

Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation

Sudhir B. Kondapaka, Sheo S. Singh, Girija P. Dasmahapatra, Edward A. Sausville, and Krishnendu K. Roy

Clinical Trials Unit, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD

Abstract

Perifosine is a novel p.o. bioavailable alkylphospholipid. Perifosine has displayed significant antiproliferative activity *in vitro* and *in vivo* in several human tumor model systems and has recently entered phase I clinical trials. Recent studies have identified that perifosine could cause cell cycle arrest with induction of p21^{WAF1/CIP1} in a p53-independent fashion; however, the basis for that effect is not known. Structurally, perifosine resembles naturally occurring phospholipids. Therefore, we hypothesized that perifosine might perturb pathways related to phospholipids modulated by growth factor action. We demonstrate here that perifosine causes dose-dependent inhibition of protein kinase B/Akt phosphorylation and thus activation at concentrations causing growth inhibition of PC-3 prostate carcinoma cells. Only the myristoylated form of Akt (MYR-Akt), which bypasses the requirement for pleckstrin homology (PH) domain-mediated membrane recruitment, abrogated perifosine-mediated decrease of Akt phosphorylation and cell growth inhibition by perifosine. We demonstrate further that perifosine decreases the plasma membrane localization of Akt, and this is substantially relieved by MYR-Akt along with relief of downstream drug effect on induction of p21^{WAF1/CIP1}. Perifosine does not directly affect phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent kinase 1, or Akt activity at concentrations inhibiting Akt phosphorylation and membrane localization. Our results demonstrate that Akt is an important cellular target of perifosine action. In addition, these studies show that the membrane translocation of certain PH domain-containing molecules can be greatly perturbed by the alkylphospholipid class of drugs and imply further that the PI3K/Akt pathway contributes to regulation of p21^{WAF1/CIP1} expression. (Mol Cancer Ther. 2003;2:1093–1103)

Received 5/27/03; revised 7/29/03; accepted 8/5/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: S.B.K. and S.S.S. equally contributed to this work.

Portions of this work were presented in abstract form in the Proc. Am. Assoc. Cancer Res., 43: Abs. 2977, 2002.

Requests for Reprints: Krishnendu K. Roy, National Institutes of Health, 10 Center Drive, Building 10, Room 6N 113, Bethesda, MD 20892-5680. Phone: (301) 496-4119; Fax: (301) 480-7456. E-mail: kr91w@nih.gov

Introduction

Phospholipid analogues are structurally novel candidate drugs with evidence of antineoplastic activity in both *in vitro* and *in vivo* model systems (1–3). Miltefosine is an alkylphospholipid that has been approved in Europe for topical treatment of cutaneous lymphomas and cutaneous metastases from breast cancer (4–6). Miltefosine has also shown promising effects against visceral leishmaniasis (7, 8). In the search for alkylphospholipids with lessened gastrointestinal side effects, perifosine [octadecyl-(1,1-dimethyl-4-piperidyl) phosphate; Fig. 1A] was noted to be a more p.o. bioavailable alkylphospholipid (9). It has recently entered phase I clinical trials (10).

Numerous activities have been ascribed to phospholipid analogues. These include modulation of signaling targets, which use naturally occurring lipid moieties as substrates or cofactors, including protein kinase C and phospholipase C (11–15). Other studies (16, 17) point to interference by these compounds with synthesis of other lipids necessary for proper membrane function. Recent studies (18) have identified that perifosine could cause cell cycle arrest in a way that correlated with induction of p21^{WAF1/CIP1} in a p53-independent fashion. To delineate the biochemical pathway leading to this outcome, we hypothesized further that control of p21^{WAF1/CIP1} expression might proceed from pathways governed by natural phospholipids, particularly those stimulated in response to the action of growth factors (19–21).

One pathway of clear importance to cell cycle progression and cell survival also related to phospholipid metabolism is controlled by phosphoinositide 3-kinase (PI3K) (22). This lipid kinase is activated in response to numerous growth factors and in turn activates a series of signaling molecules including phosphoinositide-dependent kinase (PDK) 1 and 2 and the cellular homologue of the Akt oncogene (23). The behavior of many neoplasms appears to be correlated with the expression of positive (worse prognosis) or negative (better prognosis) regulators of the PI3K/Akt signaling pathway (24–28).

PI3K activation results in accumulation of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (29–31). Pleckstrin homology (PH) domain-containing proteins including PDK1 and Akt (29, 32) then bind to the 3'-OH phosphorylated phosphatidylinositols through the PH domain. This results in targeting of Akt to the plasma membrane and provides a favorable conformation for Akt Thr308 and Ser473 phosphorylation (32–34). The upstream kinase PDK1 has been clearly shown to phosphorylate Thr308 site, but the identity of the Ser473 kinase is quite controversial (31, 35). The phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10, also called MMAC1 or TEP1) has emerged as an important regulator of Akt activation by removing the D-3 phosphate of phosphatidylinositol

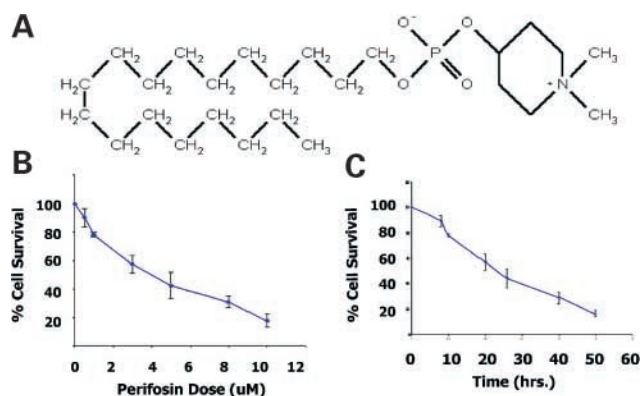


Figure 1. Effect of perifosine on cell viability. **A**, perifosine structure. **B**, effect of perifosine on the viable cell number of PC-3 prostate carcinoma cells after 24 h at the indicated concentration. **C**, PC-3 cells were exposed to 5- μ M perifosine for the indicated time, and viable cell count was determined. Points, representative of six individual measurements.

phosphates (D-3 PtdIns phosphate) and thus terminating the signal. PTEN has been found deleted in a substantial fraction of cancers, including glioblastomas, breast, prostate, and ovarian carcinomas, and several syndromes with multiple tumors incidence (36, 37). Amplification and overexpression of Akt is frequently observed in ovarian (25) and gastric tumors (38), further suggesting a role in human malignancy.

We decided to assess perifosine action on the Akt pathway in the PTEN-null human PC-3 prostate carcinoma cell line (39). Here, we present evidence that Akt is an important target of perifosine action. We demonstrate that drug concentrations, which cause cell growth inhibition and eventual cell death, cause rapid loss of Akt activity. Perifosine causes decrease in Akt Ser473 and Thr308 phosphorylation. This attenuation of Akt activity by perifosine was not restored by transfection of wild-type Akt (WT-Akt) to the cells, but overexpression of a form of Akt that is myristoylated (MYR-Akt) and thus constitutively localized to the plasma membrane overcomes perifosine inhibition of Akt activation. We demonstrate that perifosine greatly hampers translocation of WT-Akt to the cell membrane, where Thr308- and Ser473-directed kinases normally activate Akt. These data also support the idea that an Akt-related pathway affects the expression or stability of p21^{WAF1/CIP1}.

