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ORIGINAL ARTICLE

The novel Akt inhibitor, perifosine, induces caspase-dependent apoptosis and downregulates P-glycoprotein expression in multidrug-resistant human T-acute leukemia cells by a JNK-dependent mechanism

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A significant impediment to the success of cancer chemotherapy is the occurrence of multidrug resistance, which, in many cases, is attributable to overexpression of membrane transport proteins, such as the 170-kDa P-glycoprotein (P-gp). Also, upregulation of the phosphatidylinositol 3-kinase (PI3K)/Akt-signaling pathway is known to play an important role in drug resistance, and has been implicated in the aggressiveness of a number of different cancers, including T-acute lymphoblastic leukemia (T-ALL). We have investigated the therapeutic potential of the novel Akt inhibitor, perifosine (a synthetic alkylphospholipid), on human T-ALL CEM cells (CEM-R), characterized by both overexpression of P-gp and constitutive upregulation of the PI3K/Akt network. Perifosine treatment induced death by apoptosis in CEM-R cells. Apoptosis was characterized by caspase activation, Bid cleavage and cytochrome *c* release from mitochondria. The proapoptotic effect of perifosine was in part dependent on the Fas/FasL interactions and c-Jun NH_2-terminal kinase (JNK) activation, as well as on the integrity of lipid rafts. Perifosine downregulated the expression of P-gp mRNA and protein and this effect required JNK activity. Our findings indicate that perifosine is a promising therapeutic agent for treatment of T-ALL cases characterized by both upregulation of the PI3K/Akt survival pathway and overexpression of P-gp.
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Introduction

Despite improvement in chemotherapy strategies, the development of multidrug resistance (MDR) remains one of the major problems in chemotherapy.[1] MDR is a complex phenomenon, often associated with overexpression of the 170-kDa P-glycoprotein (P-gp) on the plasma membrane of tumor cells. P-gp is the product of the *ABCB1 (MDR1)* gene and belongs to the superfamily of ATP-binding cassette transporters, which actively efflux across the membranes a wide range of structurally diverse compounds used to treat cancer, thus reducing their intracellular bioavailability and toxicity.[2] P-gp overexpression correlates with poor prognosis for many human cancers.[3] In addition, it is well known that P-gp overexpression confers resistance to a wide range of caspase-dependent proapoptotic agents, not only by removing drugs from the cells, but also by inhibiting the activation of caspases, a family of proteases, which are key players in apoptotic cell death.[4] Only a few drugs are reported to overcome the MDR phenotype induced by this ATP-binding cassette transporter,[5] and most of them induce cell death in a caspase-independent manner,[6] albeit a few drugs are capable of activating caspases in P-gp-expressing cells.[7]

Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt-signaling pathway is another key factor, which plays a central role in cancer biology, conferring resistance both *in vivo* and *in vitro* to therapeutic treatments of various types of malignancies.[8–10] Upon activation by different stimuli, membrane localization of PI3K generates phosphatidylinositol-3,4,5-trisphosphate, which then activates a number of downstream substrates.[11] The serine/threonine kinase Akt is a well-characterized PI3K target, and binding of phosphatidylinositol-3,4,5-trisphosphate to the pleckstrin homology domain of Akt results in its translocation to the plasma membrane, where it undergoes phosphorylation at both Thr308 and Ser473. A fundamental negative regulator of the PI3K/Akt pathway is the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10), which removes the 3-phosphate from phosphatidylinositol-3,4,5-trisphosphate, thus yielding PtdIns 4,5 P_2.[12] Small inhibitory molecules of the PI3K/Akt pathway are currently being developed for clinical use.[13] Indeed, over the last few years, several Akt inhibitors have been synthesized, including perifosine, a novel alkylphospholipid analog, which has shown promising preclinical activity and is currently undergoing phase-I/II clinical evaluation.[14,15] Although mechanisms underlying the anticancer activity of perifosine remain to be fully elucidated, perifosine, via interference with the turnover and synthesis of natural phospholipids, disrupts membrane-linked signaling pathways at several sites, thereby inhibiting PI3K/Akt.[16] Perifosine inhibits Akt phosphorylation by its interaction with the pleckstrin homology domain of the kinase, thus preventing Akt membrane localization and phosphorylation.[14] Perifosine, in some cell types, downregulates the antiapoptotic extracellular signal-regulated kinase (ERK) 1/2 pathway[17] and activates the proapoptotic c-Jun NH_2-terminal kinase (JNK) network, thereby altering the balance between the survival- and death-signaling cascades.[18] From a clinical perspective, perifosine is the most developed PI3K/Akt inhibitor.[19] We have recently reported the cytotoxic effect of perifosine alone, or combined with etoposide, on the PTEN-null Jurkat T-acute lymphoblastic leukemia (T-ALL) cell line.[20] However, the cytotoxic activity of perifosine has not been tested on cells, which overexpress P-gp in combination with a constitutively active PI3K/Akt network.

In the following studies, we demonstrated that perifosine is cytotoxic for human T-ALL CEM-R cells, which are characterized

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by overexpression of P-gp and upregulation of the PI3K/Akt-signaling network due to absence of PTEN expression.[21] Remarkably, exposure to perifosine resulted in activation of caspases in CEM-R cells. Perifosine-dependent apoptotic cell death of CEM-R cells was also characterized by JNK activation and was partly dependent on Fas ligation and disruption of lipid raft integrity. In summary, our results indicate that perifosine is a promising drug also for treatment of P-gp-overexpressing neoplastic hematopoietic disorders characterized by PI3K/Akt signaling upregulation.

Materials and methods

Materials

Perifosine was provided by AEterna Zentaris GmbH (Frankfurt, Germany). For cell viability determination, Cell Proliferation kit I (3-[4,5-dimethylthythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)) was purchased from Roche Applied Science (Penzberg, Germany) and Annexin V staining kit was purchased from Bender MedSystem (Wien, Austria), whereas Carboxyfluorescein Fluorescent-Labeled Inhibitor of Caspases (FLICA) Apoptosis Detection kit Caspase Assay was from AbD Serotec (Oxford, UK). Antibodies were from Cell Signaling Technology (Danvers, MA, USA) (Akt, Ser473 p-Akt, Thr308 p-Akt, ERK 1/2, Thr202/Tyr204 p-ERK 1/2, JNK, Thr183/Tyr185 p-JNK, caspase-3, caspase-8, caspase-9, poly-(ADPribose)-polymerase (PARP), β-actin, cytochrome *c*, cytochrome *c* oxidase IV). The two-color fluorescence immunocytochemistry detection kit for cytochrome *c* and mitochondria was from MitoSciences (Eugene, OR, USA). Mouse monoclonal blocking antibody to human Fas (SM1/23) was from Alexis Biochemicals (Lausen, Switzerland), whereas anti-Fas antibody for immunocytochemical staining (SM1/1) was from Bender MedSystem. The cell-permeable JNK inhibitor (SP600125), the caspase-3 inhibitor (Z-DEVD-FMK) and the caspase-8 inhibitor (Z-IETD-FMK) were from Calbiochem-Novabiochem (La Jolla, CA, USA). Methyl-β-cyclodextrin (MCD), fluorescein isothiocyanate (FITC)-labeled cholera toxin B (FITC-CTx B) subunit, vinblastine, anti-P-gp antibody (clone F4) and Trypan Blue were from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

The parental drug-sensitive human T-lymphoblastic CEM cell line (CEM-S, for sensitive) and its P-gp-expressing variant (CEM-R, for resistant)[21] were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum. MDR-variant CEM-R cells were supplemented once a week with 100 ng ml^{-1} of vinblastine. To determine cell growth, 3×10^5 cells were seeded in 25-cm^2 flasks and growth curves were prepared by plotting direct counts of cells harvested for 5 days after seeding. Viable cells counts were counted by a hemocytometer using 0.2% Trypan Blue.

