

# Synergistic induction of apoptosis in human leukemia T cells by the Akt inhibitor perifosine and etoposide through activation of intrinsic and Fas-mediated extrinsic cell death pathways

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## Abstract

Perifosine is an Akt inhibitor displaying strong antineoplastic effects in human tumor cell lines and is currently being tested in phase II clinical trials for treatment of major human cancers. Several recent studies showed the apoptotic effect of perifosine alone or in combination with other anticancer agents. However, this is the first study describing the effects of combining perifosine with the commonly used chemotherapy drug etoposide in cultured human Jurkat T-leukemia cells. Low concentrations of perifosine (5  $\mu\text{mol/L}$ ) induced cell death in a synergistic fashion with etoposide if used simultaneously or immediately following exposure to etoposide (posttreatment). The increase in cell death seems to be due to an inactivation of the Akt survival pathway, where treated cells showed a complete dephosphorylation of Akt. Moreover, combined drug-induced Akt deactivation was associated with a parallel decrease in phosphorylation of FoxO1 transcription factor and in expression of antiapoptotic Bcl-xL. Furthermore, the increase in cell death was associated with a specific activation of the caspase-dependent Fas death receptor pathway. These findings might be useful when designing clinical trials where

chemotherapy is combined with perifosine for a potential broad use against hematologic malignancies in which the Akt survival pathway is frequently activated. [Mol Cancer Ther 2006;5(6):1559–70]

## Introduction

The phosphatidylinositol-3 kinase (PI3K)/Akt network is considered one of the fundamental signaling pathways in the regulation of cell cycle progression, proliferation, and apoptosis. In the last years, ample amount of evidence has shown that the constitutive activation of the PI3K/Akt axis is associated with a number of malignant diseases (1, 2). Upon activation by different growth factors and cytokines, membrane localization of PI3K generates phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P<sub>3</sub>; ref. 3], which in turn activates a number of important downstream substrates. The serine/threonine kinase Akt is a well-characterized target of PI3K, and binding of PtdIns(3,4,5)P<sub>3</sub> to the pleckstrin homology domain of Akt results in its translocation to the vicinity of the plasma membrane, where it subsequently undergoes phosphorylation within its catalytic loop at Thr<sup>308</sup> and COOH terminally at Ser<sup>473</sup> (3). The former phosphorylation step is effected by phosphoinositide-dependent kinase 1 (PDK-1), which is recruited to the proximity of the plasma membrane by PtdIns(3,4,5)P<sub>3</sub> (4, 5), whereas the latter is effected by a kinase not yet conclusively identified.

Activated Akt is known to function as an essential survival factor *in vitro* (4), where the antiapoptotic effects of Akt are accomplished by the phosphorylation of a number of targets, including the proapoptotic factors BAD and caspase-9, nuclear factor- $\kappa$ B (NF- $\kappa$ B), and forkhead (now FoxO) family members of transcription factors (2). A fundamental negative regulator of the PI3K pathway is the lipid phosphatase PTEN (6), which removes the 3-phosphate from PtdIns(3,4,5)P<sub>3</sub>, generating PtdIns(4,5)P<sub>2</sub>. Thus, loss of PTEN function results in accumulation of PtdIns(3,4,5)P<sub>3</sub> and increased signaling via the PI3K pathway, with an enhanced Akt activation as a consequence (7). The dysregulation of PTEN is often linked to malignant transformation, and PTEN is frequently found mutated or deleted in a substantial number of human cancers (8).

Phospholipids analogues compose a novel class of drugs showing proapoptotic activity in a number of cell lines (9–11). There are several clinically relevant phospholipid analogues, including edelfosine and miltefosine (12, 13). Perifosine is a new phospholipid analogue, with enhanced oral bioavailability, which has shown promising preclinical activity and is currently undergoing phase I and II clinical

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evaluation (14–16). Unlike many chemotherapeutic drugs that target the nuclear DNA, phospholipids analogues readily insert and accumulate in the outer leaflet of the plasma membrane, where they interfere with different membrane-related signal transduction events (17–20). Although the molecular mechanism underlying the antineoplastic activity of perifosine remains to be fully elucidated, several studies indicate that perifosine, via its interference with the turnover and synthesis of natural phospholipids, disrupt membrane-signaling network at several sites, resulting in the inhibition of PI3K/Akt survival pathway (20, 21), inhibition of the mitogen-activated protein kinase, and activation of the stress-activated protein kinase (SAPK)/c-Jun NH<sub>2</sub>-terminal kinase (JNK) pathway (11).

Recently, much attention has focused on multicomponent chemotherapy as a major strategy for overcoming drug resistance and improving response and cure rates. In general, agents with different targets of action are exploited in simultaneous or sequential combinations for possible biochemical synergism. We have investigated the effects of combined treatment with perifosine and the commonly used chemotherapeutic drug etoposide in human Jurkat T-leukemia cells. Etoposide is a DNA-damaging topoisomerase II targeting drug, which is commonly used for treatment of adult T-cell leukemia/lymphoma (ATL; refs. 22–24). Previous clinical reports have provided evidence that etoposide can be successfully used in polychemotherapy for treatment of ATL (25, 26).

We hypothesized that the inactivation of the PI3K/Akt pathway by perifosine may provide an opportunity to improve the response of PTEN-deficient Jurkat cells to etoposide-induced leukemic cell apoptosis. In this report, we present data that exposure to etoposide followed by a posttreatment with perifosine results in a highly synergistic action of the two agents in triggering leukemic cell death. These events seem to be related to perturbations in the Akt survival signaling pathway and activation of different proapoptotic pathways. Our work, therefore, suggests that a combination of etoposide and perifosine could represent a rational approach for efficient suppression of PI3K/Akt signaling in hematologic malignancies and advocates for their incorporation into the design of more effective anticancer treatment protocols.

## Materials and Methods

### Chemicals and Antibodies

Perifosine was provided by Zentaris GmbH (Frankfurt, Germany). Stocks (50 mmol/L) were prepared in Millipore H<sub>2</sub>O, and aliquots were stored at –20°C. Etoposide and UO126 were from Sigma-Aldrich (St. Louis, MO). Caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (LEHD-CHO) from Calbiochem (La Jolla, CA) were dissolved in DMSO as 1,000× stocks and stored at –20°C. For flow cytometry, propidium iodide (PI, DNA-Prep kit) was from Beckman Coulter Immunology (Miami, FL), whereas Annexin V staining kit was from Tau Technologies BV (Kattendijke, the Netherlands). For immunoblotting experi-

ments, the following rabbit polyclonals and reagents were from Cell Signaling Technology (Beverly, MA); Ser<sup>473</sup> phosphorylated Akt (p-Akt), Akt, extracellular signal-regulated kinase 1/2 (Erk1/2), p-Erk1/2, Ser<sup>241</sup> p-PDK, Ser<sup>9</sup> p-glycogen synthase kinase-3β (GSK-3β); p-FoxO1 (FKHR), p-SAPK/JNK, SAPK/JNK, β-actin, full-length and cleaved fragments of caspase-9, caspase-3, poly(ADP-ribose) polymerase, Bid, Bcl-xL, full-length and cleaved fragments of caspase-8 (a mouse monoclonal), horseradish peroxidase-conjugated anti-rabbit IgG, horseradish peroxidase-conjugated anti-mouse IgG, and the Phototope-Horseradish Peroxidase Western Blot Detection System. Finally, rabbit polyclonal to full length Bim was from Sigma-Aldrich; mouse monoclonal blocking antibodies anti-human Fas and anti-human Fas ligand were from Alexis Biochemicals (Lausen, Switzerland). Recombinant human tumor necrosis factor-related apoptosis-inducing ligand was from Calbiochem. The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide cell growth and viability kit I was from Roche Applied Science (Penzberg, Germany).

