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ORIGINAL ARTICLE

Proapoptotic activity and chemosensitizing effect of the novel Akt inhibitor perifosine in acute myelogenous leukemia cells

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The serine/threonine kinase Akt, a downstream effector of phosphatidylinositol 3-kinase (PI3K), is known to play an important role in antiapoptotic signaling and has been implicated in the aggressiveness of a number of different human cancers including acute myelogenous leukemia (AML). We have investigated the therapeutic potential of the novel Akt inhibitor, perifosine, on human AML cells. Perifosine is a synthetic alkylphospholipid, a new class of antitumor agents, which target plasma membrane and inhibit signal transduction networks. Perifosine was tested on THP-1 and MV 4-11 cell lines, as well as primary leukemia cells. Perifosine treatment induced cell death by apoptosis in AML cell lines. Perifosine caused Akt and ERK 1/2 dephosphorylation as well as caspase activation. In THP-1 cells, the proapoptotic effect of perifosine was partly dependent on the Fas/FasL system and c-jun-N-kinase activation. In MV 4–11 cells, perifosine downregulated phosphorylated Akt, but not phosphorylated FLT3. Moreover, perifosine reduced the clonogenic activity of AML, but not normal, CD34$^+$ cells, and markedly increased blast cell sensitivity to etoposide. Our findings indicate that perifosine, either alone or in combination with existing drugs, might be a promising therapeutic agent for the treatment of those AML cases characterized by upregulation of the PI3K–Akt survival pathway.
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Introduction

It is now well established that the reduced capacity of tumor cells of undergoing apoptosis is the main point in the pathogenesis and progression of a large number of neoplasias as well as in therapeutic treatment failure.[1,2] A signaling pathway of great importance in the regulation of cell-cycle progression, proliferation and apoptosis is the phosphatidylinositol-3 kinase (PI3K)–Akt network.[3] Indeed, over the last few years many studies have provided convincing evidence that constitutive activation of this signaling network is involved in the aggressiveness and resistance to therapeutic treatments of a large number of malignant diseases.[4–6] Accordingly, the dysregulated activation of the PI3K–Akt survival pathway is often associated with hematological malignancies, including acute and chronic human leukemias.

Once activated by different growth factors and cytokines, PI3K localizes to the plasma membrane and catalyzes the γ-phosphate transfer of ATP to the D3 position of phosphoinositides, thereby producing phosphatidylinositol[3–5] triphosphate (PIP_3), which in turn activates a number of critical downstream substrates, including the serine/threonine kinase Akt. Akt recruitment to the plasma membrane provides conformational changes, which enable the phosphorylation of both the catalytic loop at Thr 308 and the C-terminal hydrophobic tail at Ser 473. The former phosphorylation is effected by phosphoinositide-dependent kinase 1 (PDK-1);[7] whereas the latter is performed by a kinase not yet clearly identified, that could be the mammalian target of rapamycin–rictor (mTORC2) complex.[8] Activated Akt is known to function as an essential survival factor by inhibiting apoptosis through its ability to phosphorylate several targets, including Bad, FoxO transcription factors, Raf-1 and caspase-9 which are critical for cell survival.[7] Therefore, small inhibitory molecules of this pathway are currently being developed for clinical use.[9,10] Indeed, over the last few years, several Akt inhibitors have been synthesized, including perifosine, a novel phospholipid analogue which has shown promising preclinical activity and is currently undergoing phase I/II clinical evaluation.[11–14] Serum concentrations up to 16.5 μM perifosine, have been reached during clinical evaluation.[15] Although the molecular mechanisms underlying the anticancer activity of perifosine remain to be fully elucidated, it has been highlighted it has two main effects. Perifosine, via its interference with the turnover and synthesis of natural phospholipids, disrupts membrane-linked signaling pathways at several sites including lipid rafts, thereby inhibiting PI3K–Akt survival network. Perifosine capability to inhibit Akt phosphorylation depends on its interaction with the PH domain of the kinase, which prevents Akt membrane localization and phosphorylation.[16,17] In addition to inhibition of PI3K–Akt signaling, in some cell types perifosine downregulates the antiapoptotic mitogen-activated protein kinase–extracellular signal-regulated kinase (MEK–ERK) 1/2 pathway and activates the proapoptotic c-jun-N-kinase (JNK) network, thus modulating the balance between the survival and death signaling cascades, thereby inducing apoptosis.[16,18]

At present, perifosine is the most developed Akt inhibitor from a clinical point of view.[19] The PI3K–Akt pathway is activated in most acute myelogenous leukemia (AML) cases, where it markedly influences survival and chemosensitivity.[20–25] Akt is

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also activated in high-risk myelodysplastic syndrome, a hematopoietic stem cell disorder which frequently evolves into AML.[26]