Cell viability analysis

3-[4,5-Dimethylthythiazol-2-yl]-2,5-diphenyltetrazolium bromide assays were performed to assess the sensitivity of CEM cells to perifosine, as previously reported.[20] Results were statistically analyzed by GraphPadPrism Software (version 3.2; GraphPad Software Inc., San Diego, CA, USA).

Annexin V–FITC/PI staining

To assess the extent of apoptosis induction after treatment with perifosine, a flow cytometric analysis of Annexin V–FITC/propidium iodide (PI)-stained samples was performed, as reported elsewhere.[20] Samples were analyzed with the EPICS XL flow cytometer (Beckman Coulter, Miami, FL, USA) with the appropriate software (System II; Beckman Coulter). At least 15 000 events per sample were acquired.

Western blotting analysis

This was performed as reported elsewhere.[22] Quantitation of blots by scanning was performed as previously reported.[23]

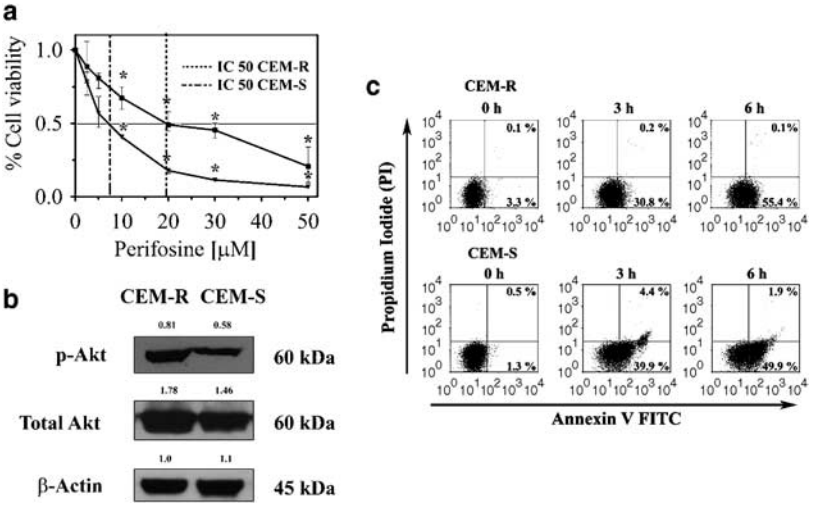

Figure 1 Perifosine cytotoxicity and apoptosis induction in CEM cells. (**a**) MTT assays of CEM-R and CEM-S cells cultured with perifosine for 24 h. Results are the mean of three different experiments ± s.e. The asterisk indicates statistically significant difference ($P<0.005$) with respect to untreated cells. (**b**) Western blot analysis showing Ser473 p-Akt and total Akt levels in CEM-R and CEM-S cells. The molecular weight of the antigens is indicated at right. The antibody to β-actin demonstrated equal loading in the lanes. The numbers indicate the results from densitometric analysis. One representative of three different experiments is shown. (**c**) Representative histograms showing flow cytometric analysis of Annexin V–FITC/PI-stained CEM cells. Cells were cultured with perifosine (20 μM for CEM-R and 7 μM for CEM-S) for the indicated time periods. The percentage of early apoptotic cells (lower right quadrant) and late apoptotic/necrotic cells (upper right quadrant) is indicated. One representative of three different experiments is shown. FITC, fluorescein isothiocyanate; MTT, 3-[4,5-dimethylthythiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI, propidium iodide.

Isolation of mitochondrial fraction

This was accomplished using the Mitochondria Isolation kit (Pierce, Rockford, IL, USA), according to the manufacturer's instructions.

Caspase activity assay

Flow cytometric assays were performed, to determine caspase activity, using the Carboxyfluorescein FLICA Apoptosis Detection kit Caspase Assay, according to the manufacturer's instructions. After treatments, cells were collected by centrifugation and washed in phosphate-buffered saline (PBS). A total of 3×10^5 cells per sample were labeled with the FLICA solution and incubated for 1 h at 37 °C and washed twice in PBS. Fluorescence resulting from the FLICA regent was measured at 488 nm by flow cytometry.

Fluorescence immunocytochemistry

Cells were collected by centrifugation at a density of 3×10^5 cells ml^{-1} and plated on electrostatically charged glass slides, using a Shandon Cytospin (Thermo Electron Corporation,

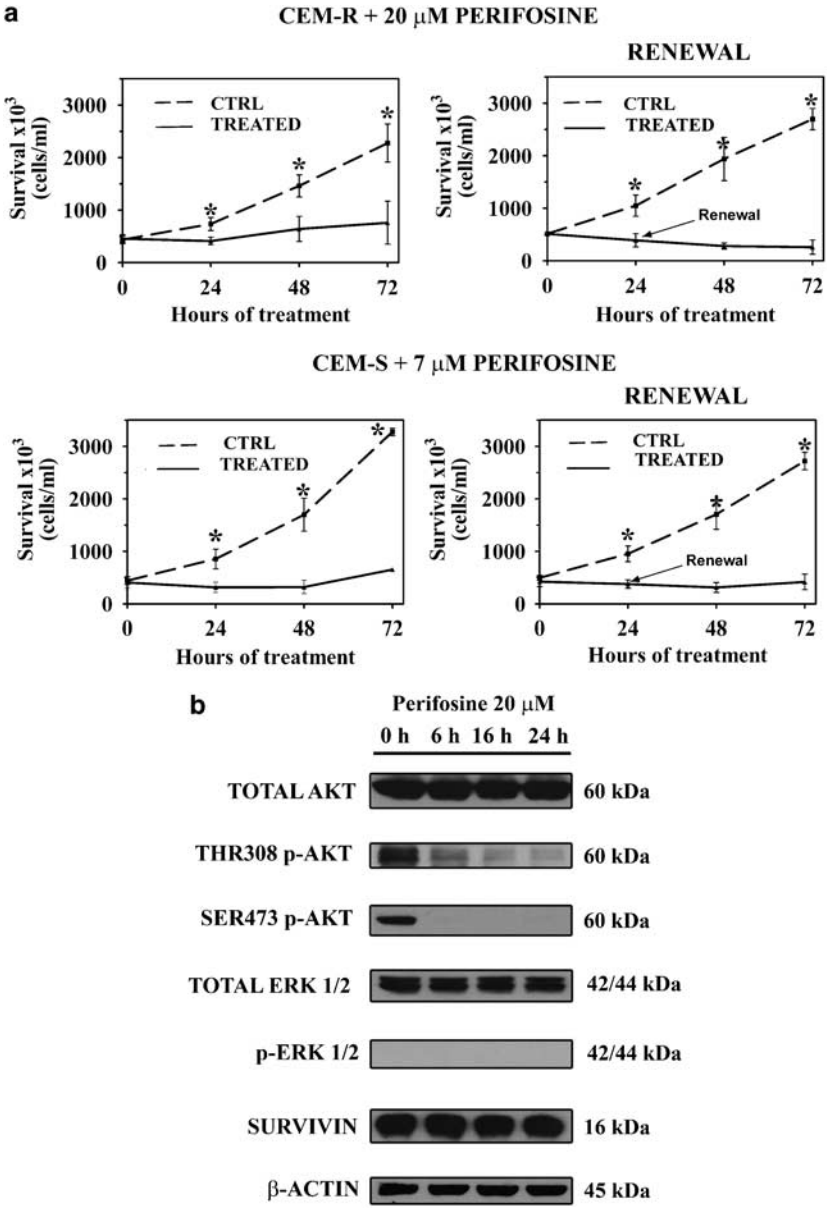

Figure 2 Perifosine negatively affects cell proliferation rates of CEM cells and p-Akt levels. (**a**) To determine cell growth, 3×10^5 cells were seeded in 25-cm^2 flasks and growth curves were generated by plotting direct count of cells harvested for 5 days after seeding. Viable cells counts were counted by a hemocytometer using 0.2% Trypan Blue. In some experiments, cells were washed with serum-free medium and incubated in complete medium to which perifosine was added to the original concentration (renewal). Results are the mean of three different experiments ± s.e. The asterisk indicates statistically significant difference ($P<0.005$) with respect to untreated cells (CTRL). (**b**) Western blot analysis. Cells were cultured for an increasing period of time in the presence of 20 μM perifosine. Whole-cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrotransferred to nitrocellulose membranes, which were then probed with the indicated antibodies. The molecular weight of the antigens is indicated at right. The antibody to β-actin demonstrated equal loading in the lanes. One representative of three different experiments is shown.