### Cell Culture

Jurkat acute human T-leukemia cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at an optimal cell density of 0.3 to 0.8 × 10<sup>6</sup> cells/mL. Cells were cultured in humidified atmosphere at 37°C and 5% CO<sub>2</sub>.

### Measurement of Cell Death and Apoptosis

Before induction of apoptosis, cells were plated with an initial cell number of 0.25 × 10<sup>6</sup>/mL in six-well plates in RPMI containing 10% fetal bovine serum and incubated 24 hours. The exponentially growing cells were exposed to indicated doses of the drugs, individually, in a combination of the two, or in sequential treatment for a total of 48 hours. Experiments involving treatment with chemotherapy always included control cells treated with an equal concentration of DMSO (≤0.1%). Untreated cells were also included as a controls. At designated time points, cells were collected by centrifugation, fixed, and permeabilized in 70% cold ethanol and incubated at –20°C overnight. Samples were washed with cold PBS and incubated with the DNA-binding dye PI (5 µg/mL) at room temperature 30 minutes before analysis. Apoptosis was also evaluated by the binding of Annexin V-FITC to phosphatidylserine exposed on the cell surface, according to the manufacturer's instruction. Moreover, a "supravital" PI staining assay was also done (exposure of PI to unfixed cells) together with Annexin V assay (27), to simultaneously detect living, apoptotic, and necrotic cells. Fluorescence resulting from FITC and PI was measured at 530 and 620 nm, respectively. All the samples were analyzed by EPICS XL flow cytometer (Beckman Coulter Immunology) equipped with dedicated software. Histograms were then analyzed with EXPO software.

### Combined Drug Effects Analysis

To characterize the interactions between perifosine and etoposide, the combination effect and a potential synergy

were evaluated from quantitative analysis of dose-effect relationships as described by Chou and Talalay (28). In this method, for every combination of two agents tested, dose-response curves are generated for each agent individually, and these data are used to analyze the results obtained from the combination treatment within the same experiment. For each perifosine/etoposide drug combination experiment, a combination index (CI) number was calculated using the formula:

$$CI = C_a/Cx_a + C_b/Cx_b,$$

where  $Cx_a$  and  $Cx_b$  are the concentrations of compound a and b alone, respectively, needed to achieve a given effect ( $x\%$ ) and  $C_a$  and  $C_b$  are the concentrations of perifosine and etoposide needed for the same effect ( $x\%$ ) when the drugs are combined. These concentrations were calculated for each experiment and for each combination experiment at a non-fixed ratio of perifosine and etoposide. A non-fixed ratio of the two drugs may better approximate what might occur in a patient treated with a constant dose of one of the drugs over a prolonged period of time. This method of analysis generally defines CI values of 0.9 to 1.1 as implying additivity, 0.3 to 0.9 as synergistic, <0.3 as strongly synergistic, whereas values >1.1 are considered to indicate antagonism.

#### Whole-Cell Extract Preparation and Immunoblotting Analysis

Cells were plated at an initial number of  $1.8 \times 10^6$  ( $0.3 \times 10^6$ /mL) and incubated 24 hours before drug treatment as described above. Cells were collected by centrifugation at 1,500 rpm for 5 minutes at 4°C and washed in PBS (4°C). Cell pellets were lysed by adding 200  $\mu$ L  $1 \times$  SDS sample buffer [62.5 mmol/L Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mmol/L DTT], incubated on ice for 10 minutes, sonicated 10 seconds, heated to 95°C for 5 minutes, cooled on ice, and centrifuged at 13,000 rpm for 10 minutes at 4°C. Protein supernatants were collected, and equal amounts of protein (60  $\mu$ g) were separated by SDS-PAGE (10% or 15% gels) and then electro-transferred to nitrocellulose membrane. Nonspecific antibody binding sites were blocked by incubation in blocking buffer [ $1 \times$  PBS, 0.1% Tween 20 (PBS/T) with 5% w/v nonfat dry milk] for 1 hour at room temperature. After PBS/T wash, membranes were incubated with primary antibodies overnight at 4°C and diluted according to the manufacturer's instructions in 10 mL PBS/T with 5% bovine serum albumin. After washes with PBS/T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted in blocking buffer for 1 hour at room temperature. Proteins were detected by incubating the membrane with LumiGLO detection reagent. The level of expression of different proteins was analyzed by using the public domain software Image J (a Java image processing program inspired by NIH Image for Macintosh). It can calculate area and pixel value statistics of user-defined selections. Briefly, it was done as follows: X-ray films were scanned and saved as 8-bit grayscale JPEG files.

The percentage of measurable pixels in the image was set (and highlighted in red) by using the adjust image threshold command. The number of square pixels in the section selected (the protein bands) was then counted by measuring the area in the binary or thresholded image.

#### Cell Viability Measured by 3-[4,5-Dimethylthiazol-2-yl]-2, 5-Diphenyltetrazolium Bromide Assay

Briefly, cells were plated in a 96-well plate ( $3 \times 10^4$  per well, 100  $\mu$ L cell suspension per well) and cultured overnight to allow for exponential growth of cells. Cells were then treated with etoposide and perifosine as described above, individually or sequentially for a total of 48 hours. At the end of the treatment, 10  $\mu$ L of  $1 \times$  3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide labeling reagent were added to each well and incubated for another 4 hours. The samples were solubilized with 100  $\mu$ L 10% SDS in 10 mmol/L HCl overnight at 37°C. The absorbance value of each well was read on a Minireader at 570 nm. Every sample was done in triplicate and in three independent experiments. The fraction of viable cells was calculated as follows: mean absorbance value treated cells / mean absorbance value control cells.

#### Statistical Evaluation

The data are shown as means  $\pm$  SD. Data were statistically analyzed by a Dunnett test after one-way ANOVA at a level of significance of  $P < 0.05$  versus control samples.

## Results

### Perifosine Inhibits the Akt Cell Survival Signaling Pathway in PTEN-Deficient Cancer Cells

Much evidence implicates that activation of the PI3K/Akt survival signaling pathway mediates resistance to chemotherapy-induced apoptosis. To address the role of Akt in conferring sensitivity to apoptosis, we initially exposed Jurkat cells to increasing concentrations of perifosine for 24 hours. Jurkat human T-leukemia cells express no detectable levels of PTEN protein because of genetic aberrations within the cell, and, as a consequence, they contain constitutively active Akt that is highly phosphorylated. The effect on cell death was analyzed by flow cytometry of PI-stained samples, and the percentage of apoptotic and necrotic cells was calculated and compared with untreated cells. The results in Fig. 1A show that perifosine induced a 50% death in Jurkat cells at a concentration of  $\sim 30 \mu$ mol/L ( $IC_{50}$ ) after 24 hours of exposure. The effect of perifosine on the Akt pathway was next determined by using an activation-specific (Ser<sup>473</sup> p-Akt) antibody in immunoblotting experiments. We also evaluated the phosphorylative state of the Akt-activating kinase (PDK-1) and a direct downstream Akt substrate whose phosphorylation is increased in response to Akt activation (GSK-3 $\beta$ ). Furthermore, activation of a member of the mitogen-activated protein kinase superfamily (Erk1/2) was also evaluated. Exposure to increasing concentrations of perifosine for 24 hours resulted in a marked decrease in Akt phosphorylation, a finding similar



