathway-independent activities.

Discussion

Perifosine selectively inhibits AKT-PH domain membrane translocation and AKT signaling. As perifosine effects on signaling, cell proliferation, and sensitization to taxanes were reversed by a membrane-targeted AKT, these effects are likely due to AKT inhibition. However, some effects of perifosine seem to be AKT independent. Nevertheless, the strong correlation between the effects of perifosine on PI3K/AKT signaling and tumor growth strongly argues that AKT inhibition is important to perifosine efficacy. Unfortunately, DOV13 and its subclones do not form tumors in vivo, precluding direct assessment of the effect of membrane AKT targeting on perifosine activity in vivo. The sensitivity of cells with spontaneous and induced genomic PI3K/AKT-activating aberrations to low perifosine concentrations suggests that such aberrations may be potential markers of human tumor perifosine responsiveness. Perifosine-induced PI3K/AKT signaling decreases strongly predict antitumor efficacy, further strengthening the potential utility of these biomarkers. We previously showed that cancer cell lines with PI3K/AKT aberrations are more sensitive to PI3K inhibition (3, 21, 23–25). These data support testing perifosine in cancers with frequent genomic PI3K/AKT anomalies. Clinical trial correlative studies may refine selection of tumors most likely to respond. Adding perifosine to paclitaxel may also overcome paclitaxel resistance in tumors with PI3K/AKT activation.

In assessing novel therapies in human cancer trials, pharmacodynamic markers of drug activity must be defined in preclinical models to make clinical development more efficient and ensure that patients are receiving a biologically relevant dose. Maximal inhibition of the target may be a more important end point than maximum tolerated dose per se. This approach may optimize efficacy, decrease toxicity, in particular off-target toxicity, and facilitate early identification of nonresponders. In this regard, perifosine-induced PI3K/AKT inhibition correlated remarkably well with tumor growth inhibition using multiple dosing schedules. Furthermore, assessment of multiple PI3K/AKT pathway members may prove superior to single markers. RPPA facilitates analysis of multiple PI3K/AKT pathway components and identification of mechanisms associated with inhibition.

RPPA (9–16, 20) is particularly applicable to assessment of on-target and off-target effects of novel therapies. It is inexpensive, high-throughput, applicable to small tissue amounts (e.g., from fine needle aspiration) and, unlike immunohistochemistry/Western blotting, can concurrently quantitate multiple markers and pathways. We expect further RPPA validation to support applicability to in vitro drug development, optimal biological dose determination, and early identification of nonresponders. RPPA also has the potential to identify and validate pathways wherein a molecular imaging approach is applicable. By quantifying functional proteomics, RPPA may prove essential in efforts to develop molecular markers for optimal biological dosing and early response detection.

When we determined perifosine IC25, IC50, and IC75 across multiple cell lines (Supplementary Table S2), there was wide variability in perifosine concentrations, likely reflecting differences in uptake, transport, and/or metabolism. Despite this variability, decreases in cell growth and PI3K/AKT signaling were concordant. Thus, it may be possible to achieve optimal personalized patient doses that account for individual differences in perifosine pharmacology and pharmacodynamics. This may improve the real but limited response rates observed in perifosine trials (1, 26). More benefit may result from individual patient dose “titration” to maximal PI3K/AKT inhibition than to maximum tolerated dose per se, potentially facilitating dose limitation, for example, in patients with sensitive cancers (e.g., having lost PTEN). Studies of this approach necessitate biopsies. However, as glucose uptake is dependent on PI3K/AKT activity, fluorodeoxyglucose-positron emission tomography may be a useful surrogate (3). We believe that such an approach is critical in clinical trials of targeted therapies.

The signaling effects of perifosine and LY294002 are attenuated by growth factors (Fig. 2A) and high FBS concentrations. Although it is difficult to determine the concentration of growth factors at the tumor interface, they are likely to be much lower than those in 10% FBS. Most of our studies were thus done in 0.5% FBS. Impaired signaling inhibition in 10% FBS markedly decreased perifosine-induced growth inhibition and apoptosis, particularly at low concentrations. Whether serum growth factor levels or perifosine binding in serum limits its effectiveness remains to be determined.

The use of cell lines to predict drug efficacy and molecular markers has many concerns. The comparative utility of two-dimensional/three-dimensional culture, representative cell lines, coculture of tumor with stroma, and appropriate growth factor concentrations remains unknown. However, the use of multiple cell lines possessing baseline and induced PI3K/AKT aberrations may alleviate some problems and render our results more applicable to human trials.

Perifosine-induced AKT inhibition was relatively specific at low drug concentrations. However, perifosine also mediates its effects through other mechanisms that become apparent at concentrations of ≥10 μmol/L in vitro. We also showed that perifosine effects on motility, invasion, and neovascularizing factors are likely PI3K/AKT independent. Although effects of perifosine on MAPK and JNK phosphorylation have been shown in other studies, we found that these changes lacked the consistency or predictive value of PI3K/AKT inhibition. However, these effects may also contribute to perifosine activity.

In contrast to LY294002, a brief perifosine incubation prevented subsequent colony formation. Furthermore, perifosine, but not LY294002, induced marked apoptosis under...
anchoragendependent conditions. The different effects of perifosine and LY294002 on cell survival remain unexplained; however, they may relate to the differential effects of targeting PI3K versus AKT or to off-target effects. Furthermore, this suggests that perifosine may show increased or broader spectrum activity in human tumors than PI3K inhibitors.

In conclusion, perifosine has significant antitumor activity that is attributable, at least in part, to AKT inhibition. Perifosine, particularly at lower concentrations, may have greater inhibitory effects on tumors with PI3K/AKT-activating genomic abnormalities. As a proof of principle, we have established the potential practical utility of novel RPPA for quantifying the pharmacodynamic effects of targeted therapies in vivo. AKTp473 and S6p240-244 are potentially the most useful pharmacodynamic markers of early efficacy and of biologically optimal perifosine dosing in human trials. Because of a very strong correlation between proportional modulation of these PI3K pathway biomarkers and quantified perifosine efficacy, measurement of such pharmacodynamic biomarkers with RPPA may maximize our ability to select patients who are responding to perifosine and guide optimal perifosine dosing. This approach requires investigation in clinical trials. Furthermore, PI3K pathway activation status may allow baseline selection of patients most likely to respond to perifosine alone or in combination with other therapies.

References
Pharmacodynamic Markers of Perifosine Efficacy