Pittsburgh, PA, USA). Samples were fixed with 4% formaldehyde in PBS, pH 7.4, for 10 min at room temperature and subsequently permeabilized with 0.2% Triton X-100 in PBS for 5 min. After two washes with PBS, the slides were saturated in PBS 5% bovine serum albumin for 1 h at room temperature.

Detection of cleaved caspase-3. Cells were treated as outlined above, and then reacted with an antibody to cleaved caspase-3 (from Cell Signaling), which was then revealed with an FITC-conjugated anti-rabbit antibody.

Two-color immunocytochemistry of cytochrome c *and mitochondrial colocalization.* This was accomplished using an anti-cytochrome *c* antibody (IgG2a isotype, which was detected by a goat anti-mouse IgG2a antibody conjugated to FITC) and by an anti-Complex V subunit monoclonal antibody (IgG2b isotype, which was detected by a goat anti-mouse IgG2b antibody conjugated to Texas Red).

Visualization of lipid rafts. Samples were incubated with 8 μg ml^{-1} of FITC-CTx B subunit diluted in PBS 1% bovine serum albumin for overnight at 4 °C in the dark, and washed three times with PBS. For colocalization experiments, FITC-CTx B subunit staining was performed as described above, but samples were incubated also with 500 ng ml^{-1} of anti-human Fas (SM1/1) IgG2a mouse monoclonal antibody at 4 °C.

Samples were further processed using Cy3-conjugated anti-mouse IgG antibody, diluted 1:500, to detect Fas. To visualize

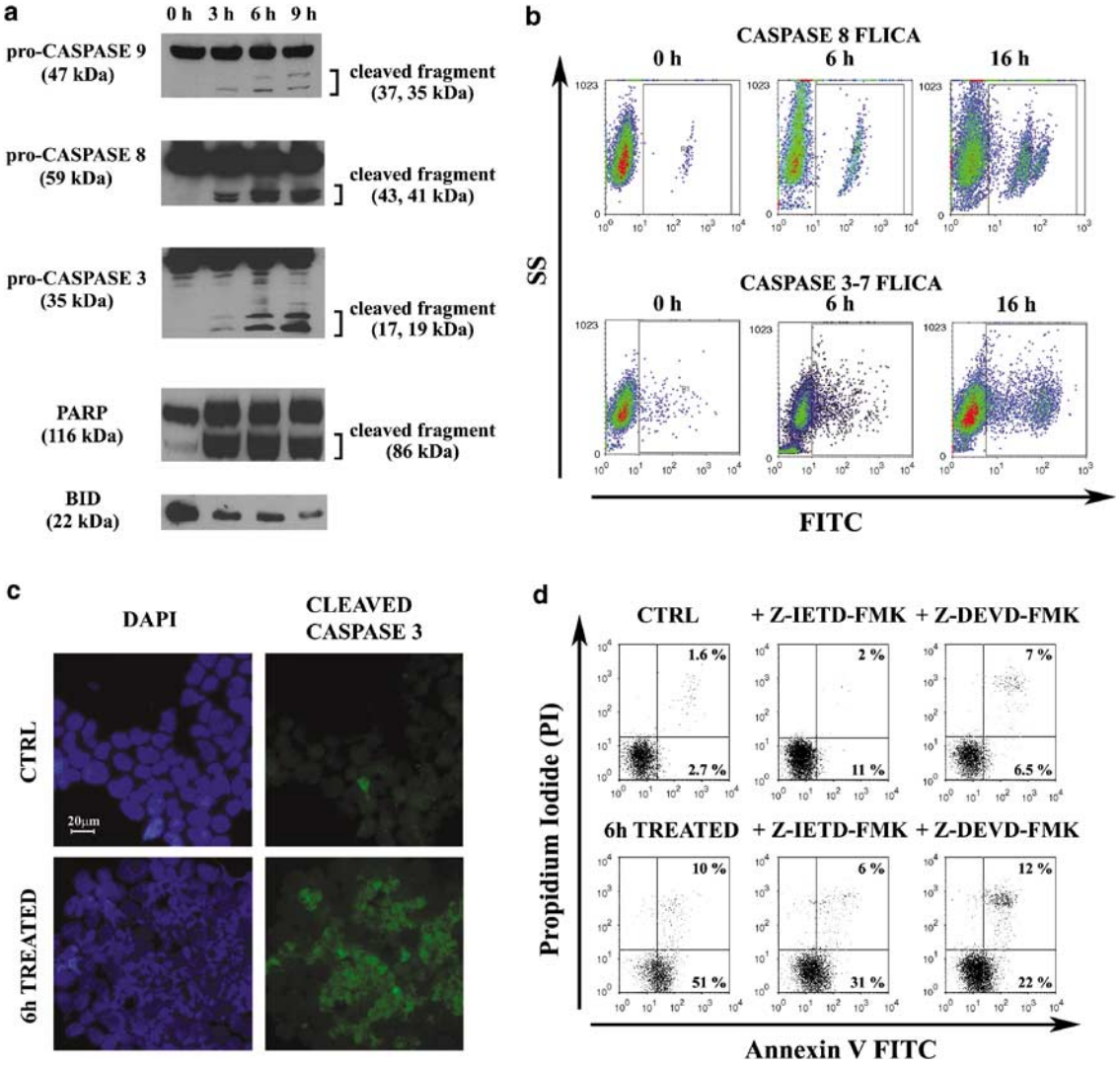

Figure 3 Perifosine activates both the extrinsic and intrinsic apoptotic pathway in CEM-R cells. (**a**) Western blot analysis showing cleavage of caspase-8, caspase-9, caspase-3, Bid and PARP in response to 20 μM perifosine. Molecular weights are indicated to the right. One representative of three different experiments is shown. (**b**) Caspase activity was analyzed by the FLICA assay. Cells were cultured for the indicated time periods in the presence of 20 μM perifosine. One representative of three different experiments is shown. (**c**) Immunofluorescence analysis demonstrating cleavage of caspase-3 in cells treated for 6 h with 20 μM perifosine. Cells were stained with an antibody, which specifically recognizes caspase-3 cleaved at Asp175, which was then revealed by an FITC-conjugated secondary antibody. Nuclei were counterstained for DNA by 4′,6-diamidino-2-phenylindole. (**d**) Caspase inhibitors decrease apoptotic cell death evoked by perifosine. Cells were preincubated for 2 h with 20 μM of either caspase-3 inhibitor Z-DEVD-FMK, or caspase-8 inhibitor Z-IETD-FMK, treated with perifosine and then subjected to flow cytometric analysis of Annexin V–FITC/PI staining. One representative of three different experiments is shown. CTRL, control; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; FLICA, Fluorescent-Labeled Inhibitor of Caspases.