Materials and Methods

Antibodies and Reagents

Antibodies against total and phosphorylated Akt (Ser473 and Thr308) were purchased from Cell Signaling Technologies (Beverly, MA). PDK1 and Akt assay kits and recombinant active Akt, inactive Akt, and active PDK1 enzymes were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Serum- and glucocorticoid-induced kinase (SGK) was also obtained from Upstate Biotechnology. Anti-PI3K- α , anti-PI3K- γ , PI3K-p85- α , p21^{WAF1/CIP1},

p27^{KIP1}, protein phosphatase 2A, and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The fluorescein-conjugated anti-hemagglutinin (HA) antibody (2364) was purchased from Cell Signaling Technologies. All cell culture media and reagents were from Life Technologies, Inc. (Rockville, MD). Perifosine was obtained from Zentaris AG (Frankfurt, Germany). 7-Hydroxystaurosporine was from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Unless otherwise noted, other signal transduction inhibitors were from Calbiochem (La Jolla, CA). Various phosphatidylinositides, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), and insulin were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and Cell Culture

Cell lines were grown in their respective culture medium as recommended by the American Type Culture Collection (Manassas, VA). In particular, human PC-3 prostate adenocarcinoma cells were maintained in RPMI medium containing 100-unit/ml penicillin G, 100- μ g/ml streptomycin medium supplemented with 10% fetal bovine serum (FBS; Life Technologies) at 37°C in an atmosphere containing 5% CO₂. Exponentially growing cells were treated with different concentrations of perifosine for 24 h. The adherent cells were treated with trypsin, washed with PBS, and collected by centrifugation at 1500 rpm for 5 min. Viable cell counts were enumerated by hemocytometer using 0.2% trypan blue.

Cell Lysis and Immunoblot Analysis

Lysis buffer consisted of 50-mM HEPES (pH 7.4), 20-mM EDTA, 0.5-mM sodium orthovanadate, 10-mM sodium glycerophosphate, 1-mM sodium fluoride, 10% glycerol, 0.5% NP40, 5- μ g/ml aprotinin, 5- μ g/ml leupeptin, and 1-mM AEBSEF. Approximately 20–40 μ g of total protein were resolved by 4–20% Tris-glycine SDS-PAGE gel. The separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Billerich, MA) in a Hooper transblotter using 25-mM Tris, 192-mM glycine, and 20% methanol. After the transfer was completed (2–3 h), the blots were blocked for 1 h in a blocking buffer containing 5% (w/v) blotto (Santa Cruz Biotechnology) in TTBS [10-mM Tris-HCl, 140-mM NaCl (pH 7.4), and 1% (v/v) Tween 20]. The membranes were washed thrice extensively in TTBS. The blots were then placed in respective primary antibodies at optimal concentrations for 1 h. After three washes with TTBS, the horseradish peroxidase-conjugated specific secondary antibodies were added and further incubated for 1 h in the presence of 5% (w/v) blotto in TTBS. The membranes were washed extensively in TTBS and detection was performed with enhanced chemiluminescence reagents (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

For insulin or other growth factor stimulation experiments, cells were serum starved for 24 h by replacing 10% FBS medium with RPMI medium. Respective growth factors and insulin (Life Technologies) were then added

to the medium and further incubated as specified in the figure legends. To assess the effect of perifosine, cells were pretreated with 5- μ M perifosine for 30 min before insulin or other growth factor addition.

PI3K Assay

Serum-starved PC-3 cells were stimulated with 100-nM insulin for 10 min. Cells were trypsinized and harvested, washed twice with cold PBS, and lysed with 20-mM Tris-HCl (pH 7.4) containing 150-mM NaCl, 1-mM EDTA, 1-mM sodium orthovanadate, 1% NP40 (v/v), 1-mM AEBSF, 5- μ g/ml leupeptine, and 5- μ g/ml aprotinin. After protein determination using Bio-Rad (Hercules, CA) kit, cellular lysates were immunoprecipitated with anti-PI3K-p85- α antibody obtained from Santa Cruz Biotechnology. To 1-mg protein sample, 5 μ l of anti-PI3K-p85- α were added and mixed in cold for 1 h followed by the addition of 50 μ l of Gamma Bind Sepharose (Pharmacia, Piscataway, NJ) and further mixed for 1 h in cold. The immunoprecipitate was washed with PBS containing 1-mM sodium orthovanadate and 1% NP40 (v/v) twice followed by two washes with 50-mM Tris containing 10-mM LiCl. The beads were then washed once with TNE (20-mM Tris-HCl [pH 7.4], 100-mM NaCl, and 1-mM EGTA). The kinase reaction was started by adding 50 μ l of kinase buffer (20-mM Tris, 100-mM NaCl, 0.3-mM EGTA, 10- μ g phosphatidylinositol, 1- μ M ATP, and 10-mCi 32 P-ATP). The reaction was performed at 30°C for 20 min. The reaction was terminated by adding 100 μ l of 0.1-M HCl. The reaction product was extracted with 200- μ l chloroform:methanol (1:1); 50:1 of the organic phase were loaded onto LK6D plates (Whatman Inc., Clifton, NJ) impregnated with 1% potassium oxalate. 32 P-labeled phospholipids were separated by TLC using chloroform:methanol:water:ammonia (45:38:8:2) mixture as the solvent system, and the reaction products were visualized by autoradiography (40).

PDK1 Assay

Recombinant active PDK1 or PDK1 immunoprecipitation kits were purchased from Upstate Biotechnology, and the enzyme assays were performed according to the manufacturer's instructions. Briefly, PDK1 enzyme was used to phosphorylate inactive SGK. The activity of phosphorylated and active SGK was then measured using 32 P-ATP incorporation in SGK/Akt substrate peptide. The incorporation of 32 P in substrate peptide was measured by applying an aliquot of the reaction mixture on a phosphocellulose paper followed by three washes with 0.75% phosphoric acid and once with acetone to remove unreacted ATP. The dried paper was subjected to liquid scintillation counting for quantitation.

Akt Kinase Assay

A recombinant active Akt assay kit was purchased from Upstate Biotechnology. The assay was performed according to the manufacturer's instructions using the SGK/Akt substrate peptide. Akt kinase assay kit was also purchased from Cell Signaling Technologies to perform IP kinase assays from PC-3 cells. Lysates from control and perifosine-treated PC-3 cells were prepared using lysis buffer provided in the kit supplemented with 50-nM okadaic acid

and 50-nM calyculin A. Kinase reaction protocol was as per manufacturer's instruction using glycogen synthase kinase-3 (GSK-3) fusion protein as substrate. The degree of GSK-3 fusion protein phosphorylation was determined using phospho-GSK-3 antibody to determine the Akt kinase enzyme activity. In few experiments, Akt kinase activity was performed according to Murga *et al.* (41) using 32 P-ATP and histone H2B (Roche Molecular Biochemicals, Indianapolis, IN) as substrate.

Integrin-Linked Kinase Assay

Integrin-linked kinase (ILK) was immunoprecipitated from serum-stimulated PC-3 cells using anti-ILK monoclonal antibody (Upstate Biotechnology), and kinase assays were performed using S6 peptide (Upstate Biotechnology) and 32 P-ATP according to Persad *et al.* (42). Incorporation of 32 P in the peptide was quantitated using the phosphocellulose paper as described before.

Src Kinase Assay

Active Src (recombinant enzyme), kinase reaction buffer, and Src kinase substrate peptide were obtained from Upstate Biotechnology. Src kinase assays were performed according to the manufacturer's instructions. Incorporation of 32 P from 32 P-ATP into the substrate peptide was quantitated using the phosphocellulose paper as described in PDK1 assay system.

Plasmid Constructs and Transfections

An expression vector for HA-tagged different Akt constructs (pCEFL-HA-Akt1) have been reported elsewhere (41). The distinct constructs encoding WT-Akt called pCEFL-HA-WT-Akt1, the constitutively membrane localized Akt called pCEFL-HA-MYR-Akt1 (which contains an amino-terminal sequence that allows myristoylation but which lacks a PH domain), the kinase dead (KD) mutant of Akt (Akt with a K197M mutation) called pCEFL-HA-K197M-Akt1, and the Ser473 and Thr308 mutant construct in which both sites have been mutated to alanine (AA-Akt1) called pCEFL-HA-AA-Akt1 have all been described previously (43). pCEFL-enhanced green fluorescent protein (EGFP)-AH-Akt1 construct, the PH domain of the mouse WT-Akt spanning the PH motif, was obtained by PCR as a *Bam*HI-*Not*I insert, including a four glycine residue cross-linker after the *Bam*HI restriction site. The previous construct was then subcloned into the pCEFL-EGFP (43). The previous construct was then subcloned into the pCEFL-EGFP (43). Plasmids encoding different forms of PI3K γ were made in pcDNA III for transient expression experiments. WT-PI3K γ is designated as pcDNA III-WT-PI3K γ , constitutively active (CA) PI3K γ is designated as pcDNA III-CAAX-PI3K γ (CA-PI3K), and the cDNA for the dominant negative (DN) mutant of PI3K γ is designated as pcDNA III-KR799-PI3K γ (DN-PI3K). These also have been described previously (44, 45).