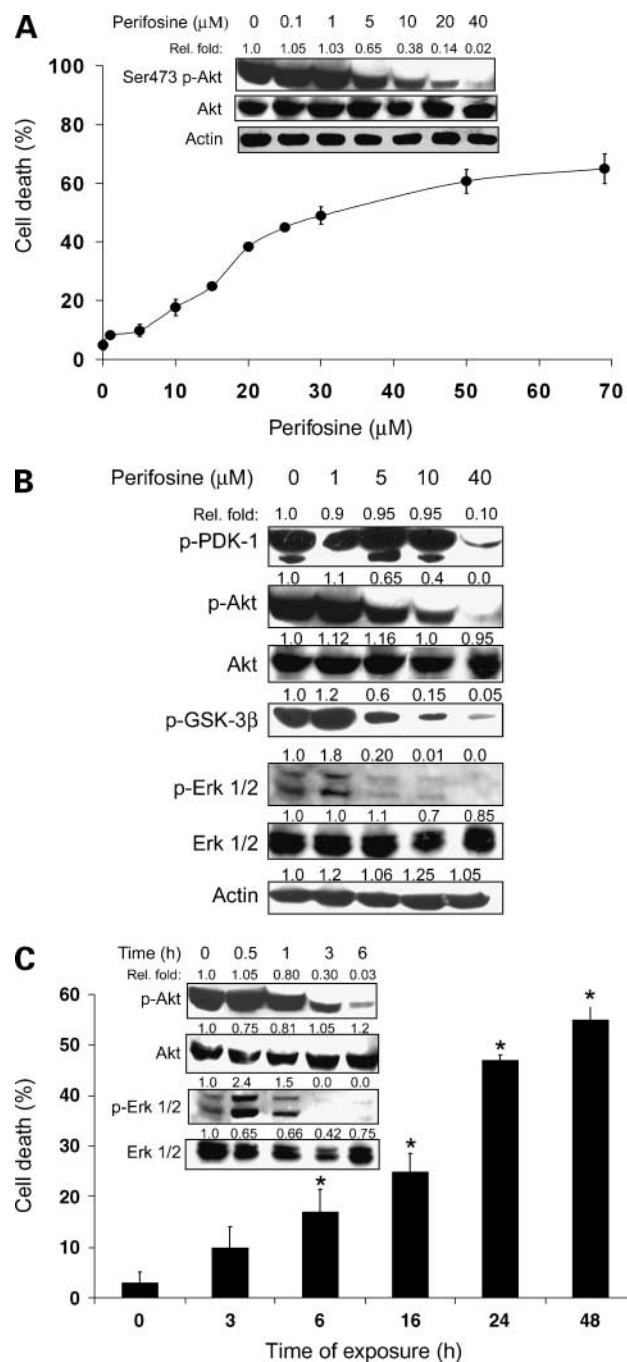
to what has been previously reported by others in different cell lines (20). As seen in *inset* in Fig. 1A, the immunoblot analysis of cells exposed to a subtoxic dose of 5  $\mu\text{mol/L}$  perifosine resulted in about 40% decrease in Akt phosphorylation, without affecting the amount of total Akt. Exposure to 40  $\mu\text{mol/L}$  perifosine, resulting in about 50% cell death, led to virtually a complete dephosphorylation of Akt, still without affecting total amount of Akt. The dephosphorylation of Akt in the presence of 5  $\mu\text{mol/L}$  perifosine seems to be functionally significant because a

40% decrease in the phosphorylation of GSK-3 $\beta$  was observed, while having no effect on the upstream kinase PDK-1 at a site necessary for its activity (Fig. 1B). The dose-response studies revealed that low concentrations of perifosine (1  $\mu\text{mol/L}$ ) moderately enhanced Erk1/2 phosphorylation, indicating an activation of the mitogen-activated protein kinase survival pathway, whereas higher concentrations of perifosine resulted in lower to abolished levels of p-Erk1/2, this without affecting the total levels of Erk1/2. A time course study revealed that at an early interval (<1 hour), exposure to perifosine resulted in an inactivation of Akt in parallel to a 2-fold activation of Erk1/2 (Fig. 1C, *inset*). After 3 hours of exposure to perifosine, about 70% of Akt was dephosphorylated, this even before the onset of extensive apoptosis was observed (Fig. 1C). Moreover, no Erk1/2 activation was detected after 3 hours of treatment. Total levels of Akt or Erk1/2 was mainly unaffected by perifosine treatment.

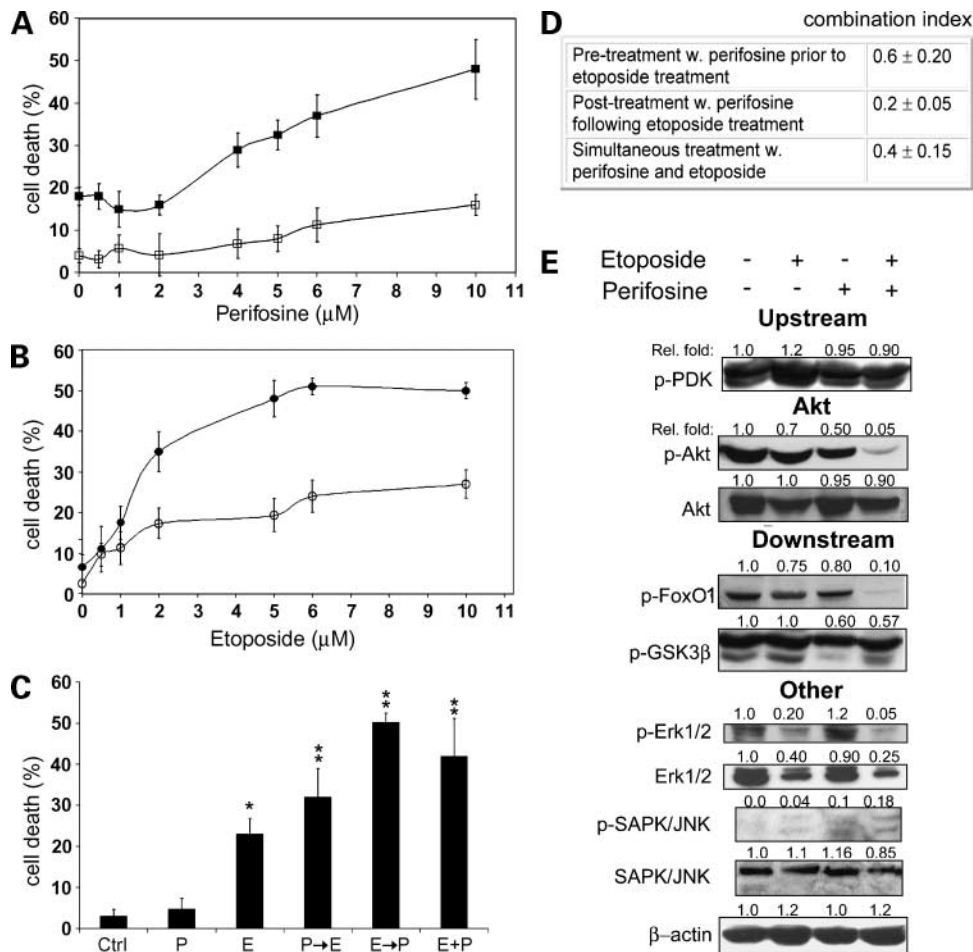
**Down-Regulation of Akt Activity Provides Sensitivity to Chemotherapy Drugs and Sequential Treatment Increases Induction Rate of Apoptosis**

Based on these results, we next explored the effect of inhibition of the Akt survival pathway in enhancing etoposide-induced apoptosis. A potential synergistic effect of perifosine and etoposide on cell death was evaluated in dose-response studies. The combination effect and synergy was evaluated from the multiple drug-effect analysis (28), where CI values were calculated as described in Materials and Methods. CI is considered as positive (synergistic) when  $CI < 0.85$  and negative (antagonistic) when  $CI > 1.1$ . Values close to 1 were considered to indicate an additive effect.