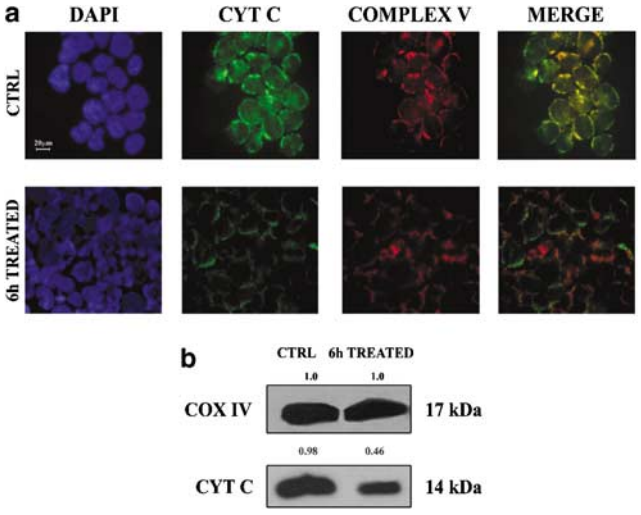

Figure 4 Perifosine induces cytochrome *c* release from the mitochondria of CEM-R cells. (**a**) Double immunofluorescence analysis. Control (CTRL) and perifosine-treated (20 μM for 6 h) cells were fixed and were permeabilized. They were then immunostained with an antibody to cytochrome *c* (cyt. *c*, green) and an antibody to complex V (red). The merged image shows colocalization of the two fluorochromes (yellow-orange). Nuclei were counterstained for DNA by 4′,6-diamidino-2-phenylindole. (**b**) Western blot analysis performed on the purified mitochondrial fraction. The blots were probed with antibodies to the mitochondrial marker complex IV (cytochrome *c* oxidase IV) and cytochrome c (cyt. *c*). Molecular weights are indicated to the right. The numbers indicate the results from densitometric analysis. One representative of three different experiments is shown. DAPI, 4′,6-diamidino-2-phenylindole.

nuclei, samples were incubated for 1 min with 4′,6-diamidino-2-phenylindole (0.5 μg ml^{-1}, DAPI). Finally, slides were examined under epifluorescent illumination. Images were taken with a Zeiss Axio Imager.Z1 microscope coupled to a computer-driven Zeiss AxioCAM digital camera (MRm), using Zeiss AxioVision software (version 4.5) and the Zeiss colocalization module with constant settings of exposure.

Electrophoretic mobility-shift assay

This was performed according to Faenza *et al*.[24] A double-stranded oligonucleotide containing the consensus binding site for Activator Protein 1 (AP1), 5′-CGCTTGATGACTCAGCCG GAA-3′ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a probe for the assay. The probe was labeled with [γ-32P]dATP (3000 Ci mmol^{-1}; Perkin Elmer, Whaltam, MA, USA) using T4 polynucleotide kinase. A 10-μg weight of nuclear extract was used for each binding reaction. Where appropriate, the extract was preincubated for 10 min at room temperature with 100-fold molar excess of unlabeled AP1 oligonucleotide; for supershift analysis, 2 μg of antibodies against c-Jun and c-Fos (Santa Cruz Biotechnology) were added for 1 h at room temperature. Subsequently, binding to 10 nmol of labeled AP1 oligo was carried out in a reaction mixture containing 1 μg of poly(dI–dC), 10 mM Tris, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM EDTA for 30 min at room temperature.

Real-time reverse transcriptase-PCR (RT-PCR)

The expression of *MDR1* gene was determined using a TaqMan based real-time PCR method. To analyze and quantify the levels of the *MDR1* gene, we used a TaqMan isoform-specific probe (assay no. Hs00184491_m1; Applied Biosystems, Foster City, CA, USA). Amplification was performed using a total reaction volume of 25 μl in a MicroAmp Optical 96-well reaction plate. All real-time PCR reactions were carried out with TaqMan Universal Master Mix. Reactions were run on the ABI Prism 7300 sequence detection system (Applied Biosystems) using the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantification analysis was performed by the $\Delta\Delta C_t$ method and statistically analyzed by GraphPad Prism software.

siRNA experiments

CEM-R cells were transfected with a nucleofection device (Amaxa Inc., Cologne, Germany), using kit C and program X-001, according to the manufacturer's instructions. A total of 5×10^6 cells were transfected with 2 μg JNK small interfering RNA (siRNA) (sc-29380; Santa Cruz Biotechnology) or aspecific siRNA (siCONTROL riscfree no. 1; Dharmacon, Lafayette, CO, USA). After 72 h, cells were partly analyzed for real-time RT-PCR, and partly lysed for western blotting to assess the level of JNK expression.

Flow cytometric detection of Ser63 p-c-Jun levels

This was performed as reported by others,[25] using an anti-Ser63 p-c-Jun antibody (Cell Signaling), which was then detected with an FITC-conjugated anti-rabbit IgG (Dako, Glostrup, Denmark).

Flow cytometric analysis of P-gp expression and P-gp direct dye efflux assay

These were performed essentially as described.[21]

Results

Perifosine reduces cell viability and induces apoptosis in wild-type and P-gp-expressing T-ALL CEM cells

CEM cells contain constitutively elevated amounts of activated Akt, since they lack the PTEN gene.[26] To determine whether perifosine treatment would result in diminished survival and increased apoptosis, CEM-R and CEM-S cells were incubated in the presence of increasing concentrations of the drug for 24 h and viability was evaluated by MTT assays. As shown in Figure 1a, perifosine induced a dose-dependent decrease in viability in both cell lines. At 24 h, the IC_{50} was 6.8 μM for CEM-S cells, while it was 19.3 μM for CEM-R cells. The difference in sensitivity to perifosine could be due to a different level of Akt activation in these two cell lines. In fact, by western bloting and densitometric scanning we observed that CEM-R cells had much higher amounts of Ser473 p-Akt than CEM-S cells (Figure 1b). Thus, for all subsequent experiments, we used perifosine concentrations of 7 μM for CEM-S and 20 μM for CEM-R. It was next investigated whether decreased survival was related to apoptosis. In response to treatment with 7 μM perifosine for 6 h, approximately 50% of CEM-S cells were positive for Annexin V (early apoptotic cells), whereas 2% were positive for both Annexin-V and PI, and they represented late apoptotic/secondary necrotic cells (Figure 1c). Also CEM-R cells exhibited early apoptotic features when stained with Annexin V–FITC/PI. In fact, after 6 h of treatment, 55% of the cells were early apoptotic, whereas a 0.1% was late apoptotic/secondary necrotic (Figure 1c). The effect of perifosine on proliferation was analyzed at longer incubation times by direct cell counting.