Src and ILK constructs were purchased from Upstate Biotechnology (Allelic Pack Catalogues 17-268 and 17-307, respectively). In brief, different forms of Src cDNA were constructed in pUSEamp vector. WT (Catalogue 21-114), CA (Catalogue 21-115), and DN (Catalogue 21-154) forms of Src cDNA are represented as Src-WT, Src-CA,

and Src-DN, respectively. Similarly, different forms of ILK constructs were also constructed in pUSEamp vectors. WT (Catalogue 21-184), kinase hyperactive (S343D; Catalogue 21-185), and kinase inactive (R211A; Catalogue 21-182) forms of ILK are represented as ILK-WT, ILK-CA, and ILK-KD, respectively.

Different forms of human PDK1-cDNA construct were generously provided by Drs. Sunhong Kim and Jongkyeong Chung (Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Republic of Korea). WT *myc*-tagged PDK1 constructed in pcDNA III vector is designated as PDK1-WT (PDK1-WT-*myc*-pcDNA III), while KD PDK1-cDNA in pcDNA III is indicated by PDK1-KD (PDK1-KD-*myc*-pcDNA III). A CA membrane localized form of PDK1 was made in pBJ5 vector with myristoylation motif and is designated as PDK1-CA (pBJ5-MYR-PDK1-FLAG). Unless otherwise indicated, cells were cotransfected with the same quantity of plasmids for the different signaling molecules (5 μ g each/90 mm dish). The total DNA amount was kept constant in all the transfection experiments, including appropriate amounts of empty vector. Unless otherwise indicated, cells were cotransfected with the same quantity of plasmids coding for Akt and for the different molecules such as PDK1 and PI3K (5 μ g each/90 mm dish). Transfections were performed using LipofectAMINE 2000 (Life Technologies) according to the manufacturer's instructions. At 24 h post-transfection, the cells were either serum starved or grown in regular medium for 24 h followed by treatment as indicated in the figure legends. Experiments were performed in triplicate.

Immunofluorescence

PC-3 cells were plated onto glass coverslips at a density of 5000 cells/well in 24-well dishes. After 24 h, the cells were transfected by the LipofectAMINE 2000 (Life Technologies) technique with 1–3 μ g of the relevant plasmid. On the day after transfection, cells were rendered quiescent by incubation in serum-free medium for 24 h and then treated with 5.0- μ M perifosine for 30 min followed by induction with 100-nM insulin for 10 min. The cells were then fixed in 3.7% paraformaldehyde for 15 min and permeabilized for 5 min in 0.2% Triton X-100 for 10 min. The coverslips were washed and blocked as recommended (Cell Signaling Technologies). Briefly, coverslips were washed with PBS, and nonspecific antibody binding sites were blocked by incubation with PBS containing 3% BSA. The coverslips were then incubated with fluorescein-conjugated anti-HA antibody diluted in PBS-BSA (1/2000) overnight and washed five times with PBS. Control staining was performed without antibody. Cells were then incubated for 30 s with 4',6-diamidino-2-phenylindole for nuclear staining. After extensive washes in PBS, coverslips were mounted in Aquamount and examined under epifluorescent illumination. The cells were examined in an inverted microscope under a 63 \times oil immersion magnification objective using Olympus microscope. For quantification, 50–100 cells/coverslip were counted.

In another set of experiments, PC-3 cells were transfected with PH domain conjugated to GFP (pCEFL-EGFP-

AH-Akt1 construct). At 24 h post-transfection, the cells were either serum starved or grown in regular medium for 24 h followed by treatment as indicated in the figure legends.

Results

Perifosine Inhibition of PC-3 Cell Growth

Perifosine has a similar structure to naturally occurring phospholipids (Fig. 1A). Perifosine (5 μ M) causes growth inhibition of PC-3 prostate carcinoma cells by 50% (GI_{50}) in 24 h (Fig. 1B). Continued exposure to this concentration (Fig. 1C) causes virtually all cells to die by 48 h. Cleavage of poly(ADP-ribose) polymerase is evident (data not shown) on incubation of 2- μ M perifosine at 24 h, indicating that pronounced cell death occurred. Perifosine inhibits the growth of other cell types (data not shown), with GI_{50} values of 1–10 μ M [K-562, SUDHL-7, HL-60, PC-3, Colo-205, and MESA-SA (CRL-1976)], 11–30 μ M [MG-63 (CRL-1427), H-3386, RD (CCL-136), HT-29, MDA-435, SW-620, CCRF-7, H-1355, SAJA-1 (CRL-2098), and DU-145], and >30 μ M [MCF-7, Hos (CRL-1543), and LnCAP]. We elected to further characterize early signaling events affected by perifosine in PC-3 cells, as these cells are known to be mutated in PTEN and therefore would be expected to have relative activation of the Akt pathway.

The Akt Pathway Is a Target for the Antiproliferative Effect of Perifosine

We first investigated the effect of perifosine on the phosphorylation status of Akt on Ser473 and Thr308. Fig. 2A shows that exposure of PC-3 cells to 5- μ M perifosine for only 0.5 h causes virtually complete loss of phosphorylation of Akt on Thr308 and Ser473, without affecting the amount of Akt. This loss of phosphorylation was also seen in almost all cell lines tested at their GI_{50} concentration (data not shown). Fig. 2B shows that in PC-3 cells loss of phosphorylation of both known regulatory phosphorylation sites of Akt (compare with Fig. 1B) occurs at similar perifosine concentrations.

As it is well documented that the phosphorylation on Thr308 and Ser473 is a prerequisite for the catalytic activity of Akt, we next assessed perifosine's effects on Akt kinase activity in treated PC-3 cells. Fig. 2C shows that when PC-3 cells are treated with different concentrations of perifosine for 1 h and Akt protein is immunoprecipitated to measure its catalytic activity using SGK as substrate, perifosine's effect on Akt phosphorylation state paralleled exactly its inhibition of Akt kinase activity. Maximum inactivation of its kinase catalytic activity was observed within 1 h at 5 μ M. Taken together, these data suggest that loss of phosphorylation of both Thr308 and Ser473 results in perifosine-induced inactivation of Akt at very early times after addition of drug.

Reversible Effect of Perifosine and Relevance to Several Growth Factors

As perifosine is a lipid, it is possible that its interactions with cells will be detergent like. However, after 24 h of

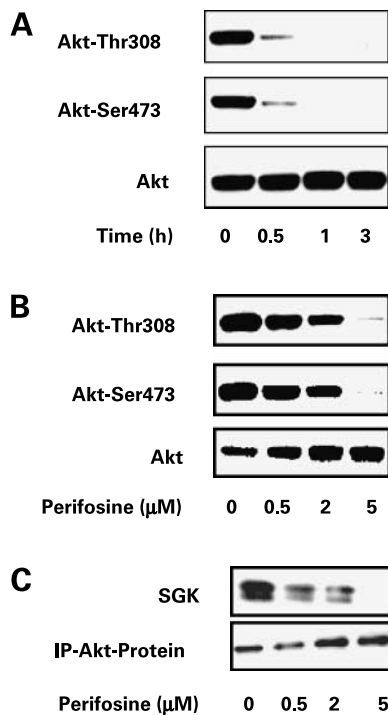


Figure 2. Effect of perifosine on Akt phosphorylation and activity. Exponentially growing PC-3 cells were treated with 5- μ M perifosine for up to 3 h (A) or with varying concentrations of perifosine for 1 h (B). Cells were lysed, and equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, probed, and reprobed with phosphospecific as well as total Akt antibodies as described in "Materials and Methods." C, cells were treated with indicated concentrations of perifosine for 1 h, and cell lysates were used to immunoprecipitate endogenous Akt protein with anti-Akt antibody and its kinase activity was determined using SGK/Akt substrate peptide as described in "Materials and Methods."

perifosine (5 μ M) treatment, PC-3 cells when replaced with fresh medium revert to their original morphology and the growth rate after 24 h is comparable with that of control cells (data not shown). Therefore, we next tested withdrawal of perifosine effect on Akt phosphorylation with respect to time. Fig. 3A shows that PC-3 cells exposed to 5- μ M perifosine for 3 and 12 h, washed, and exposed to fresh medium demonstrated substantial recovery of Akt phosphorylation level within 1 h. After 3 h, there was a full recovery of phosphorylation when compared with control. Removal of perifosine even after exposure of 12 h to PC-3 cells resulted in complete restoration of Akt phosphorylation level within 6 h. Next, PC-3 cells were also stimulated with a variety of different growth factors to activate the PI3K/Akt pathway following pretreatment with perifosine for 30 min. Stimulated Akt phosphorylation by serum, insulin, IGF-I, EGF, and PDGF was strongly inhibited by perifosine (Fig. 3B). In contrast to effect on Akt, preliminary results suggest that analogous concentrations of perifosine actually cause activation of p38 mitogen-activated protein kinase, p42/44 Erk kinase, and stress-activated protein kinase/c-Jun N-terminal kinase pathway (data not shown).