In Fig. 2, flow cytometry analysis of PI-stained samples showed that perifosine (Fig. 2A,  $\square$ ) and etoposide (Fig. 2B,  $\circ$ ) were minimally toxic during a 48-hour exposure, when given individually at concentrations ranging from 1 to 10  $\mu\text{mol/L}$ . The sequential exposure to perifosine before etoposide exposure (5  $\mu\text{mol/L}$ ) for a total of 48 hours



**Figure 1.** Effect of perifosine on Jurkat leukemic cell growth, and Akt phosphorylation and activity. **A**, exponentially growing Jurkat cells were exposed to increasing concentrations of perifosine for 24 h, after which the percentage of apoptotic/dead cells was determined by PI staining and flow cytometry analysis as described in Materials and Methods. *Inset*, immunoblot analysis of a similar set of samples treated as described above. Whole-cell extracts (60  $\mu\text{g}$  per lane) were probed with Ser<sup>473</sup> p-Akt and total Akt antibodies as described in Materials and Methods. Detection of  $\beta$ -actin was done to ensure equal loading. **B**, immunoblot analysis of Jurkat cells treated with the indicated perifosine concentrations for 24 h and analyzed for the expression of p-PDK-1, p-Akt, p-GSK-3 $\beta$ , and p-Erk1/2 as well as total Akt, Erk1/2, and actin. **C**, induction of cell death in Jurkat cells after exposure to 20  $\mu\text{mol/L}$  perifosine for 3 to 24 h analyzed by flow cytometry of PI-stained samples. *Columns*, mean from three experiments; *bars*, SD. \*,  $P < 0.01$ , samples show significant difference compared with untreated (control) cells. *Inset*, an immunoblot analysis of cells treated with 20  $\mu\text{mol/L}$  perifosine for the same interval as above, showing a decrease in p-Akt levels, accompanied by a temporary stimulation of Erk1/2 activity. Total Akt and Erk1/2 are shown as loading controls. Immunoblots were scanned, and band intensities were quantified by Image J (NIH) densitometry analysis as described in Materials and Methods. Band intensities of control was normalized to 1, and treated samples were expressed as fraction of control.



**Figure 2.** Synergistic induction of cell death by etoposide and perifosine in Jurkat leukemic cells results in a striking decrease in p-Akt and p-FoxO1 levels. **A**, Jurkat cells were exposed for 48 h to the indicated concentration of perifosine alone (□) or in sequential combination with 5 μmol/L etoposide added after 24 h of exposure to perifosine (■), after which the percentage of apoptotic/dead cells was evaluated by PI staining and flow cytometry analysis. **B**, exponentially growing Jurkat cells were exposed for 48 h to the indicated concentrations of etoposide alone (○) or in sequential combination with 5 μmol/L perifosine added after an initial 24 h exposure to etoposide (●), after which the percentage of dead cells was evaluated as described above. **C**, synergistic induction of cell death by sequential or simultaneous exposure to perifosine (5 μmol/L) and etoposide (5 μmol/L) for a total of 48 h. *Ctrl*, control; *P*, perifosine; *E*, etoposide. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ , significant difference compared with untreated (control) cells. **D**, CI with sequential or simultaneous treatment with perifosine (5 μmol/L) and etoposide (5 μmol/L) for a total of 48 h. Based on the growth inhibition curve for each single drug, the effects of the drug combinations were analyzed using the method of Chou and Talalay as described in Materials and Methods. Mean ± SD from three independent experiments. **E**, immunoblot analysis of phosphorylation levels of PDK-1, Akt, FoxO1, GSK-3β, Erk1/2, SAPK/JNK, and β-actin (as loading control). Total cell extracts (60 μg per lane) were probed with phosphorylation specific and total protein antibodies as described in Materials and Methods. Expression of proteins were quantified by using the Image J software as described in Materials and Methods. Band intensities of control was normalized to 1, and treated samples were expressed as fraction of control.

(Fig. 2A, ■) resulted in moderate increase in cell death when compared with treatment with either drug individually. However, in the reverse sequence, where cells were exposed to etoposide for 24 hours followed by a posttreatment with a subtoxic concentration of perifosine (5 μmol/L) for an additional 24 hours (Fig. 2B, ●), a marked increase in cell death was observed. As seen in Fig. 2C, the sequential exposure to etoposide (5 μmol/L) followed by perifosine (5 μmol/L) resulted in more than a 50% increase in cell death, whereas the reverse sequence of addition or simultaneous addition of the two drugs resulted in a 32% and 40% increase in cell death, respectively. Then, by applying the Chou-Talalay method,

we calculated that the strongest synergism was indeed achieved when Jurkat cells were sequentially exposed to etoposide followed by posttreatment with perifosine (CI = 0.20 ± 0.04). The pretreatment with perifosine before etoposide exposure resulted in a moderate synergism, closer to an additive effect (CI = 0.60 ± 0.20), whereas simultaneous treatment with the two drugs resulted in an intermediate combination index (CI = 0.40 ± 0.15), indicating synergism to moderate synergism (Fig. 2D).

Next, we examined the expression and activation of signaling proteins in the Akt survival pathway after combined exposure to etoposide and perifosine (Fig. 2E). The activation state of PDK-1, Akt, GSK-3β, and FoxO1 was

analyzed by immunoblotting, and band intensities were quantified by densitometry analysis. GSK-3 $\beta$  is rapidly emerging as a critical regulator of cell cycle (29), whereas FoxO1 is involved in the control of Fas ligand gene expression (30). Furthermore, the activation state of the survival protein Erk1/2 and the proapoptotic SAPK/JNK pathway was followed, which previously have been reported to be affected by perifosine treatment (20).

As shown in Fig. 2E, the exposure to etoposide (5  $\mu$ mol/L) for 48 hours resulted in a minor decrease in the phosphorylation level of Akt, although when combining data from three independent immunoblotting experiments (see Table 2), no reduction in p-Akt levels was detected (mean ratio of expression =  $1.06 \pm 0.2$ ). Exposure to perifosine (5  $\mu$ mol/L) for 24 hours resulted in a 50% decrease in p-Akt levels, and the Akt activation was essentially abrogated in cells exposed to etoposide followed by posttreatment with perifosine (>90% decreased phosphorylation), while having no effect on total Akt levels. The combined drug treatment did not alter the levels of PDK-1 or GSK-3 $\beta$  to any greater extent than what either drug did individually. Neither did cells treated individually with etoposide or perifosine at these concentrations display any major change in the phosphorylation levels of FoxO1. However, sequential treatment with etoposide followed by perifosine resulted in virtually a complete loss of FoxO1 phosphorylation. As described previously, the phosphorylation of FoxO1 by Akt results in inactivation and affects its nuclear localization. Thus, drug-induced Akt dephosphorylation results in FoxO activation and nuclear translocation (31) and thereby activation of transcription of proapoptotic proteins. The sequential treatment also resulted in a down-regulation of p-Erk1/2 levels, owing to the fact that etoposide caused a 2-fold reduction in total Erk1/2 levels. Lastly, we examined if individual and combined treatment induced SAPK/JNK activation in Jurkat cells. As seen in Fig. 2E, etoposide or perifosine, when used under these conditions, did not induce SAPK/JNK phosphorylation to any significant extent. The combined treatment showed only some slight SAPK/JNK activation.