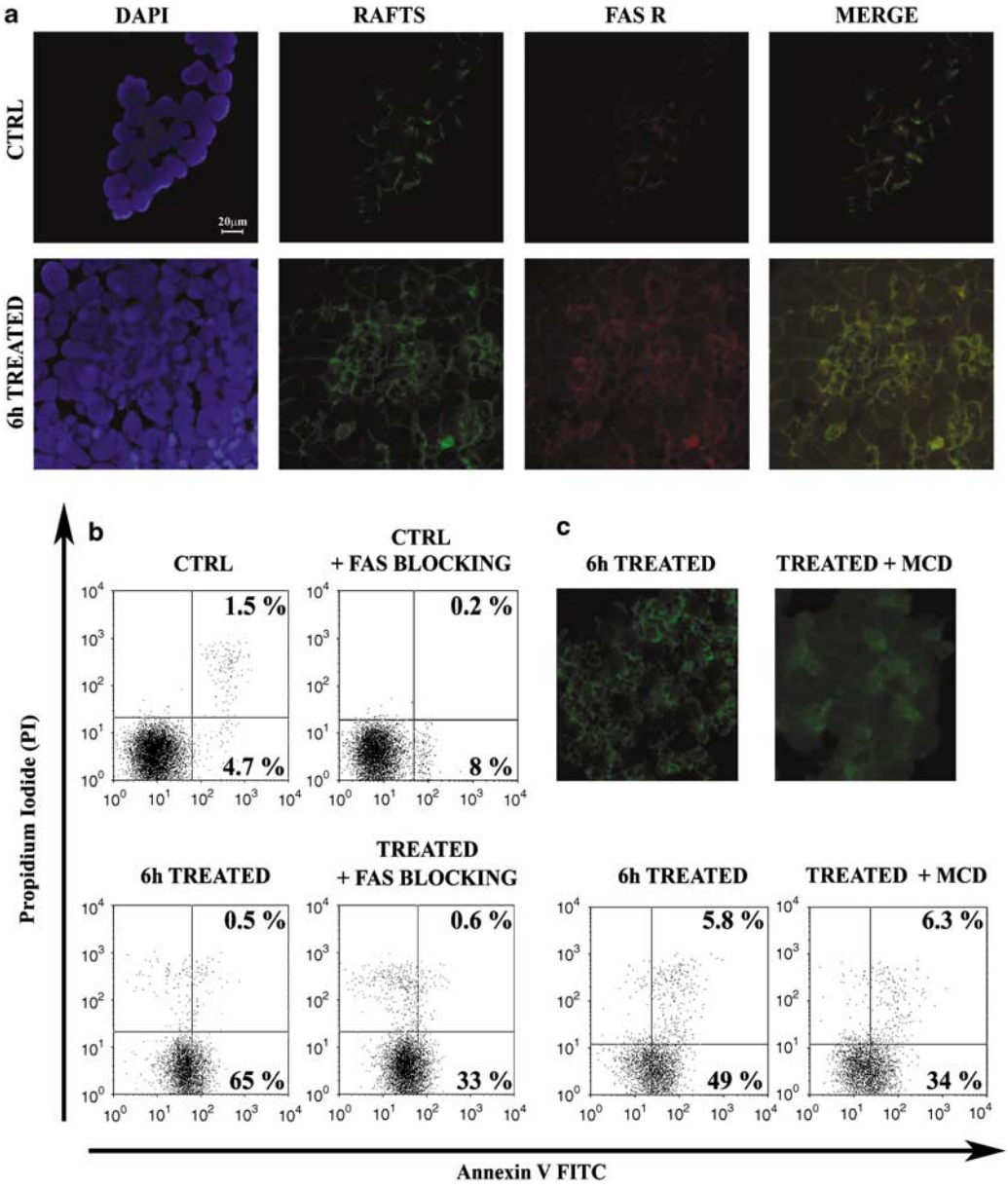

Figure 5 Perifosine-evoked apoptosis is partly dependent on Fas/FasL interactions and lipid raft integrity. (**a**) Immunofluorescence analysis. Control (CTRL) and perifosine-treated (20 μM for 6 h) CEM-R cells were stained with FITC-CTx B subunit (green), and then samples were incubated with 500 ng ml^{-1} of anti-human Fas (SM1/1) IgG2a mouse monoclonal antibody for overnight at 4 °C, which was detected by a Cy3-conjugated secondary antibody (red). Merging of the two fluorescent signals results in a yellow-orange color. Nuclei were counterstained for DNA by 4′,6-diamidino-2-phenylindole. (**b**) Representative histograms showing flow cytometric analysis of Annexin V–FITC/PI-stained cells. Cells were cultured with perifosine (20 μM) for 6 h in the presence or absence of a mouse monoclonal blocking antibody to human Fas (SM1/23, 2 μg ml^{-1}). The percentage of early apoptotic cells (lower right quadrant) and late apoptotic/necrotic cells (upper right quadrant) are indicated. One representative of three different experiments is shown. (**c**) Immunofluorescence analysis showing disruption of lipid rafts in cells treated with perifosine (20 μM) for 6 h in the presence or absence of methyl-β-cyclodextrin (15 μg ml^{-1}). The dot plots show Annexin V/PI staining in cells treated as above. The percentage of early apoptotic cells (lower right quadrant) and late apoptotic/necrotic cells (upper right quadrant) are indicated. One representative of three different experiments is shown. DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; FITC-CTx B, FITC-labeled cholera toxin-B; MCD, methyl-β-cyclodextrin; PI, propidium iodide.

Exponentially growing cells were treated with perifosine for 72 h and viable cells were counted every day. If the drug was not added again after 24 h of incubation, there was a slight increase in the cell number in both cell lines beginning at 24 h (Figure 2a). However, if cells where washed with serum-free medium and the drug was re-added, we observed that perifosine was able to completely block cell proliferation in both cell lines up to 72 h.

Perifosine treatment results in downregulation of p-Akt levels

We then focused our attention on whether the drug influenced the expression and phosphorylation levels of Akt in CEM-R cells. Western blotting analysis with phosphospecific Akt antibodies demonstrated complete dephosphorylation of Akt at Ser473 already after 6 h of incubation with 20 μM perifosine, whereas slight phosphorylation of Thr308 was still observable at 24 h of

incubation (Figure 2b). In CEM-R cells, we did not detect p-ERK 1/2. Perifosine did not affect ERK 1/2 phosphorylation positively, at variance with other reports,[20,27] and total levels of ERK 1/2 were unaffected by drug treatment (Figure 2b).

Perifosine treatment induces caspase-dependent cell death in CEM-R cells

It is well established that P-gp overexpression impairs caspase activation.[4] Our previous results highlighted that perifosine induces caspase-dependent cell death in Jurkat T-ALL cells.[20] Thus, we examined the possible activation of caspases in perifosine-treated CEM-R cells using various approaches. Western blotting analysis revealed that cleavage of apical procaspase-8 into the characteristic 43/41-kDa fragments was already evident after 3 h of treatment with perifosine, and then increased. Also, cleavage of apical procaspase-9 became evident at 3 h of treatment and increased in a time-dependent manner. Cleavage of procaspase-3 into the characteristic 17/19-kDa fragments was already detectable at 3 h of treatment with the inhibitor and displayed a time dependent increase. Caspase activation was accompanied by cleavage of the caspase-3 substrate, PARP, and by Bid cleavage (Figure 3a), and it was also examined by flow cytometric analysis of FLICA-stained samples. This technique confirmed activation of caspase-8 and caspase-3/7 by perifosine (Figure 3b). Also, immunofluorescence staining with cleaved caspase-3-specific antibody demonstrated caspase-3 upregulation at 6 h of treatment (Figure 3c). Moreover, the relevance of caspase activation for perifosine-induced apoptosis was demonstrated by pretreatment of CEM-R cells with selective pharmacological inhibitors of either caspase-8 or caspase-3, which markedly attenuated apoptosis elicited by perifosine (Figure 3d). These inhibitors induced some cytotoxicity in untreated cells. This is consistent with a previous report indicating that caspase inhibitors alone could induce a modest apoptosis in T-ALL cell lines.[28]

Perifosine induces cytochrome c *release from mitochondria*

Caspase-9 and Bid cleavage suggested that the intrinsic (mitochondrial) pathway of apoptosis could be upregulated by perifosine in CEM-R cells. To demonstrate the involvement of the mitochondrial pathway, we analyzed cytochrome *c* release from mitochondria. Double immunofluorescence staining demonstrated that in untreated cells, cytochrome *c* and complex V colocalized at the mitochondrial level (Figure 4a). In contrast, in perifosine-treated cells, the two signals (green for cytochrome c and red for complex V) were completely separated, indicating release of cytochrome *c* from mitochondria.