Effect on PI3K Activity

Immunoprecipitated PI3K complex obtained from PC-3 cell lysates does not show any change in its kinase activity on direct incubation of perifosine even up to 20 μ M (Fig. 4A), under conditions where wortmannin completely inhibited the PI3K-mediated D-3 PtdIns phosphate generation. Next, we investigated whether perifosine inhibits growth factor-mediated activation of PI3K. Serum-starved PC-3 cells pretreated with or without perifosine (10 μ M) for 30 min were exposed to insulin (100 nM) for another 10 min or were left unstimulated. PI3K activity was determined in anti-p85- α -PI3K immunoprecipitates, as described previously. Immunoprecipitates obtained from insulin-stimulated cell lysates showed a 5.5–7.5-fold increase of PI3K activity compared with extracts from non-insulin-stimulated countercontrol cells (Fig. 4B). Perifosine treatment of cells before addition of insulin, which prevented activation of Akt, did not inhibit D-3 PtdIns phosphate generation, while wortmannin (20 μ M) totally inhibited production of D-3 PtdIns phosphate (in the same Fig. 4B). Perifosine did not inhibit PDK1, ILK, and Src kinases (data not shown).

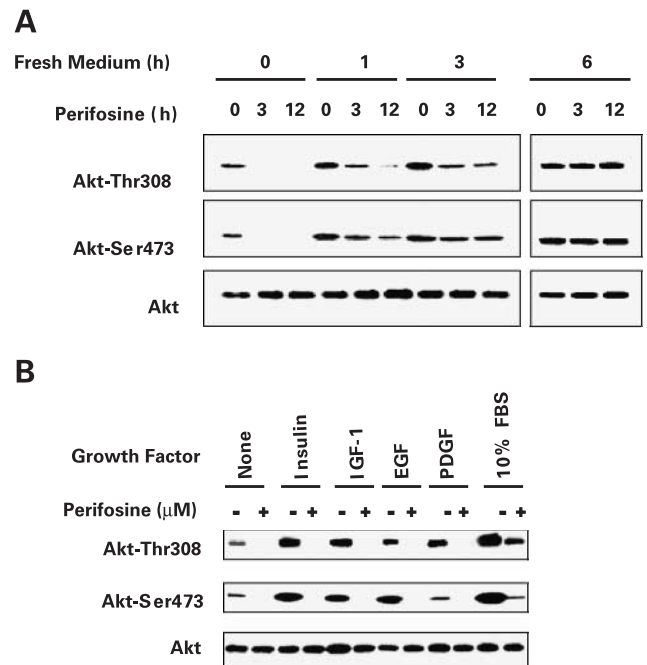


Figure 3. Perifosine-induced inhibition of Akt phosphorylation is reversible and affects the action of several growth factors. A, PC-3 cells were treated with 5- μ M perifosine for 0, 3, and 12 h. At the end of each treatment, cells were washed and further grown in fresh medium. Total protein isolated was separated by SDS-PAGE and analyzed by immunoblotting with indicated antibodies. B, exponentially growing PC-3 cells were serum starved for 20–24 h. Then, the cells were treated with 5- μ M perifosine for 30 min and induced with indicated growth factors for an additional 10 min. The growth factors were insulin (50 nM), IGF-I (50 ng/ml), EGF (50 ng/ml), PDGF (10 ng/ml), and 10% FBS. At the completion of experiment, cell lysates were prepared and analyzed by immunoblotting with respective antibodies. Results are representative of three different experiments.

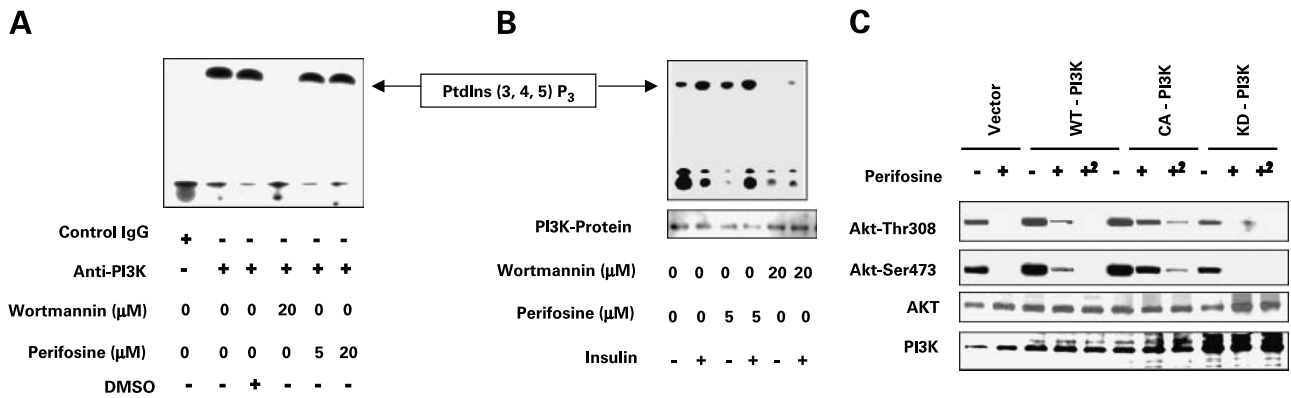


Figure 4. Relation of PI3K to perifosine action. **A**, *in vitro* PI3K enzymatic activity was measured using immunoprecipitated PI3K as an enzyme of PC-3 cells. Serum-starved (24 h) cells were stimulated with insulin (50 nM) for 15 min. The protein isolated was immunoprecipitated with anti-PI3K-p85- α subunit as described in detail in "Materials and Methods." The lipid products were fractionated and resolved by TLC followed by autoradiography. **B**, exponentially growing PC-3 cells were starved and treated independently with perifosine (5 μM) and wortmannin (20 μM), respectively, for 30 min followed by induction without (control) or with insulin (100 nM) for another 15 min. After the completion of the experiment, cells were lysed and immunoprecipitated with anti-PI3K-p85- α antibody to isolate the PI3K protein for measuring its enzymatic activity. **C**, subconfluent PC-3 cells were transfected (1 $\mu\text{g}/\text{plate}$) with WT-PI3K γ , CA-PI3K γ , and KD-PI3K γ constructs, respectively. At 24 h post-transfection, respective plates were treated without (control) or with 5 and 10 μM perifosine for 30 min. Lysates were subjected to immunoblotting with specific antibodies. Results were repeated at least thrice.

Although growth factor-mediated signaling leading to PI3K activation is not affected by perifosine, but Akt phosphorylation clearly is diminished (Fig. 2), we hypothesized that perifosine may interfere with recruitment of Akt or PDK1 to the cell membrane. Increased *in situ* generation of D-3 PtdIns phosphate might therefore overcome perifosine action. We approached this question by transfecting the PI3K catalytic subunit (PI3K-p110 α cDNA) of the holoenzyme. When PC-3 cells are transfected with the CA-PI3K-p110 α (CA-PI3K), partial protection from the effect of 5- μM perifosine was observed (Fig. 4C), but the effect of 10- μM perifosine on Akt phosphorylation at both Thr308 and Ser473 was equivalent to the effect of 5 μM on the WT enzyme. Under the same conditions, KD-PI3K-p110 α (KD-PI3K) has no influence on perifosine action on Akt phosphorylation, serving as a negative control. These results therefore suggest that perifosine might affect normal recruitment of molecules binding D-3 PtdIns phosphates, including Akt.