To evaluate to what level PI staining represented the apoptotic cell population, a simultaneous detection of apoptosis by supravital PI and Annexin V staining was done as described previously and is shown in Fig. 3 (27). After combined treatment with etoposide and perifosine, nearly all PI-positive cells (38%) were also positive for FITC-Annexin V (30%; Fig. 3D). Furthermore, all viable cells, which excluded PI, were not stained by FITC-Annexin V. This strongly indicates that both techniques identify the same apoptotic populations.

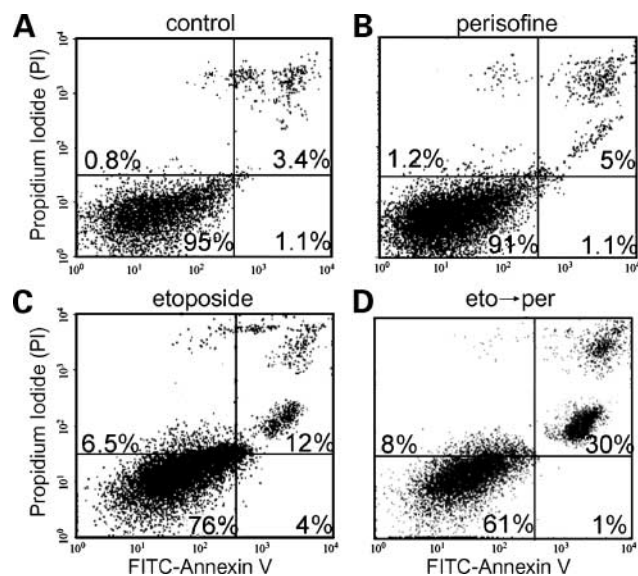
#### Perifosine Increases the Apoptotic Rate in Cells Committed to Apoptosis after Etoposide Exposure

Cell cycle distribution analysis was done on cells treated individually with etoposide and perifosine, or sequentially and simultaneously exposed to both drugs for 24, 30, and 48 hours (Table 1). Beside its well-known effects on G<sub>2</sub>-M phase of the cell cycle, etoposide has been shown to have an inhibitory mechanism also on S-phase progression,

retaining cells in the latter half of the S phase (32, 33). Accordingly, our data showed that etoposide (5  $\mu$ mol/L) induced an accumulation of cells in both S phase (47%) and G<sub>2</sub>-M phase (35%). Perifosine given alone at this subtoxic concentration (5  $\mu$ mol/L) did not markedly disturb cell cycle progression. As shown in Table 1, exposure to etoposide for 24 hours followed by exposure to perifosine for an additional 24 hours resulted in a substantial induction of apoptosis in cells accumulated in G<sub>2</sub>-M phase (38–51%). The induction rate was greater than that of exposure to etoposide alone (17–24%) or perifosine alone (4–10%). In the reverse sequence, where cells were exposed to perifosine before etoposide, a 1.5- to 2-fold increased accumulation of cells in S and G<sub>2</sub>-M phase was observed, whereas the apoptotic population was proportionally 1.5- to 2-fold decreased. Taken together, these results suggest that inactivation of the Akt survival pathways sensitizes cells to the apoptotic stimuli induced by etoposide chemotherapy, resulting in an increased rate of apoptosis.

#### Combined Treatment with Etoposide and Perifosine Results in Caspase-8, Caspase-9, and Caspase-3 Activation as well as Down-Regulation of Antiapoptotic Bcl-xL

As the Akt pathway is known to affect susceptibility to apoptosis, we next wanted to assess to what extent



**Figure 3.** Supravital PI and Annexin V assays identify the same population of apoptotic cells after exposure to etoposide and/or perifosine. **A**, untreated, control Jurkat cells were maintained in medium 48 h and stained with PI and Annexin V simultaneously and analyzed by flow cytometry as described in Materials and Methods. **B**, cells treated with 5  $\mu$ mol/L perifosine for 48 h and then analyzed for PI and Annexin V positivity. **C**, Jurkat cells exposed to 5  $\mu$ mol/L etoposide alone for 48 h. **D**, Jurkat cells exposed to sequential addition of 5  $\mu$ mol/L perifosine (*per*) after 24 h with etoposide (*eto*) for a total of 48 h. Percentage of PI<sup>+</sup>/Annexin V<sup>-</sup>, top left quadrant; PI<sup>+</sup>/Annexin V<sup>+</sup>, top right quadrant; PI<sup>-</sup>/Annexin V<sup>+</sup>, bottom right quadrant; PI<sup>-</sup>/Annexin V<sup>-</sup>, bottom left quadrant.



**Table 1. Cell cycle perturbation and apoptosis induced by perifosine and etoposide in Jurkat cells**

	24 h				30 h				48 h			
	G <sub>0</sub> -G <sub>1</sub>	S	G <sub>2</sub> -M	Apoptosis	G <sub>0</sub> -G <sub>1</sub>	S	G <sub>2</sub> -M	Apoptosis	G <sub>0</sub> -G <sub>1</sub>	S	G <sub>2</sub> -M	Apoptosis
Control	63	23	13	1.6	64	24	11	1	69	19	10	2
Etoposide (5 μmol/L)	3.8	47	34	15	5	46	32	17	6	21	49	24
Perifosine (5 μmol/L)	61	23	14	1.7	60	23	14	4	55	23	13	10
Etoposide → perifosine	—	—	—	—	12	41	10	38	12	26	9	51
Perifosine → etoposide	—	—	—	—	35	28	16	22	10	38	20	32
Etoposide + perifosine	3.5	44	34	18	6	25	43	27	8.3	27	25	40

NOTE: The apoptotic population percentages were determined by measuring the sub-G<sub>1</sub> phase by flow cytometry analysis after collecting the cell samples at various times following drug exposure. The data presented are the mean percentage from three independent experiment.

etoposide and perifosine exposure resulted in activation of the cell death mechanisms. We investigated potential cellular targets of etoposide and perifosine with respect to mitochondrial injury, caspase activation, and expression of various proapoptotic and antiapoptotic proteins.