This finding was corroborated by western blot analysis performed on the isolated mitochondrial fraction, which demonstrated robust decrease of cytochrome *c* remaining in mitochondria, whereas the amount of the mitochondrial marker cytochrome *c* oxidase IV[29] did not change (Figure 4b).

Perifosine cytotoxicity in CEM-R cells is dependent on lipid raft integrity and Fas ligation

Very recent results have highlighted that in multiple myeloma cells, perifosine-induced apoptosis was dependent on recruitment of Fas/CD95 death receptor, Fas-associated death domain-containing protein and procaspase-8 into lipid rafts, leading to formation of the death-inducing signaling complex.[16] Thus, we determined whether this occurred in CEM-R cells. By immuno-

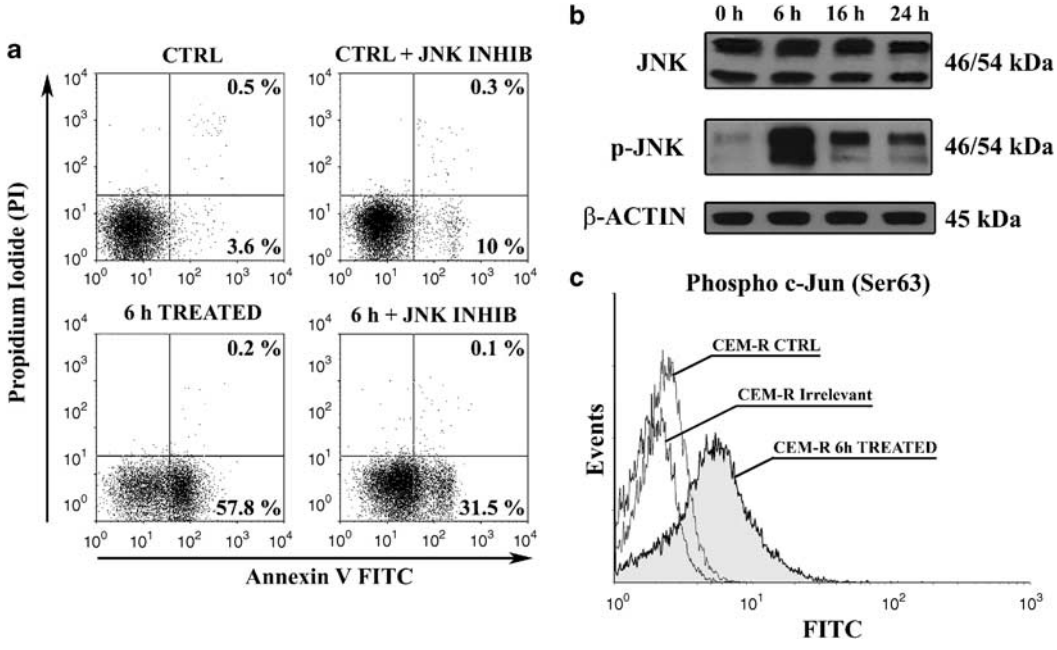

Figure 6 Perifosine cytotoxicity is dependent on JNK and c-Jun activation in CEM-R cells. (**a**) Representative histograms showing flow cytometric analysis of Annexin V–FITC/PI-stained cells. Cells were cultured with perifosine (20 μM) for 6 h in the presence or absence of the JNK-selective inhibitor SP600125 (10 μM). The percentage of early apoptotic cells (lower right quadrant) and late apoptotic/necrotic cells (upper right quadrant) are indicated. One representative of three different experiments is shown. (**b**) Western blot analysis. Whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose, which were then probed by antibody to total JNK and Thr183/Tyr185 p-JNK. Molecular weights are indicated to the right. One representative of three different experiments is shown. (**c**) Flow cytometric detection of Ser63 p-c-Jun. CEM-R cells were treated with perifosine for 6 h, reacted with a primary antibody to p-c-Jun (Ser63) and then a secondary FITC-conjugated secondary antibody was used. At least 15 000 events were analyzed. CTRL, untreated cells; FITC, FITC, fluorescein isothiocyanate; JNK, c-Jun NH_2-terminal kinase; PI, propidium iodide.

cytochemical staining, we demonstrated that, in response to perifosine treatment, there was an increase in colocalization of FITC-CTx B subunit, which binds ganglioside GM1 mainly found in rafts (see Schon and Freire[30]), with Fas (Figure 5a). Moreover, a Fas-blocking monoclonal antibody markedly reduced perifosine-dependent cell death (Figure 5b). In addition, we analyzed the effect of lipid raft disruption on perifosine-mediated apoptosis. Methyl-β-cyclodextrin treatment resulted in disruption of lipid rafts as indicated by fluorescence microscopy analysis of FITC-CTx B subunit-stained samples (Figure 5c). Methyl-β-cyclodextrin interfered with protein association with lipid rafts by cholesterol depletion,[31] and resulted in a decrease in the number of apoptotic cells (Figure 5c). Taken together, these findings strongly suggested that in CEM-R cells, apoptotic cell death is, at least in part, dependent on lipid raft integrity and Fas/Fas ligand (FasL) interactions.

Perifosine activates JNK and c-Jun in CEM-R cells

Since previous findings hinted that JNK could be another important mediator of perifosine cytoxicity in multiple myeloma cells,[27] it was investigated whether this was true also for CEM-R cells. In cells preincubated with the JNK-selective inhibitor SP600125 and then treated for 6 h with perifosine, there was a marked decrease in apoptosis, as revealed by Annexin-V/PI staining (Figure 6a). Moreover, perifosine dramatically increased JNK phosphorylation at Thr183/Tyr185, as indicated by western blot analysis (Figure 6b). Perifosine induced c-Jun phosphorylation at Ser63, a JNK-specific target, as demonstrated by flow cytometric analysis of CEMR cells (Figure 6c).

Perifosine causes downregulation of MDR1 gene expression and enhances the DNA-binding activity of AP1 through JNK activity

We determined whether perifosine affected the expression of P-gp. Real-time RT-PCR analysis demonstrated that transcription of the *MDR1* gene was significantly reduced by perifosine treatment at 6 h. In contrast, treatment with the JNK inhibitor significantly opposed the downregulation of P-gp mRNA caused by perifosine (Figure 7a). Moreover, in cells treated with siRNA specific for JNK, the perifosine-dependent decrease in P-gp mRNA levels was not detected (Figure 7b). Downregulated JNK expression by siRNA was verified by western blot analysis (Figure 7c). The *MDR1* gene promoter was previously reported to contain an AP1-binding site, which functions as a negative element for its regulation.[32,33] We investigated AP1 activation by electrophoretic mobility-shift assay in response to perifosine treatment. As shown in Figure 7d, after 6 h of treatment with perifosine, AP1-binding activity increased when compared with untreated cells, whereas the JNK inhibitor opposed perifosine-mediated increase in AP1 activity. Moreover, we performed gel supershift assays. As expected, the band disappeared by addition of the c-Jun antibody, and was strongly reduced by addition of c-Fos antibody (Figure 7d).

