Perifosine is a novel ether alkylphospholipid demonstrating antitumor activity in human trials (1). Perifosine inhibits tumor growth through multiple mechanisms; however, recent studies suggest that perifosine inhibits the phosphatidylinositol-3'-kinase (PI3K)/AKT pathway by preventing cell membrane recruitment of the AKT pleckstrin homology domain (2). Many effects of perifosine can be bypassed by enforced membrane AKT targeting, further implicating AKT inhibition in its activity. The PI3K/AKT pathway is activated more frequently by genomic anomalies than other signaling pathways in cancer, making it a potentially exciting therapeutic target (3–7). Other than rapamycin and its analogues, perifosine is the only PI3K/AKT inhibitor in clinical trials, making methods to determine if perifosine inhibits PI3K/AKT signaling, if patients receive a biologically relevant dose, and select likely responders crucial.

The PI3K/AKT pathway regulates many “hallmarks of cancer,” including survival, proliferation, and metastasis (3). PDK1, TSC2, glycogen synthase kinase 3 (GSK3), mTOR, p70S6 kinase, and S6 mediate many effects of PI3K/AKT and participate in feedback loops that regulate pathway homeostasis. These feedback loops may play a critical role in patient management and S6 mediate many effects of PI3K/AKT and participate in feedback loops that regulate pathway homeostasis. These feedback loops may play a critical role in patient management.
to perifosine than lines without such aberrations. Enforced P3K/AKT pathway activation also renders cells more sensitive to perifosine. Thus, perifosine may be particularly useful in treating tumors with P3K/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 P3K/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 (S240/244), phosphorylated MEK (Thr202/204), phosphorylated c-Jun-NH2 kinase (JNK; Thr183/Tyr185), phosphorylated epidermal growth factor receptor (Tyρ123/42), Jun, p38, and cleaved caspase-7. Rabbit anti-IN1K 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 transcription – PCR (Titan One Tube RT-PCR System from Roche Applied Science). The myristylation site (ATG-CCG-AGC-AGC-AGG-AAG-CCC-AAC) and HA-tagged sequence (ATG-TAC-CCA-TAC-GAT-GTT-CCA-CAT-TAC-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 found to be without error. Adenovirus-containing kinase inactive (dominant-negative) AKT (K179M) was provided by Dr. Ho-Young Lee (M. D. Anderson Cancer Center). Myristylation 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 found to be 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-PLC-γ1-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 P3K pathway aberrations) were cultured by Keryx Pharmaceuticals. Isogenic pairs of DOV13 cell lines were established by stable transfection with different cDNA plasmids containing human active PI3K p110 catalytic domain or active or kinase inactive AKT2 sequences using Fugene 6 transfection reagent (Roche Molecular Biochemicals) and Nucleofector (Amaxa Biosystems). Eighteen hours later, cells were seeded in 96-well plates (5,000/well) and serum starved overnight before incubation with or without perifosine in 0.5% FBS 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 with 4′,6-diamidino-2-phenylindole. GFP fusion protein localization was observed using a fluorescence microscope.

**AKT localization assay.** GFP fusion proteins containing the AKT-PH or PLC-γ1-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 μmol/L) or LY294002 (20 μmol/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 with 4′,6-diamidino-2-phenylindole. GFP fusion protein localization was observed using a fluorescence microscope.

**Cell proliferation assay.** Cells were seeded in 96-well plates (5,000/well) and serum starved overnight before incubation with or without perifosine in 0.5% FBS for 72 h, followed by 3-h incubation with MTS reagents (Promega). Alternatively, cells were washed at the end of treatment with ice-cold PBS and stained with 0.5% crystal violet containing 20% methanol for 30 min at room temperature followed by dissolution of the dye in Sorenson’s buffer for 1 h. For some experiments, cells were seeded in 96-well plates and infected with adenovirus containing dominant-negative AKT. Forty-eight hours after infection, cells were washed and incubated in medium supplemented with 0.5% FBS for 72 h, followed by 3-h incubation with MTS reagents. In all proliferation assays, triplicates were done. The quantity of 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.
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 administrated orally as 200 mg/kg weekly 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; nex, 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 1.33u, 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

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**Fig. 1.** Specific inhibition of AKT-PH domain membrane localization by perifosine. The localization of the GFP-AKT-PH domain fusion construct is primarily cytoplasmic in unstimulated transfected OVCAR3 cells; after stimulation with EGF, the construct protein relocates to the plasma membrane. Perifosine inhibits EGF-induced membrane localization of AKT in a concentration-dependent manner. The PI3K inhibitor LY294002 is shown as a positive control. In contrast, perifosine does not inhibit membrane localization of a GFP-PLCγ-PH domain construct.
phosphatidylinositol (PI)(3,4,5)P3, and of PLCγ, which preferentially binds PI(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.

PPI3K/AKT pathway activation was also explored the ability of perifosine to alter signaling through multiple pathways using RPPA (9–16, 20) 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 (AKTp473) and Thr308 (AKTp308) 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 undefined 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.

**Fig. 2.** Inhibition of PI3K/AKT signaling by perifosine. A, perifosine abrogates basal and EGF-induced phosphorylation (p) of AKT (Ser473 and Thr308), p70S6K (Thr389), S6 ribosomal protein (Ser235/236), and GSK3α/β (Ser21/27) in both Western blots and RPPAs. The effects of the PI3K inhibitor LY294002 (10 μmol/L) are shown as a positive control.

RPPA also has the potential to provide quantitative analysis. Perifosine at 5 μmol/L reduced AKTp473 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 AKTp473 per cell, respectively (21). Thus, 5 μmol/L perifosine reduces AKTp473 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). Baseline AKTp473 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 AKTp473 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 AKTp473 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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).
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\(^{473}\) (AKTp473) and Thr\(^{308}\) (AKTp308) but not of mitogen-activated protein kinase (MAPKp) pathway components in three cell lines incubated for 4 h with 1.25, 5, and 20 \(\mu\)mol/L perifosine followed by EGF stimulation and cell lysis. In each case, the Y axis represents quantified phosphorylation relative to control. S6p240-244 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 administrated orally 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.

**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_{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

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_{50}s followed by lysis, RPPA, quantitation, and load controlling of AKT phosphorylation (Ser^{473}), the percentage inhibition of AKT phosphorylation at Ser^{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 (Fig. 5A and B; phosphoprotein levels were corrected for protein loading; see Table 1). 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

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**Table 1. Treatment groups**

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<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1</td>
<td>Control</td>
</tr>
<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>9</td>
<td>78.75 mg/kg four times weekly orally × 4</td>
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<tr>
<td>10</td>
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</tr>
<tr>
<td>11</td>
<td>45 mg/kg daily orally × 28</td>
</tr>
<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 \textit{in vitro} (AKT$p^{473}$, S6$p^{240-244}$). 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 \textit{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 AKT$p^{473}$ 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 AKT$p^{473}$, 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, AKT$p^{308}$ and AKT$p^{473}$ were significantly decreased at 7 days, whereas activation of PI3K/AKT pathway components downstream from AKT (e.g., S6$p^{235-236}$, S6$p^{240-244}$) 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.

\textit{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 cells 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 IC_{25}, IC_{50}, and IC_{75} 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
anchorage-dependent 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. AKT$p^473$ and S6$p^{240/244}$ 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


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