Jurkat cells exposed to etoposide (5 μmol/L) showed an increased cleavage of caspase-8 and caspase-3, whereas exposure to perifosine did not markedly increase caspase activation (Fig. 4, lanes 2 and 3). A 2-fold increased cleavage of procaspase-8, and procaspase-9 into active fragments was observed with sequential exposure to etoposide followed by perifosine (Fig. 4, lane 4), and an almost complete cleavage (90%) of procaspase-3 was observed. Moreover, sequential drug treatment also resulted in increased degradation of the DNA repair enzyme poly(ADP-ribose) polymerase. Next, we investigated the expression of the Bcl-2 family of intracellular proteins (e.g., Bid, Bax, Bim, and Bad), which function as central regulators of caspase activation through opposing functions of their antiapoptotic and proapoptotic members (34). A slight decrease in full-length BID expression was observed with sequential drug treatment, presumably reflecting cleavage/activation of BID. Furthermore, sequential treatment also resulted in cleavage of pBIM<sub>EL</sub>, generating truncated BIM having high proapoptotic activity (35). Finally, we examined the expression of antiapoptotic Bcl-xL and found that sequential treatment with etoposide and perifosine results in virtually a complete reduction of Bcl-xL expression. Interestingly, Bcl-xL contains NF-κB-binding sites in its promoter region (36), and it is possible that a drug-induced Akt deactivation causes inactivation of NF-κB, resulting in a reduced expression of Bcl-xL. Neither combined nor individual exposure to these agents modified expression of β-actin. The relative expression of each protein is indicated above each band in Fig. 4, and mean values ( $n = 2$ ) are summarized in Table 2.

#### Main Apoptotic Cascade for Synergistic Cell Death by Etoposide and Perifosine Is Fas/Fas Ligand/Caspase-8 Dependent

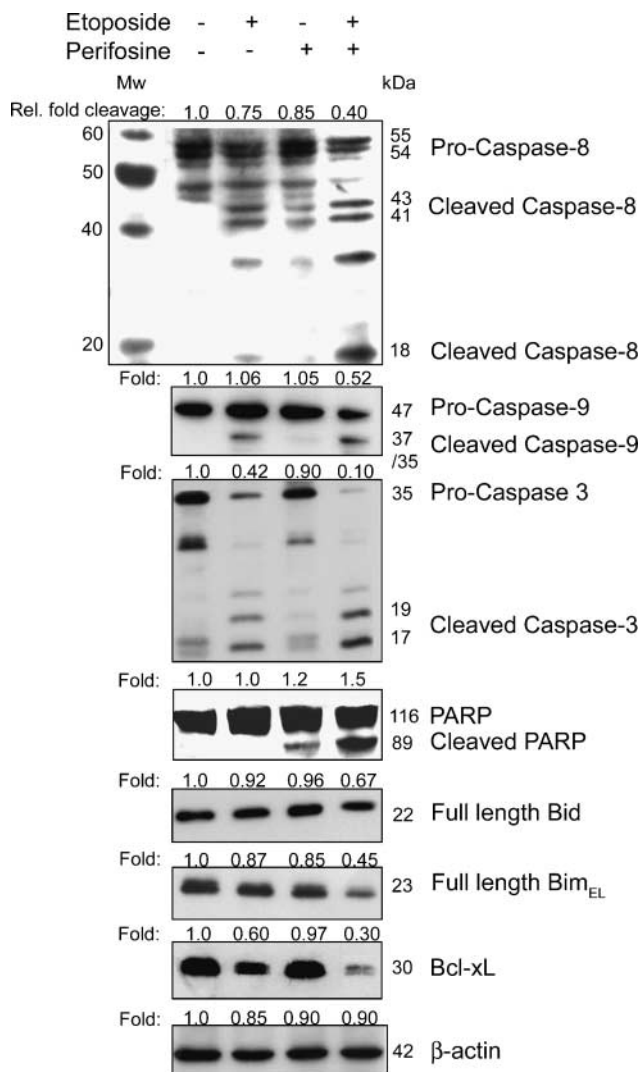
Next, we wanted to evaluate the relative importance of death receptor-mediated versus mitochondrial-mediated

apoptosis in cells exposed to chemotherapy and perifosine, in individual or sequential treatment. First, we evaluated the importance of the Fas death receptor pathway in etoposide- and perifosine-induced apoptosis, using anti-Fas and anti-Fas ligand blocking antibodies (37–39). Exponentially growing Jurkat cells were treated with etoposide and perifosine, individually or in sequence as described above, in the presence or absence of blocking monoclonal antibodies (2 μg/mL) to human Fas or Fas ligand or a control IgG1 mouse monoclonal antibody. After 48 hours, cell growth was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay, which reflects both cell proliferation and viability.

As seen in Fig. 5A, cell death induced after exposure to etoposide or perifosine was inhibited by blocking the Fas/Fas ligand pathway. Jurkat cells exposed to etoposide in the presence of anti-Fas and anti-Fas ligand resulted in a 20% to 30% increase in cell viability. Cell viability was promoted to the same extent in perifosine treated cells, where the presence of anti-Fas or anti-Fas ligand blocking antibodies completely inhibited the cytotoxic effects of perifosine. When treated sequentially with both etoposide and perifosine in the presence of anti-Fas ligand and anti-Fas blocking antibodies, cells showed a moderate 10% increase in viability ( $P > 0.05$ ). The specificity of the anti-Fas ligand and anti-Fas blocking antibodies was investigated in a following experiment in which the addition of blocking antibodies (2 μg/mL) before treatment with increasing amounts of tumor necrosis factor-related apoptosis-inducing ligand for 24 hours (1, 2.5, or 5 μg/mL) did not inhibit tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in Jurkat cells (data not shown). Lastly, flow cytometry analysis of a similar set of samples suggested that the amplification in the number of viable cells observed in the presence of anti-Fas or anti-Fas ligand blocking antibodies was most likely due to the inhibition of induction of cell death on live cells (as observed with 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide viability assay) and not via inhibiting the increase in the number of apoptotic/necrotic cells as detected by PI staining (data not shown).

We next investigated the relative importance of caspase-8 and caspase-9 in etoposide- and/or perifosine-induced

cell death. In the presence of selective inhibitors of caspase-8 (Z-IETD-FMK) or caspase-9 (LEHD-CHO), before etoposide and/or perifosine exposure, an increase in cell viability was observed (Fig. 5B). A moderate 10% ( $7 \pm 3\%$  to  $10 \pm 3\%$ ) increase in cell viability in the presence of caspase inhibitors was observed in etoposide-treated cells. On the other hand, inhibition of caspase-8 offered an



**Figure 4.** Combined treatment with etoposide and perifosine results in a marked increase in caspase activation, mitochondrial injury, and changes in expression of Bcl-2 family members. Jurkat cells were exposed to etoposide ( $5 \mu\text{mol/L}$ ) and perifosine ( $5 \mu\text{mol/L}$ ) alone or in sequential combination for a total of 48 h, after which whole-cell extracts were prepared, and immunoblot analysis was done as described in Materials and Methods. Cell extracts ( $60 \mu\text{g}$  per lane) were probed with antibodies to full-length and cleaved fragments of caspase-8, caspase-9, caspase-3, and PARP. In addition, expression of full-length Bid, Bim, and Bcl-xL was followed. Blots were also probed with antibodies to  $\beta$ -actin to ensure equivalent loading and transfer. Representative results from an additional experiment. Immunoblots were scanned, and band intensities were determined as described in Materials and Methods. Band intensities of control was normalized to 1, and treated samples were expressed as fraction of control.

almost 100% protection against perifosine-induced cell death ( $94 \pm 10\%$  viable cells), whereas inhibition of caspase-9 was less effective ( $80 \pm 8\%$  viable cells). Addition of caspase-8 or caspase-9 inhibitor during sequential treatment with etoposide and perifosine resulted in a 10% increase in viable cells ( $42 \pm 1\%$  and  $37 \pm 0.5\%$ , respectively;  $P < 0.05$ ). Conclusions from these findings support the concept that etoposide and perifosine are selective in their effects, requiring specific activation of the Fas/Fas ligand/caspase cascade, and is not merely broad nonselective effects on cell death following drug exposure. The fact that cells undergo apoptosis even in the presence of caspase inhibitors may indicate caspase-independent parallel pathways of cell death, triggered by cotreatment with etoposide and perifosine.