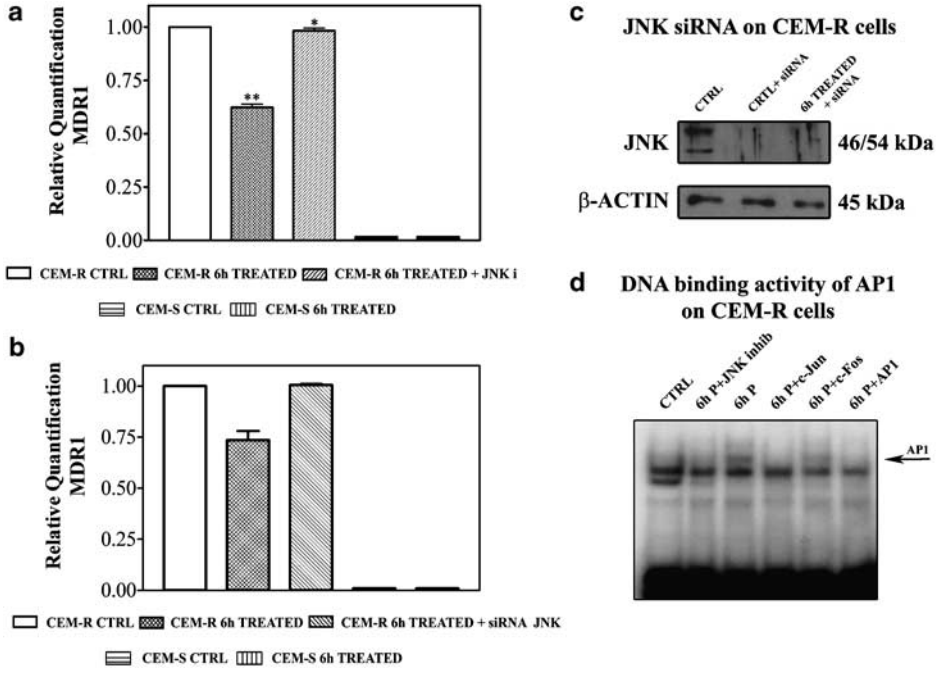

Figure 7 Perifosine downregulates P-gp mRNA in CEM-R cells. (**a**) Real-time RT-PCR. Results are from three different experiments ± s.e. One asterisk indicates statistically significant difference ($P<0.001$) with respect to untreated cells (CTRL), whereas two asterisks indicate statistically significant difference ($P<0.001$) with respect to cells treated with perifosine alone. (**b**) Real-time RT-PCR of cells treated with JNK siRNA. In this panel, CEM-R CTRL and CEM-R 6 h TREATED were transfected with aspecific siRNA. Results are from two different experiments ± s.e. (**c**) Western blot analysis of JNK downregulation by siRNA. CTRL, cells were transfected with 2 μg of aspecific siRNA for 72 h; ctrl. + siRNA, cells were transfected for 72 h with 2 μg of JNK-specific siRNA; 6 h treated + siRNA, cells were transfected for 72 h with 2 μg of JNK-specific siRNA and then treated 6 h with perifosine. A 20-μg weight of protein from total cell lysate was analyzed by western blotting with the indicated antibodies. (**d**) Effect of perifosine on AP1 DNA-binding activity by electrophoretic mobility-shift assay. CTRL, untreated cells; 6 h P + JNK inhib: perifosine-treated cells preincubated with JNK inhibitor; 6 h P, cells treated with perifosine alone for 6 h; 6 h P + c-Jun, nuclear extracts from perifosine-treated cells were preincubated with c-Jun antibody; 6 h P + c-Fos, nuclear extracts from perifosine-treated cells were preincubated with c-Fos antibody; 6 h P + AP1, nuclear extracts from perifosine-treated cells were preincubated with 100-fold molar excess of unlabeled AP1 oligonucleotide. One representative of three different experiments is shown. JNK, JNK, c-Jun NH_2-terminal kinase; RT-PCR, reverse transcriptase-PCR; siRNA, small interfering RNA.

Perifosine downregulates P-gp protein expression and activity

Western blot analysis corroborated real-time RT-PCR assay, showing strong decrease in P-gp protein levels at 24 h of treatment (Figure 8a), whereas at 16 h there was a slight decrease. Moreover, surface expression of P-gp was also diminished at 16 h, as indicated by flow cytometric analysis (Figure 8b). Overall, these findings indicated that perifosine is capable of downmodulating P-gp expression at both mRNA and protein levels, and that JNK activity plays an important role in this phenomenon. Finally, a functional assay for P-gp activity, based on Rhodamine 123 extrusion, demonstrated that already at 6 h of perifosine treatment, there was a marked decrease in P-gp activity (Figure 8c).

Discussion

Perifosine is an alkylphospholipid, a novel class of antitumor agents. Alkylphospholipids have three main effects, which might explain their cytotoxicity. First, they interfere with multiple cellular processes, including phospholipid turnover and lipid-dependent signal transduction pathways. Second, they induce stress signaling and apoptosis through caspase activation. Third, they inhibit survival and proliferation pathways, including those for PI3K/Akt and ERK 1/2.[23] In phase-I/II studies for advanced solid tumors, perifosine did not cause significant hematological toxicity.[15,34] This is consistent with our recent results, which showed no cytotoxic effect of perifosine in $CD34^+$ hematopoietic precursors from healthy donors.[22] Here, we have addressed perifosine efficacy in decreasing cell survival of PTEN-negative CEM T-ALL cells, including a subclone, which overexpresses P-gp. Perifosine, in a dose-dependent manner, decreased survival of both CEM-R and CEM-S cells. The IC_{50} was lower in CEM-S than in CEMR cells, 6.8 vs 19.3 μM, respectively, and this could have resulted from the lower level of Akt expression in CEM-S than in CEM-R. It should be emphasized here that 19.3 μM perifosine corresponds to 8.9 mg ml^{-1}.[17] Interestingly, during a phase-I trial of perifosine, the maximum tolerated dose was determined to be 150 mg for six doses load and 100 mg once daily maintenance.[15] This schedule resulted in a steady-state plasma concentration of perifosine, which was approximately 8.8 mg ml^{-1}. Hence, the *in vitro* perifosine bioactive range for CEM-R cells is within the