A CA Form of Akt Overcomes Perifosine Inhibition

We next asked whether forced overexpression of exogenous Akt could abrogate the effect of perifosine on Akt activating phosphorylations. Fig. 5A presents results from transiently overexpressing different forms of Akt. Only MYR-Akt completely overcomes perifosine inhibition of Akt phosphorylation. Interestingly, in the same setting, WT-Akt overexpression failed to overcome 5- μM perifosine inhibition of Akt regulatory phosphorylation.

As we previously (Fig. 4C) had consistently noticed that CA forms of PI3K could also overcome perifosine inhibition, we cotransfected WT PI3K, PDK1, and Akt constructs together rather than the CA construct forms. Fig. 5B demonstrates that only WT-PI3K-p110 α and WT-Akt in combination could overcome the perifosine action on regulatory Akt phosphorylations. Any other combination has no significant effect on perifosine

inhibition action on Akt phosphorylation. These data are consistent with the idea that *in situ* generation of sufficient D-3 PtdIns phosphates with an abundance of Akt can overcome the perifosine inhibition of Akt phosphorylation.

Perifosine Markedly Alters WT-Akt and Also Akt-PH Domain Membrane Translocation

If it is true that perifosine alters the membrane recruitment of Akt to allow juxtaposition to regulatory "upstream" kinases such as PDK1, altered capacity of Akt to associate with membranes of perifosine-treated cells might be evident. We therefore transiently expressed different forms of HA-tagged Akt1 (HA-Akt1) construct in PC-3 cells and examined the changes of its subcellular localization with immunofluorescence imaging. Fig. 6A demonstrates clearly that in non-insulin-treated cells, both WT-HA-Akt1 and KD-HA-Akt1 are localized to the cytoplasm, while MYR-HA-Akt1 is distributed to the membrane. After insulin treatment, WT-HA-Akt and KD-HA-K179A-Akt1 as well as MYR-HA-Akt1 localize to the membrane. Perifosine abolishes both WT-HA-Akt and KD-HA-K179A-Akt1 membrane localization but failed to inhibit the MYR-HA-Akt1 membrane localization (Fig. 6A).

We next examined perifosine effect on the intracellular localization of pCEFL-EGFP-AH-Akt1 plasmid. pCEFL-EGFP-AH-Akt1 plasmid, in which GFP is fused to AH domain of the WT form of amino-terminal regulatory portion of Akt1. This amino-terminal regulatory region includes the PH domain. The pCEFL-EGFP-AH-Akt1 plasmid construct was transiently expressed in PC-3 cells and then stimulated with insulin in the presence of perifosine. Serum-starved PC-3 cells containing pCEFL-EGFP-AH-Akt1 plasmid on insulin stimulation translocated to plasma membrane very rapidly (Fig. 6B), whereas perifosine treatment abolished this effect, with GFP mainly remaining in the cytosol even with insulin stimulation.

These results confirm that inhibition of membrane localization of the PH domain of Akt can be targeted by perifosine, accounting for interference by the drug with the normal membrane localizing activity of Akt.

Perifosine Up-Regulation of p21^{WAF1/CIP1} Is Opposed by MYR-Akt1

As recent studies have shown that cell cycle arrest on exposure of HN12 cells to perifosine was associated with increase of endogenous p21^{WAF1/CIP1} (18), we asked whether forced membrane localization of Akt altered the capacity of perifosine to induce p21. Fig. 7A indicates that, consistent with prior studies (16), 5- μ M perifosine increases p21^{WAF1/CIP1} after 3 h and reaches maximum effect by 6 h, while p27^{KIP1} did not change through the course of treatment. Transfection of MYR-Akt1 overcame the effect

of perifosine on Akt phosphorylation, as there was no increase of p21^{WAF1/CIP1} expression in presence of perifosine (Fig. 7B). In contrast, WT-Akt1 and KD-K179A-Akt1 still demonstrated increase in p21^{WAF1/CIP1}.

Discussion

The experiments presented in this paper demonstrate that perifosine, an alkylphospholipid, causes inhibition of PC-3 prostate carcinoma cell growth (GI₅₀ by 5 μ M in 24 h) associated with rapidly decreased Akt activation, as assessed by Thr308 and Ser473 phosphorylation, and assay of enzymatic activity. Evidence is presented that perifosine inhibits recruitment of Akt to the cell membrane but without significant inhibition of PI3K as well as PDK1, ILK, and Src (experiments not described in detail). Perifosine-mediated inhibition of Akt phosphorylation is substantially relieved by introduction of MYR-Akt, which bypasses the requirement for PH domain-mediated membrane recruitment, particularly in the context of coexpression of the PI3K catalytic subunit. Further evidence for the functional importance of Akt in perifosine-mediated signaling is the abrogation of perifosine-induced increase in p21^{WAF1/CIP1} previously associated in a causal sense with perifosine-mediated cell cycle arrest (18) by concomitant overexpression of CA-PI3K and MYR-Akt. Taken together, these experiments point to the Akt pathway as a prominent participant in the cellular effects of perifosine.

Perifosine is structurally related to miltefosine (46), approved for use in Europe as a topical treatment for cutaneous neoplasms. The latter compound's limited p.o. bioavailability and tendency to produce hemolysis has prevented miltefosine's further evaluation for cancer treatment, but even limited p.o. bioavailability apparently allows sufficient activity on the part of miltefosine in the treatment of visceral leishmania infection (7).

Numerous mechanisms have been proposed to explain the antiproliferative activity of miltefosine, including inhibition of protein kinase C, alteration of phospholipase activity, and immune mechanisms (47). Likewise, other phospholipid and ether lipid analogues have been studied in recent years (15, 48), and similar pleiotropy of potential mechanisms for inhibiting cellular proliferation has been documented. For example, edelfosine inhibits G protein-mediated phospholipase C activation (49) as well as affect metabolism of membrane lipid turnover. In the case of perifosine, previous studies (18) had documented that cell cycle arrest after exposure to the drug correlated with arrest of cells in G₂ and G₁, with loss of the S-phase compartment. This in turn correlated with the p53-independent accumulation of p21^{WAF1/CIP1}. In fact, cells from p21^{WAF1/CIP1}^{-/-} animals did not display this response to drug.

We therefore were encouraged to consider signal transduction pathways, which might affect cell cycle progression, mediate cell survival, and could be modulated by lipid-related metabolites structurally similar to perifosine. As PI3K/PDK1/Akt signaling clearly can mediate nutritional state and growth factor-related effects on cell

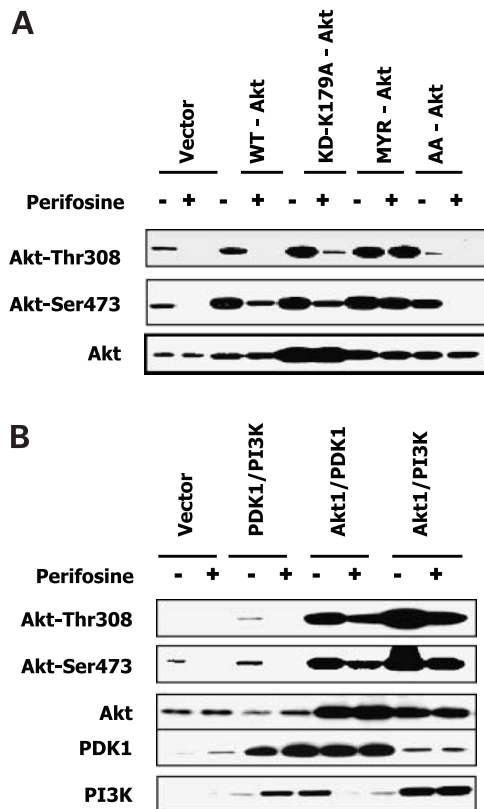


Figure 5. Overexpression of activated Akt and cotransfection of WT Akt/PDK1, Akt/PI3K, and PDK1/PI3K transfection on perifosine action. Subconfluent PC-3 cells were transfected (1 μ g/plate) with cDNA each using LipofectAMINE 2000 as per manufacturer's instruction. **A**, cells were transfected with different forms of Akt construct represented as WT-Akt1 (WT), K179A-Akt1 (KD), MYR-Akt1 (CA), AA-Akt1 (Ser308 and Thr473 are mutated to alanine), and vector alone, respectively. **B**, effect of perifosine on Akt phosphorylation on cotransfection of WT PDK1/PI3K γ , Akt1/PDK1, and Akt1/PI3K γ . Subconfluent PC-3 cells were transfected with a total of 1 μ g/plate (equal amount of each) of cDNA. In all cases, 24 h post-transfected cells were treated with (5 μ M) or without (control) perifosine for 30 min; then, the cells were lysed and equal amounts of protein were subjected to SDS-PAGE followed by immunoblotting with specific antibodies. Data are representative of three or more independent experiments.