## Discussion

Ample amount of evidence has implicated PI3K-mediated Akt activation in promoting tumor cell proliferation and survival (1). In fact, several components of the PI3K/Akt pathway have been found to be deregulated in different human cancers, including PI3K gene amplification and/or mutation, Akt gene amplification or Akt protein overexpression, and PTEN gene deletion or inactivating mutations (8, 40, 41). Over the last years, several studies have linked active Akt to enhanced resistance to chemotherapy in a variety of unrelated human cancers. It has been shown that agents that interfere with signal transduction pathways are able to lower the threshold for chemotherapy-induced cell death, providing effective treatment in cancer therapy (42–44). The membrane permeable phospholipid analogue perifosine is a signal transduction modulator that has been reported to interfere with activation of the Akt pathway (20). In the present study, we report for the first time that perifosine, when used in combination therapy with etoposide in human acute T-leukemia cells having a constitutively active PI3K/Akt pathway (45), results in a marked decrease in p-Akt levels, increased mitochondrial injury, increased caspase activation, and the onset of extensive apoptosis. Such findings are consistent with the results of several recent reports showing the capacity of perifosine to synergize with radiation or other anticancer compounds (46–49). Furthermore, etoposide, which is commonly used for treatment of ATL (22–24), has successfully been used for polychemotherapy in ATL patients (25, 26). Interestingly, recent findings have shown decreased PTEN expression levels in neoplastic cells from ATL patients, which related with up-regulated Akt phosphorylation (50). Therefore, our *in vitro* experimental model (PTEN-deficient Jurkat cells) quite closely mimics the human disease.

Perifosine given alone to exponentially growing Jurkat cells displayed a dose-dependent antiproliferative and cell death-inducing activity ( $\text{IC}_{50}$  was around  $30 \mu\text{mol/L}$  at 24 hours). Interestingly, early clinical studies indicate perifosine to be effective at serum concentrations of 5 to  $20 \mu\text{mol/L}$  (14, 16); thus, the concentrations studied here



**Table 2. The relative expression of p-Akt, its downstream targets, and antiapoptotic and proapoptotic proteins after combined etoposide and perifosine treatment**

Relative expression of	Etoposide	Perifosine	Drugs combined
p-PDK1	0.95	1.0	0.90
p-Akt	1.06	0.55	0.07
Akt	0.90	1.05	0.80
p-FoxO1	0.95	1.0	0.20
p-GSK-3 $\beta$	1.0	0.55	0.60
p-SAPK/JNK	0.04	0.1	0.18
SAPK/JNK	1.1	1.1	0.85
p-Erk1/2	0.20	1.2	0.05
Erk1/2	0.40	0.90	0.25
Procaspace-8	0.80	0.90	0.40
Procaspace-9	1.1	1.1	0.5
Procaspace-3	0.40	1.0	0.15
Cleaved PARP	0.0	0.30	0.50
Bid	1.0	1.0	0.75
Bim	0.9	0.85	0.50
BclxL	0.60	1.0	0.25
$\beta$ -Actin	0.80	0.90	0.90

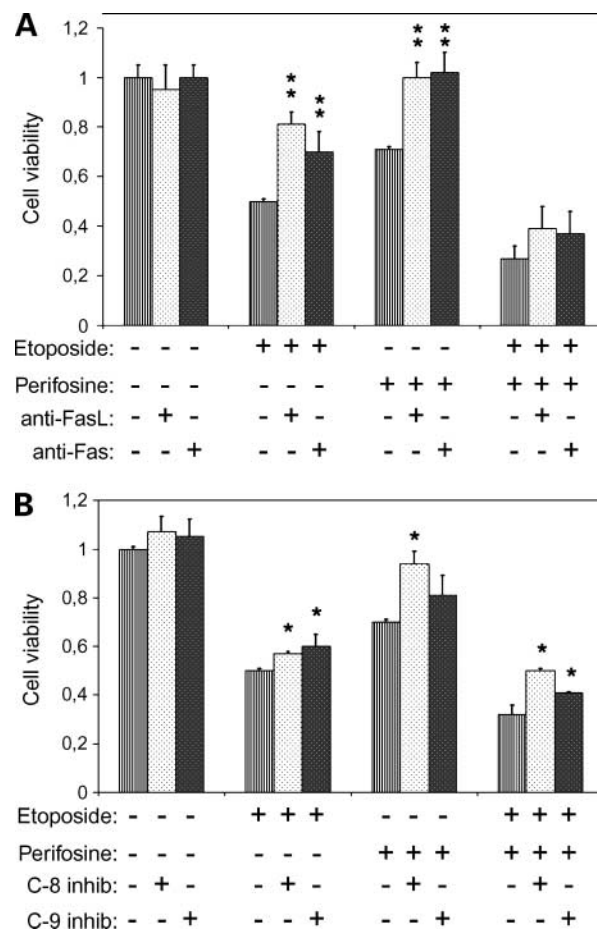
NOTE: The relative expression of the proteins (mean ratios calculated from two or more different immunoblotting experiments) were determined by quantifying the band intensities of the immunoblots presented in Figs. 2 and 4 (additional blots not shown) by using Image J software as described in Materials and Methods.

Abbreviation: PARP, poly(ADP-ribose) polymerase.

are clearly within clinically achievable range. The increase in cell death was associated with a marked reduction in Ser<sup>473</sup> p-Akt levels, in accordance to what has been reported previously (20). Perifosine apparently inhibits the membrane translocation of Akt, which in turn prevents its activation by PDK-1 or other kinase(s) (21). Although perifosine treatment resulted in reduced levels of p-Akt and its downstream targets, it had no discernible effect on PDK-1 intrinsic activity. Previous reports have shown that exposure to low concentrations of perifosine, in certain cell types, resulted in a temporary stimulation of Erk1/2 (49, 51, 52). Similarly, we found that exposure to low perifosine concentrations, or short exposure times, resulted in a brief stimulation of the mitogen-activated protein kinase pathway and higher levels of p-Erk1/2 as a consequence. It is possible that perifosine-induced inactivation of Akt eliminates the negative regulation Akt exerts on Raf-1, allowing for activation of the mitogen-activated protein kinase pathway to compensate for the loss of Akt survival effects, thereby delaying drug-induced cell death (53).