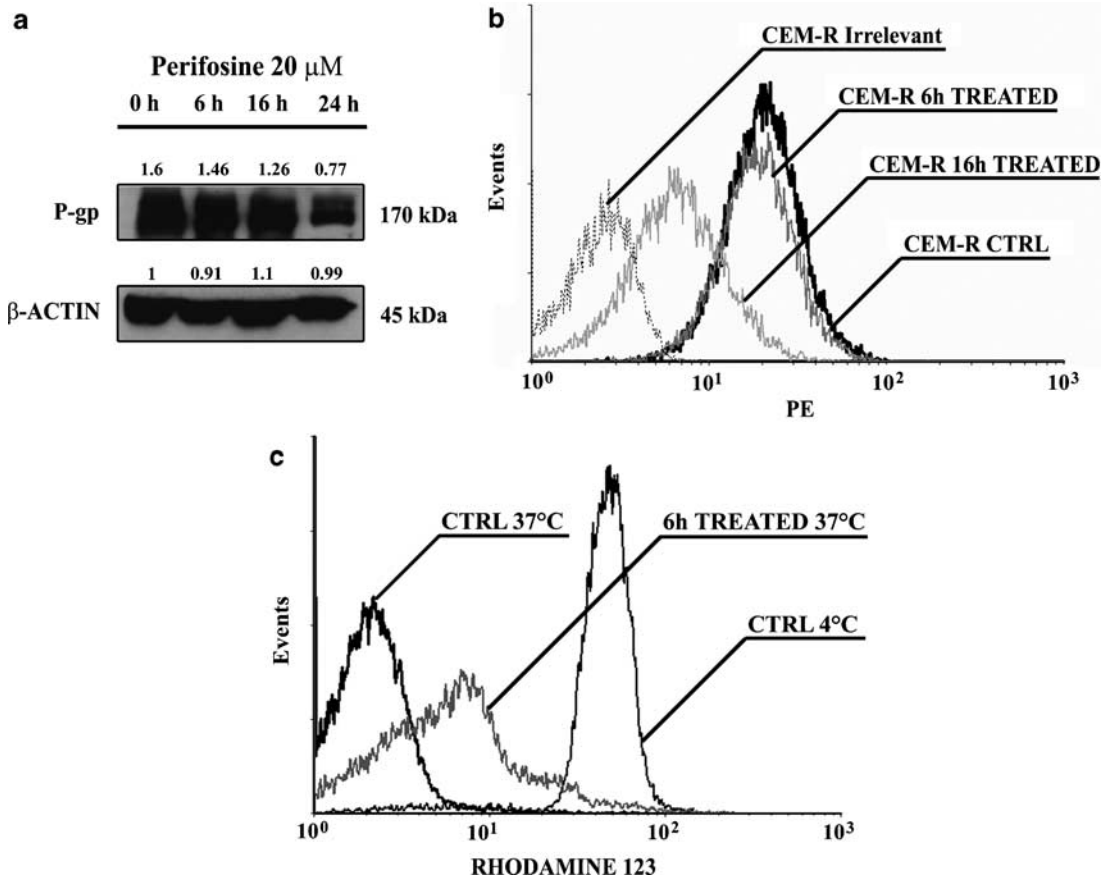

Figure 8 Perifosine downregulates P-gp protein expression in CEM-R cells. (**a**) Western blot analysis. Whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, which were then probed with antibody to P-gp or β-actin. Molecular weights are indicated to the right. The values indicate the results from densitometric analysis. One representative of three different experiments is shown. (**b**) Flow cytometric detection of surface P-gp. CEM-R cells were treated with perifosine for 6 or 16 h. A phycoerythrin-conjugated monoclonal antibody to P-gp was used. Analysis was carried out on healthy cells, which were gated by physical parameters (side and forward scatter). (**c**) Flow cytometric functional efflux activity of 170 kDa P-gp in CEM-R cells. The assay is based on extrusion of the fluorescent P-gp substrate, Rhodamine 123. The efflux activity of P-gp is highly temperature-sensitive; P-gp functions optimally at 37 °C, but is inactive at 4 °C. When preloaded with Rhodamine 123 and incubated at 4 °C (CTRL), CEM-R cells retained the dye and consequently exhibited high fluorescence. Conversely, cells incubated at 37 °C (CTRL) effluxed the dye. CEM-R cells treated with perifosine (6 h treated 37 °C), showed strong decrease in the pump activity. PE, phycoerythrin; P-gp, P-glycoprotein.

concentrations, which could be reached *in vivo*. The decreased cell survival was due to apoptosis, as indicated by flow cytometric analysis of samples stained with Annexin-V/PI. In CEM-R cells, we observed a marked dephosphorylation of Akt on both Ser473 and Thr308. Perifosine did not result in ERK 1/2 activation in CEM-R cells although in some previous studies upregulated ERK 1/2.[20,27]

A remarkable finding of our investigation is that perifosine treatment resulted in activation of multiple caspases in CEM-R cells including apical caspase-8 and caspase-9, and executioner caspase-3. Caspase-3 activation was accompanied by cleavage of the caspase-3 substrate, PARP. Usually, cells overexpressing P-gp display impaired caspase activation.[4] In our experiments, selective caspase-3 and caspase-8 pharmacological inhibitors attenuated the percentage of CEM-R cells undergoing apoptosis in response to perifosine. This finding indicates the relevance of caspases in perifosine-induced cell death, but it also suggests that caspase-independent cell death signaling pathways are activated by the drug in CEM-R cells, as reported for other cell types, including Jurkat T-ALL cells[20] and non-small lung carcinoma cell lines.[18] Cleavage of both caspase-8 and caspase-9 demonstrated that perifosine activated the extrinsic as well as the intrinsic apoptotic pathway. A linkage between the two pathways could occur via Bid. Indeed, it is well known that active caspase-8 cleaves Bid and that truncated Bid migrates to mitochondria where it induces cytochrome *c* release. Consistently, perifosine induced Bid cleavage in CEM-R cells. Moreover, cytochrome *c* release from mitochondria in perifosine-treated CEM-R cells was demonstrated by immunofluorescence staining and western blot analysis performed on the purified mitochondrial fraction, thus indicating the intrinsic apoptotic pathway activation. Our findings suggest that lipid raft integrity is important for perifosine-mediated apoptosis in CEM-R cells. However, in multiple myeloma cells, perifosine-mediated cytotoxicity was FasL independent,[16] whereas in CEM-R cells, the perifosine proapoptotic effect was at least partly dependent on FasL, because a blocking monoclonal antibody, which abrogates Fas/FasL-mediated killing, significantly enhanced survival of cells treated with the drug. Our findings highlight that JNK activation is also important for perifosine-mediated cytoxicity in CEM-R cells, as perifosine increased JNK phosphorylation and a selective pharmacological inhibitor of JNK markedly enhanced survival in perifosine-treated cells.

We have also shown for the first time that perifosine was capable of reducing P-gp mRNA and protein expression. Interestingly, this phenomenon could be reverted by a JNK inhibitor. Also, siRNA downregulation of JNK, impeded the perifosine-evoked P-gp mRNA decrease. Albeit mRNA downregulation was detected as early as after 6 h of treatment, the decrease in P-gp protein was slightly apparent at 16 h and was extremely evident at 24 h. This most likely depends on the fact that a P-gp half-life as long as 15–18 h has been reported in some MDR cancer cell lines.[33,35] Thus, our results are consistent with reports highlighting that in solid tumor cell lines and K562 erythroleukemia cells, P-gp mRNA downregulation required the catalytic activity of JNK and was mediated by the c-Jun transcription factor.[33] Accordingly, in our experiments, perifosine resulted in c-Jun phosphorylation on Ser63 and activation of AP1 transcriptional activity, which could be counteracted by a selective JNK inhibitor. At present, the transcriptional regulation of *MDR1* gene is poorly understood. The *MDR1*-gene promoter has been reported to contain a negative binding site of the transcription factor activator protein AP1.[32] Besides AP1, other transcription factors involved in the regulation of *MDR1* gene transcription include HSF1 and MEF1. Also, tumor-suppressor protein p53 has been both positively or negatively implicated in regulating *MDR1* transcription.[1]

The finding that P-gp activity was downregulated by perifosine at 6 h (that is, a time point where P-gp protein expression levels were not affected by the drug) is puzzling. However, it is established that P-gp exists in lipid rafts,[36] where it displays a more active state than when localized outside rafts.[37] Since perifosine specifically accumulates in lipid rafts,[38] it might be that this alkylphospholipid would somehow interfere with P-gp activity and/or localization in lipid rafts, ensuing in downregulation of the pump activity. Further experiments are clearly required to investigate this issue. Activation of the PI3K/Akt pathway is frequently observed in T-ALL patients (56%) and likely contributes to drug resistance.[39,40] In some T-ALL cases, increased PI3K/Akt signaling could be due to PTEN-gene hypermethylation resulting in diminished expression of the PTEN gene.[41] P-gp overexpression is also detected in T-ALL patients (24%) and negatively correlates with complete remission achievement.[42] Therefore, it is likely that some T-ALL patients would exhibit PI3K/Akt upregulation together with P-gp overexpression, even if this has not been shown in a conclusive manner. In conclusion, our pre-clinical studies support the concept that inhibition of PI3K/Akt signaling with perifosine may have clinical application for treatment of drug resistant disease or elimination of leukemic stem cells. At the same time, it is difficult to state whether perifosine will have a clinical future, since the drug has not yet been studied extensively in preclinical models or phase-I/II trials for malignant hematological disorders.

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