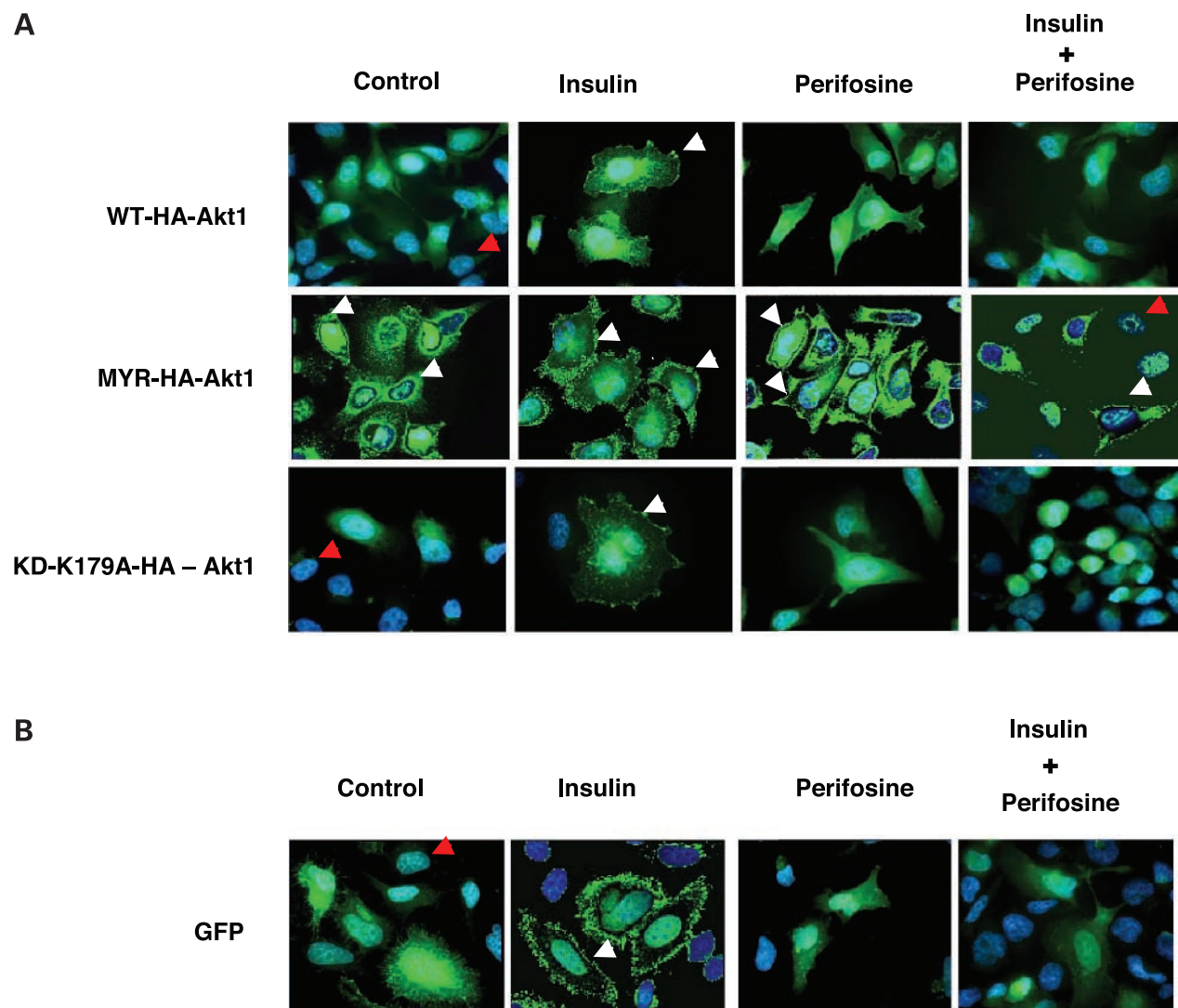


Figure 6. Perifosine affects membrane distribution of HA-Akt1 and Akt1-PH domain constructs. **A**, at 24 h post-transfection with the indicated construct, cells were starved for another 24 h and then treated with perifosine (5 μ M) for 30 min followed by insulin (50 nM) stimulation for another 5 min. The cells were blocked and probed with FITC-conjugated HA as described in "Materials and Methods." The cells were observed under 63 \times magnification using Olympus microscope. *Red arrows*, nontransfected cells and nuclear staining with 4',6-diamidino-2-phenylindole. *White arrowheads*, membrane localization of Akt. **B**, subconfluent PC-3 cells grown on coverslips were transfected with pCEFL-EGFP-AH-Akt1. At 24 h post-transfection, cells were starved for another 24 h and then treated with perifosine (5.0 μ M) for 30 min followed by induction with insulin (50 nM) for another 5 min. The cells were fixed and slides were prepared as described in "Materials and Methods." GFP immunofluorescence images were monitored on PC-3 cells transfected with pCEFL-EGFP-AH-Akt1. The *green fluorescence* was observed under 63 \times magnification.

proliferation, consideration of this pathway as a potential new target for perifosine action led to the experiments reported here. Quite strikingly, perifosine rapidly down-modulates signaling through Akt and does so in a way that to our knowledge has not been described previously, most probably by decreasing the association of PH domain-containing Akt with the plasma membrane, thus leading to its functional inactivation. As perifosine is a variant phospholipid, one hypothesis is that perifosine might actually interfere with the ability of PH domains to interact with cell membranes. This would be consistent with a mechanism of drug action where perifosine itself physically

occupied the PH domain or interfered with the proper combination of natural D-3 PtdIns phosphates with PH domains. Further experiments must address the question directly with purified components. Our results are concurrent with those of Ruiter *et al.* (16) who presented while this manuscript was in preparation experiments demonstrating that edelfosine, miltefosine, and perifosine could all inhibit Akt Ser473 phosphorylation, with activation of stress-activated protein kinase/c-Jun N-terminal kinase activity. Future experiments must clarify the contribution of Akt inhibition, influence of PTEN status, and activation of PI3K activity to perifosine-induced growth inhibition.

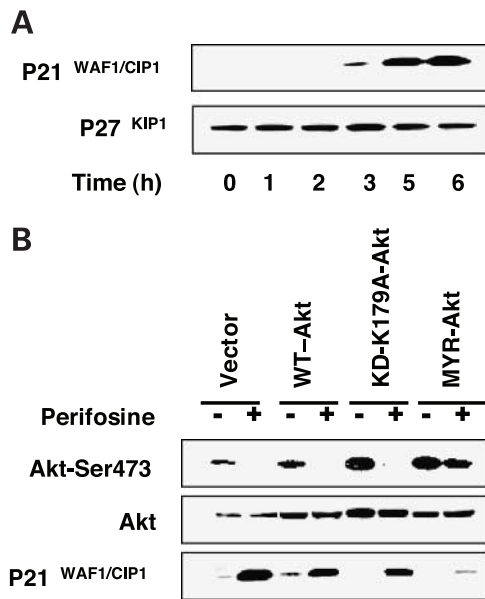


Figure 7. MYR-Akt1 expression abrogates perifosine-mediated up-regulation of p21^{WAF1/CIP1}. **A**, PC-3 cells were grown in 60-mm dishes overnight and treated with 5- μ M perifosine for different time points up to 6 h. The cells were washed with PBS and lysed for protein extraction. Total protein was estimated, separated on SDS-PAGE, and immunoblotted for p21^{WAF1/CIP1} and p27^{KIP1}. **B**, PC-3 cells grown in 60-mm dishes were transfected with different forms of Akt1 constructs [WT, MYR, and KD (K179A)] using LipofectAMINE 2000 as per manufacturer's instructions. Post-transfected cells were treated with perifosine (5 μ M) for 6 h. The protein isolated was immunoblotted for Akt and p21^{WAF1/CIP1}.