Combination therapy with etoposide and a subtoxic concentration of perifosine led to a synergistic interaction between the two drugs, resulting in a 2-fold increase in apoptotic rate and a marked increase in cell death. Etoposide is a topoisomerase II-targeting anticancer drug (54), whose inhibitory mechanism on S-phase progression results in late S-phase and G<sub>2</sub>-M phase arrest, indicative of a DNA repair process in progress (32). Moreover, it is possible that in addition to the G<sub>2</sub>-M arrest, which results in mitotic cell death, a parallel commitment to apoptosis

occurs in leukemic cells (55). A possible explanation to the increased apoptotic rate observed in cells posttreated with perifosine is provided by recent data implicating active Akt in the transition through G<sub>2</sub>-M phase of the cell cycle (56). An increased Akt activity has been observed in cells entering the G<sub>2</sub> phase (57), and Akt has been shown to inhibit G<sub>2</sub>-M DNA checkpoint proteins and other proapoptotic proteins (58, 59). Thus, it is possible that drug-induced



**Figure 5.** Specific inhibition of caspase-8 and caspase-9 and Fas/Fas ligand-dependent killing. **A**, for measurement of Fas-dependent killing by etoposide or perifosine, 2  $\mu$ g of either anti-Fas or anti-Fas ligand monoclonal blocking antibody, inhibiting apoptosis mediated through the Fas/Fas ligand pathway, was added 1 h before addition of drugs. As a control, an isotype-matched control monoclonal antibody (2  $\mu$ g) was used. Cells were incubated with 5  $\mu$ mol/L etoposide and 5  $\mu$ mol/L perifosine individually or in sequential combination as described previously for a total of 48 h. \*\*,  $P < 0.001$ , significant difference compared with untreated (control) cells. **B**, to determine the proapoptotic activity of caspase-8 and caspase-9, 10  $\mu$ mol/L of specific inhibitors to caspase-8 (Z-IETD-FMK) or caspase-9 (LEHD-CHO) was added 1 h before exposure to the drugs, individually or in combination as described above. As a control, a vehicle of DMSO was added at a final concentration of 0.1  $\mu$ mol/L. Effects of the caspase and Fas/Fas ligand pathway inhibitors were estimated by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide cell growth and viability assay 48 h after beginning of the treatment, as described in Materials and Methods. Cell viability was expressed as fraction of the control. Columns, mean; bars, SD. \*,  $P < 0.05$ , significant differences compared with control samples.

inactivation of Akt subsequent to G<sub>2</sub>-M arrest allows for full activation of the DNA repair machinery at the G<sub>2</sub>-M check point, consequently leading to apoptotic cell death. Furthermore, we observed, by Western blot analysis, that combined therapy resulted in an even more pronounced decrease in p-Akt levels and p-FoxO1. This indicates that the two drugs converge upstream of Akt, resulting in dephosphorylation Akt and thus Akt inactivation. Dephosphorylation of FoxO family members allows for their nuclear translocation and transcription of proapoptotic genes, such as *Fas ligand* and *Bim* (31).

Exposure to etoposide alone or in combination with perifosine resulted in a loss of Erk1/2 expression and activation. At present, we do not know whether this reflects down-regulated gene expression or proteolytic cleavage. It should be reminded here that whereas Akt could be cleaved during apoptosis, Erk1/2 apoptotic cleavage has never been reported thus far (60).

Therefore, we investigated if the loss of Erk1/2 survival effects could contribute to the perifosine-induced increase in cell death by exposing Jurkat cells to 5  $\mu$ mol/L of the specific Erk1/2 inhibitor UO126 and 5  $\mu$ mol/L perifosine in sequential treatment as described before. However, no synergism was observed between the Erk1/2 and Akt inhibitors (data not shown). Thus, when used under these conditions, the inhibition of Erk1/2 signaling did not augment perifosine-induced cytotoxicity, at variance with results of others (61). It has been proposed that anticancer drugs, such as etoposide, can induce the expression of Fas and/or Fas ligand (62, 63), thereby initiating the Fas death receptor pathway and apoptosis. To further delineate the convergence in apoptosis signaling, we have identified effector caspases, and proapoptotic and antiapoptotic proteins that are affected by treatment with perifosine and etoposide. Caspase activation can be initiated by two distinct mechanisms: (a) one mediated by the formation of a death-inducing signaling complex at the cytoplasmic side of death receptors (64) and (b) the other by signals that initially results in the release of apoptogenic molecules from the mitochondria (i.e., cytochrome *c*; refs. 65, 66). In each case, the activation of a proximal caspase, such as caspase-8 for receptor-mediated apoptosis and caspase-9 for mitochondrial-mediated apoptosis, results in a cascade where downstream caspases are activated. Certain cells (type I) employs only death receptor-initiated apoptotic cell death, whereas in other cells (type II), including Jurkat, even death receptor-initiated apoptotic signals may be dependent on the mitochondria to induce cell death (67). We observed an increased caspase-8 and caspase-3 activation in response to etoposide treatment, whereas caspase-9 processing was minor. A subtoxic dose of perifosine caused a minor caspase-8 activation, but no significant caspase-3 or caspase-9 activation. Combined treatment resulted in a two-fold increase in caspase-8 activation, as well as marked increase in caspase-9, caspase-3, and poly(ADP-ribose) polymerase processing. Furthermore, combined treatment resulted in changes in Bid, Bim, and Bcl-xL expression, suggesting that mitochondrial participation is essential for

cell death response. Caspase-8 and caspase-3 can cleave cytosolic Bid (68, 69), where active Bid fragments interact with proapoptotic protein Bax at the mitochondria to induce cytochrome *c* release (70). Moreover, caspase-3 has also been shown to induce cleavage of Bim, generating truncated Bim with high proapoptotic activity (35), antagonizing the antiapoptotic functions of Bcl-2 and Bcl-xL through direct interaction. Finally, the reduced levels of p-Akt observed after combined treatment is found in parallel to a selective decrease in Bcl-xL levels. Interestingly, Bcl-xL expression might be regulated by Akt via I $\kappa$ K and NF- $\kappa$ B (71) through its NF- $\kappa$ B-binding sites in the promoter region (36). Drug-induced Akt deactivation could result in NF- $\kappa$ B down-regulation, which causes a reduced expression of antiapoptotic Bcl-xL, resulting in release of cytochrome *c* from mitochondria and activation of caspase-9, and caspase-3.

Both etoposide and perifosine toxicity was reduced after inhibition of the Fas/Fas ligand signaling pathway. This indicates that there is a convergence in apoptotic signaling downstream of Fas. However, whereas perifosine toxicity was strongly inhibited by the caspase-8 inhibitor, and to a lesser extent the caspase-9 inhibitor, etoposide-induced cell death was barely affected by addition of the caspase inhibitors. Apparently, Fas signaling can diverge at caspase-8, with one branch of the pathway resulting directly in effector-caspase activation and the other branch communicating with the mitochondria (68, 70). Our findings are supported by other results that suggest that anticancer drugs can induce apoptosis by activating Fas death receptor system signaling (62, 63). Although, this is contradictory to other reports indicating that no Fas ligand synthesis was observed in Jurkat cells in response to etoposide treatment, nor was drug-induced apoptosis blocked by interaction of Fas with its ligand (72, 73).

In conclusion, we have shown here that etoposide and perifosine cooperate to induce leukemic cell death by inactivation of Akt and activation of FoxO1 and the Fas/Fas ligand death receptor pathway and causing a marked increase in mitochondrial dysfunction. Moreover, the factors regulating etoposide/perifosine-mediated apoptosis in neoplastic cells may involve increased activation of caspase-8, caspase-9, caspase-3, Bid, and Bim activation and decreased expression of Bcl-xL. Our results imply that in Jurkat cells, apoptotic signaling induced by etoposide and perifosine converges with a Fas-induced pathway downstream of caspase-8, with the essential participation of a mitochondria-dependent caspase complex. Our work suggests that a combination therapy with etoposide and perifosine could represent a rational approach for efficient suppression of PI3K/Akt signaling in ATL patients with low PTEN levels and provides support for their incorporation into the design of more effective anticancer treatment protocols.

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