Clinical trials are now underway to test perifosine efficacy in AML patients (see for example, http://clinicaltrials.gov/ct/show/NCT00391560;jsessionid=E974BADE9686AD752E4E88859929DAC5?order=4). Surprisingly, however, there is only one preclinical study that has addressed the issue of perifosine cytotoxicity on AML cells. In that investigation, a synergism between perifosine and histone deacetylase inhibitors was observed in U937 and HL60 human leukemia cell lines.[27] With the above in mind, we undertook this study aimed at better evaluating the *in vitro* efficacy of perifosine on AML cell lines and blasts. We demonstrate that perifosine negatively affects survival of both THP-1 and MV 4-11 AML cells. Perifosine-mediated cytotoxicity was accompanied by a dephosphorylation of Akt and ERK 1/2, and caspase activation. Decreased cell survival induced by perifosine could be counteracted by treatment with antibodies blocking either Fas/CD95 or Fas ligand (FasL). Perifosine also decreased the clonogenic activity of $CD34^+$ cells from AML patients with active Akt, and increased etoposide sensitivity of AML blasts. Therefore, our findings suggest that perifosine, either alone or in combination with existing drugs, could represent an effective approach for PI3K–Akt and ERK 1/2 signaling downregulation in AML and advocate for its use in therapeutic treatments of this hematological malignant disorder.

Materials and methods

Chemicals and antibodies

Perifosine was kindly provided by AEterna Zentaris GmbH (Frankfurt, Germany). The powder was dissolved in H_2O to provide a final concentration of 50 mM. Aliquots were stored at −20 °C. For cell viability determination, Cell Viability Kit I (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)) was purchased from Roche Applied Science (Penzberg, Germany). Propidium iodide (PI, DNA-Prep kit) was from Beckman Coulter Immunology (Miami, FL, USA). The Annexin V-FITC (fluorescein isothiocyanate) staining kit was from Tau Technologies BV (Kattendijke, The Netherlands), while carboxyfluorescein fluorescent-labeled inhibitor of caspases (FLICA) Apoptosis Detection Kits for caspase activity assay were from AbD Serotec (Oxford, UK). Etoposide, granulocyte–macrophage-colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and IL-6, were from Sigma-Aldrich, St Louis, MO, USA. LY294002, PD98059 and the JNK inhibitor SP600125 were from EMD Biosciences, La Jolla, CA, USA. The following antibodies, all from Cell Signaling Technology, Danvers, MA, USA, were employed for western blot analysis: to Akt (catalog no. 9272); to Ser 473 p-Akt (9271); to Thr 308 p-Akt (9275); to ERK 1/2 (9102); to Thr 202/Tyr 204 p-ERK 1/2 (9106); to Ser 221 p-MEK 1/2; to p70S6K (2708); to Thr 389 p-p70S6K (9206); to caspase-2 (2224); to caspase-3 (9665); to caspase-6 (9762); to caspase-7 (9492); to caspase-8 (9746); to caspase-9 (9502); to poly (ADP-ribose) polymerase (PARP, 9542); to mTOR (2972); to Raptor (4978); to Rictor (2114); to Ser 241 p-PDK-1; to Thr 423 p-PAK-1 (p21 activated kinase-1); to actin (4967). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology, while SuperSignal West Pico Chemiluminescent Substrate was from Pierce Biotechnology (Rockford, IL, USA). For flow cytometric analysis: anti-Ser 112 p-Bad; Alexa Fluor 488 conjugate anti-Tyr 591 p-FLT3 (3549), Alexa Fluor 647 conjugate anti-Ser 473 p-Akt (2337), Alexa Fluor 647 conjugate anti-Thr 202/Tyr204 p-ERK 1/2, and Alexa Fluor 647 conjugate anti-Thr183/Tyr 185 p-JNK conjugate were from Cell Signaling Technology. FITC-conjugated anti-Fas and anti-FasL mouse monoclonal antibodies were from Ancell, Bayport, MN, USA. FITC-conjugated mouse IgG1 was from Chemicon, Temecula, CA, USA. Blocking antibodies to either Fas or FasL were from Alexis Biochemicals, Lausen, Switzerland.

Cell culture

THP-1 human acute monocytic leukemia and MV 4–11 human biphenotypic B myelomonocytic leukemia cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at a cell density between 4×10^5 and 1×10^6 cells per ml. Cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2.

Cell viability analysis by MTT assay

An MTT assay was employed to analyze cell growth and viability. Briefly, cells were plated in a 96-well plate (3×10^4 cells per well, 100 μl cell suspension per well) and cultured overnight in 10% serum to allow for exponential growth of cells. Cells were then treated with perifosine for the indicated times. At the end of the treatment, 10 μl 1 × MTT labeling reagent was added to each well and incubated for another 4 h. The samples were solubilized with 100 μl 10% sodium dodecylsulfate in 10 mM HCl, overnight at 37 °C. The optical density absorbance value of each well was read on a Minireader at 570 nm. Every sample was performed in triplicate and in three independent experiments. The fraction of viable cells was calculated as follows: mean optical density value treated cells/mean optical density value control cells.