The mechanism for activation of Akt is well characterized at a molecular level. PDK1 is one kinase immediately upstream of Akt and phosphorylates Thr308. Several findings support the theory that phosphatidylinositol 3,4,5-trisphosphate binding is important for membrane localization of PDK1 and subsequently facilitates its kinase activity (50, 51). Measurements of commercially activated PDK1 activity in the presence of perifosine strongly suggest that it has no influence on PDK1 phosphotransferase activity (data not shown). Perifosine-treated cells from which PDK1 is subsequently immunoprecipitated also show no alteration of enzymatic activity (data not shown).

Akt inactivation is selective, because PDK1 along with ILK (data not shown), the immediate upstream kinases, remain unchanged in the presence of perifosine. The reversal of PC-3 cell growth inhibition on withdrawal of perifosine follows reversal of the inhibition of Akt phosphorylation. This event also suggests that perifosine is not acting simply as a detergent, as commitment to cell death is to a point reversible in PC-3 cells. Because reduction in Akt phosphorylation occurs very early (within 30 min at the GI₅₀ for cell growth at 24 h), this effect likely does not result from induction of a cell cycle arrested state but instead could conceivably cause growth arrest as a consequence of these effects.

The molecular basis for inhibition of Akt activation remains to be established. One hypothesis is that following

insertion into the cell membrane, perifosine inhibits the normal association of PH domain-containing proteins with the 3' phosphorylated products of PI3K action. Indeed, immunofluorescence imaging supported this interpretation of the transfection experiments, as WT-Akt transfectant shows hampered membrane localization after perifosine treatment despite insulin stimulation. The finding that perifosine does not affect PDK1 activity but does alter Akt localization is concordant with the observations of others (52, 53) that the affinity of the PH domain of PDK1 is much greater than that of Akt for lipid-linked membrane-associating moieties. Our immunofluorescence imaging observation is also in agreement with the observation that the membrane-targeted MYR-Akt form efficiently overcomes perifosine inhibition.

Recently, Patel *et al.* documented that perifosine promotes cell cycle arrest at either G₁-S or G₂-M with concomitant up-regulation of p21^{WAF1/CIP1} in a p53-independent manner (18). Our results here extend those findings by demonstrating that overexpression of MYR-Akt not only overcome perifosine inhibition on Akt phosphorylation but also cause inhibition of perifosine-induced p21^{WAF1/CIP1} expression in PC-3 cells. This finding suggest a mechanistic link between Akt signaling and regulation of p21^{WAF1/CIP1}, which must be defined more clearly in future experiments, underscoring the potential for perifosine in affecting an important pathway in cell growth regulation through its effects on Akt. In this context, it has recently been suggested that the Akt survival pathway modulates p21^{WAF1/CIP1} through phosphorylation of p21^{WAF1/CIP1} on residues Thr145 and Ser146 (54–56). This phosphorylation has been linked to altered subcellular localization (54), regulation of the binding of proliferating cell nuclear antigen binding and complexation of cyclin/cyclin-dependent kinases (55), and p21^{WAF1/CIP1} stability. Perifosine clearly has the potential to modulate each of these processes.

In summary, we have demonstrated here that perifosine inhibits Akt phosphorylation and activation in cells growing exponentially in serum-containing medium and after growth factor stimulation. Direct inhibition of the phosphotransferase activity of the kinases known to regulate Akt could not be demonstrated, but interference with Akt plasma membrane localization and membrane localization of Akt suggests an as yet undescribed mechanism for this aspect of the drug's action: interference with the proper membrane localization of target proteins for their phosphorylation and activation. Biochemical data that support this mechanism are presented, as forced overexpression of WT-PI3K in combination with WT-Akt opposes perifosine-induced inactivation of Akt and overexpression of MYR-Akt greatly augments this effect along with relief of downstream indicators of drug effect such as induction of p21^{WAF1/CIP1}. Thus, we have highlighted Akt, a multifunctional kinase, the constitutive activation of which is associated with resistance to cell death and tumorigenesis, as an important target of perifosine. Further exploitation of these findings in the design of novel agents may lead to improved approaches to cancer treatment.

Acknowledgments

We thank Dr. Silvio J. Gutkind (National Institute of Dental Research, NIH) for providing the different forms of Akt, PI3K, and p85- α expression vector plasmids. We also thank Drs. Sunhong Kim and Jongkyeong Chung (Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Republic of Korea) for the kind gift of different PDK1 expression plasmids.

References

- Unger, C., Damenz, W., Flee, E. A., Kim, D. J., Breiser, A., Hilgard, P., Engel, J., Nagel, G., and Eibl, H. Hexadecylphosphocholine, a new ether lipid analogue. Studies on the antineoplastic activity *in vitro* and *in vivo*. *Acta Oncol.*, **28**: 213–217, 1989.
- Berdel, W. E., Bausert, W. R., Fink, U., Rastetter, J., and Munder, P. G. Anti-tumor action of alkyl-lysophospholipids (Review). *Anticancer Res.*, **1**: 345–352, 1981.
- Principe, P., Sidoti, C., Coulomb, H., Broquet, C., and Braquet, P. Tumor cell kinetics following long-term treatment with antineoplastic ether phospholipids. *Cancer Detect. Prev.*, **18**: 393–400, 1994.
- Leonard, R., Hardy, J., van Tienhoven, G., Houston, S., Simmonds, P., David, M., and Mansi, J. Randomized, double-blind, placebo-controlled, multicenter trial of 6% mitofosine solution, a topical chemotherapy in cutaneous metastases from breast cancer. *J. Clin. Oncol.*, **19**: 4150–4159, 2001.
- Terwogt, J. M., Mandjes, I. A., Sindermann, H., Beijnen, J. H., and ten Bokkel Huinink, W. W. Phase II trial of topically applied mitofosine solution in patients with skin-metastasized breast cancer. *Br. J. Cancer*, **79**: 1158–1161, 1999.
- Smorenburg, C. H., Seynaeve, C., Bontenbal, M., Planting, A. S., Sindermann, H., and Verweij, J. Phase II study of mitofosine 6% solution as topical treatment of skin metastases in breast cancer patients. *Anticancer Drugs*, **11**: 825–828, 2000.
- Jha, T. K., Sundar, S., Thakur, C. P., Bachmann, P., Karbwang, J., Fischer, C., Voss, A., and Berman, J. Mitofosine, an oral agent, for the treatment of Indian visceral leishmaniasis. *N. Engl. J. Med.*, **341**: 1795–1800, 1999.
- Eibl, H. Mitofosine for visceral leishmaniasis. *N. Engl. J. Med.*, **342**: 894–895, 2000.
- Hilgard, P., Klenner, T., Stekar, J., Nossner, G., Kutscher, B., and Engel, J. D-21266, a new heterocyclic alkylphospholipid with antitumor activity. *Eur. J. Cancer*, **33**: 442–446, 1997.
- Woo, E. W., Messmann, R., Sausville, E. A., and Figg, W. D. Quantitative determination of perifosine, a novel alkylphosphocholine anticancer agent, in human plasma by reversed-phase liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. B Biomed. Sci. Appl.*, **759**: 247–257, 2001.
- Arthur, G. and Bittman, R. The inhibition of cell signaling pathways by antitumor ether lipids. *Biochim. Biophys. Acta*, **1390**: 85–102, 1998.
- Maly, K., Uberall, F., Schubert, C., Kindler, E., Stekar, J., Brachwitz, H., and Grunicke, H. H. Interference of new alkylphospholipid analogues with mitogenic signal transduction. *Anticancer Drug Des.*, **10**: 411–425, 1995.
- Brachwitz, H. and Vollgraf, C. Analogues of alkyllysophospholipids: chemistry, effects on the molecular level and their consequences for normal and malignant cells. *Pharmacol. Ther.*, **66**: 39–82, 1995.
- Kley, J. T., Unger, C., and Massing, U. Inhibition of 14-kDa PLA2 by 2-acylamino-alkylphospholipids: the influence of amide acidity. *Biochim. Biophys. Acta*, **1392**: 193–201, 1998.
- Berkovic, D. Cytotoxic etherphospholipid analogues. *Gen. Pharmacol.*, **31**: 511–517, 1998.
- Ruiter, G. A., Zerp, S. F., Bartelink, H., Van Blitterswijk, W. J., and Verheij, M. Anti-cancer alkyl-lysophospholipids inhibit the phosphatidylinositol 3-kinase-Akt/PKB survival pathway. *Anticancer Drugs*, **14**: 167–173, 2003.
- Leroy, A., de Bruyne, G. K., Oomen, L. C., and Mareel, M. M. Alkylphospholipids reversibly open epithelial tight junctions. *Anticancer Res.*, **23**: 27–32, 2003.
- Patel, V., Lahusen, T., Sy, T., Sausville, E. A., Gutkind, J. S., and Senderowicz, A. M. Perifosine, a novel alkylphospholipid, induces p21(WAF1) expression in squamous carcinoma cells through a p53-independent pathway, leading to loss in cyclin-dependent kinase activity and cell cycle arrest. *Cancer Res.*, **62**: 1401–1409, 2002.
- Okumura, E., Fukuhara, T., Yoshida, H., Hanada Si, S., Kozutsumi, R., Mori, M., Tachibana, K., and Kishimoto, T. Akt inhibits Myt1 in the signaling pathway that leads to meiotic G₂-M-phase transition. *Nat. Cell Biol.*, **4**: 111–116, 2002.
- Burging, B. M. and Kops, G. J. Cell cycle and death control: long live Forkheads. *Trends Biochem. Sci.*, **27**: 352–360, 2002.
- Falck, J., Mailand, N., Syljuasen, R. G., Bartek, J., and Lukas, J. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature*, **410**: 842–847, 2001.
- Brennan, P., Mehl, A. M., Jones, M., and Rowe, M. Phosphatidylinositol 3-kinase is essential for the proliferation of lymphoblastoid cells. *Oncogene*, **21**: 1263–1271, 2002.
- Vivanco, I. and Sawyers, C. L. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat. Rev. Cancer*, **2**: 489–501, 2002.
- Shayesteh, L., Lu, Y., Kuo, W. L., Baldocchi, R., Godfrey, T., Collins, C., Pincel, D., Powell, B., Mills, G. B., and Gray, J. W. PIK3CA is implicated as an oncogene in ovarian cancer. *Nat. Genet.*, **21**: 99–102, 1999.
- Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., et al. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int. J. Cancer*, **64**: 280–285, 1995.
- Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K., and Testa, J. R. Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc. Natl. Acad. Sci. USA*, **93**: 3636–3641, 1996.
- Ruggeri, B. A., Huang, L., Wood, M., Cheng, J. Q., and Testa, J. R. Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas. *Mol. Carcinog.*, **21**: 81–86, 1998.
- Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., Said, J. W., Isaacs, W. B., and Sawyers, C. L. Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc. Natl. Acad. Sci. USA*, **95**: 5246–5250, 1998.
- Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tschlis, P. N. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*, **81**: 727–736, 1995.
- Toker, A. and Cantley, L. C. Signaling through the lipid products of phosphoinositide-3-OH kinase. *Nature*, **387**: 673–676, 1997.
- Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science*, **277**: 567–570, 1997.
- Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tschlis, P. Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene*, **17**: 313–325, 1998.
- Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. Role of translocation in the activation and function of protein kinase B. *J. Biol. Chem.*, **272**: 31515–31524, 1997.
- Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.*, **15**: 6541–6551, 1996.
- Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B α . *Curr. Biol.*, **7**: 261–269, 1997.
- Di Cristofano, A. and Pandolfi, P. P. The multiple roles of PTEN in tumor suppression. *Cell*, **100**: 387–390, 2000.
- Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaris, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, **275**: 1943–1947, 1997.
- Staal, S. P. Molecular cloning of the *akt* oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc. Natl. Acad. Sci. USA*, **84**: 5034–5037, 1987.
- McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. R. Loss of PTEN expression in paraffin-embedded primary

- prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res.*, **59**: 4291–4296, 1999.
40. Murga, C., Fukuhara, S., and Gutkind, J. S. A novel role for phosphatidylinositol 3-kinase β in signaling from G protein-coupled receptors to Akt. *J. Biol. Chem.*, **275**: 12069–12073, 2000.
41. Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A., and Gutkind, J. S. Activation of Akt/protein kinase B by G protein-coupled receptors. A role for α and β γ subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinase. *J. Biol. Chem.*, **273**: 19080–19085, 1998.
42. Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J. T., Leung, D., Yan, J., Sanghera, J., Walsh, M. P., and Dedhar, S. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. *J. Biol. Chem.*, **276**: 27462–27469, 2001.
43. Montaner, S., Sodhi, A., Pece, S., Mesri, E. A., and Gutkind, J. S. The Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor promotes endothelial cell survival through the activation of Akt/protein kinase B. *Cancer Res.*, **61**: 2641–2648, 2001.
44. Bondeva, T., Pirola, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R., and Wymann, M. P. Bifurcation of lipid and protein kinase signals of PI3K γ to the protein kinases PKB and MAPK. *Science*, **282**: 293–296, 1998.
45. Leopoldt, D., Hanck, T., Exner, T., Maier, U., Wetzker, R., and Nurnberg, B. G β γ stimulates phosphoinositide 3-kinase- γ by direct interaction with two domains of the catalytic p110 subunit. *J. Biol. Chem.*, **273**: 7024–7029, 1998.
46. Zeisig, R., Arndt, D., Stahn, R., and Fichtner, I. Physical properties and pharmacological activity *in vitro* and *in vivo* of optimized liposomes prepared from a new cancerostatic alkylphospholipid. *Biochim. Biophys. Acta*, **1414**: 238–248, 1998.
47. Hilgard, P., Klenner, T., Stekar, J., and Unger, C. Alkylphosphocholines: a new class of membrane-active anticancer agents. *Cancer Chemother. Pharmacol.*, **32**: 90–95, 1993.
48. Mareel, M., Bracke, M., Bruyneel, E., Van Larebeke, N., and De Mets, M. Invasion and metastasis control: implications for increased therapeutic index of antitumor drugs. *Cancer Treat. Rev.*, **17**: 335–338, 1990.
49. Strassheim, D., Shafer, S. H., Phelps, S. H., and Williams, C. L. Small cell lung carcinoma exhibits greater phospholipase C- β 1 expression and edelfosine resistance compared with non-small cell lung carcinoma. *Cancer Res.*, **60**: 2730–2736, 2000.
50. Vanhaesebroeck, B. and Alessi, D. R. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.*, **346** (Pt 3): 561–576, 2000.
51. Belham, C., Wu, S., and Avruch, J. Intracellular signaling: PDK1—a kinase at the hub of things. *Curr. Biol.*, **9**: R93–R96, 1999.
52. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.*, **68**: 965–1014, 1999.
53. Lemmon, M. A., Ferguson, K. M., and Abrams, C. S. Pleckstrin homology domains and the cytoskeleton. *FEBS Lett.*, **513**: 71–76, 2002.
54. Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., and Hung, M. C. Cytoplasmic localization of p21^{Cip1/WAF1} by Akt-induced phosphorylation in HER-2/*neu*-overexpressing cells. *Nat. Cell Biol.*, **3**: 245–252, 2001.
55. Rossig, L., Jadidi, A. S., Urbich, C., Badorff, C., Zeiher, A. M., and Dimmeler, S. Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. *Mol. Cell. Biol.*, **21**: 5644–5657, 2001.
56. Li, Y., Dowbenko, D., and Lasky, L. A. AKT/PKB phosphorylation of p21^{Cip1/WAF1} enhances protein stability of p21^{Cip1/WAF1} and promotes cell survival. *J. Biol. Chem.*, **277**: 11352–11361, 2002.