Flow cytometric detection of apoptosis

Apoptosis was determined by the binding of Annexin V-FITC to phosphatidylserine exposed to the cell membrane according to the manufacturer's instructions. A PI (5 μg ml^{-1}) staining was also performed to simultaneously detect different populations of living, apoptotic and necrotic cells. Briefly, 1×10^6 cells per sample were collected by centrifugation, washed in phosphate-buffered saline (PBS, pH 7.4), and incubated with Annexin V-FITC alone or in combination with PI. Fluorescence resulting from both FITC and PI was measured at 530 and 620 nm, respectively. Samples were analyzed by EPICS XL flow cytometer supported by dedicated software (Beckman Coulter Immunology).

Whole-cell lysate preparation and western blot analysis

THP-1 cells were plated in RPMI complete medium at an initial concentration of 5×10^5 cells per ml and incubated for 24 h before drug treatment, as reported above. Cells were collected by centrifugation and washed in PBS. Cell pellets were then lysed in 150–200 μl of M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology) supplemented with Na_2VO_3 (200 mM) and Protease Inhibitor Cocktail (40 μl ml^{-1}; Roche Applied Science), and incubated on ice for 10 min. Cell debris were removed by centrifugation at 13 000 r.p.m. for 15 min at 4 °C in a microfuge. Protein supernatants were collected, heated to 95 °C for 5 min, cooled on ice and were used for further analysis or stored at −80 °C. Equal amounts of protein samples (40 μg) were separated on sodium dodecylsulfate-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes. Membranes were briefly washed in PBS-0.1% Tween-20 (PBS-T) and nonspecific binding sites were blocked in

5% non-fat dry milk in PBS-T for 1 h at room temperature with gentle agitation. After PBS-T washes, membranes were incubated overnight at 4 °C with primary antibodies diluted according to the manufacturer's instructions. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 in 5% non-fat dry milk in PBS-T for 1 h at room temperature with gentle agitation. Antibody binding was detected by the SuperSignal West Pico Chemiluminescent Substrate.

Immunoprecipitation of mTORC complexes

This was accomplished as reported elsewhere.[28]

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. FLICA assay uses a cell permeable inhibitor sequence of caspases linked to a green (carboxyfluorescein) fluorescent probe. If there is an active caspase enzyme, it will form a covalent bond with FLICA and retain the fluorescent signal within the cell.

THP-1 cells were plated and treated with perifosine as mentioned above. After 24 h, cells were collected by centrifugation and washed in 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 were washed two times in PBS. Fluorescence resulting from the FLICA reagent was measured at 488 nm by flow cytometry (EPICS XL flow cytometer).

Flow cytometric detection of p-FLT3, p-Akt, p-Bad, p-ERK 1/2, p-JNK, Fas and FasL

This was performed essentially as reported previously.[29] Briefly, cells were fixed with Reagent 1 of the Intraprep kit, according to the manufacturer's instructions (Beckman Coulter Immunology), permeabilized with saponin-base reagent 2, and incubated at 4 °C overnight with Alexa Fluor 488 conjugate antibody to Tyr 591 p-FLT3 and Alexa Fluor 647 conjugate antibody to Ser 473 p-Akt. Both antibodies (2 µl per 10^5 cells) were diluted in PBS/0.5% bovine serum albumin. Alexa Fluor 647 conjugate antibodies to p-ERK 1/2 or p-JNK were used at the same concentration. For p-Bad detection, the primary antibody was diluted 1:100 and was revealed by a FITC-conjugated anti-rabbit antibody. After incubation with the antibodies, cells were washed with PBS and analyzed on dual-laser FC500 flow cytometer (Beckman Coulter Immunology). Anti-Fas and anti-FasL antibodies (final concentration: 10 µg ml^{-1}) were employed on fresh, unfixed cells (5×10^5), according to the manufacturer's procedure. Then cells were washed three times with PBS, fixed with 0.5% paraformaldehyde in PBS, and washed again three times in PBS. At least 5000 events were analyzed for each sample in all flow cytometric analyses.

Colony assay of AML cells

Samples were obtained from patients at presentation of AML and from healthy donors at the Policlinico S.Orsola-Malpighi Hospital. Informed consent was obtained from all patients and healthy donors before obtaining the samples, according to Institutional guidelines. Bone marrow or peripheral blood mononuclear cells were isolated by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density-gradient centrifugation. $CD34^+$ progenitor cells were then isolated using immunomagnetic cell separation (Miltenyi Biotec, Bergisch Gladbach, Germany). They were seeded at a density of 1000 per six-well plate in Methocult Methylcellulose medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 20 ng ml^{-1} each of GM-CSF, IL-3 and IL-6. Plates were incubated for 16 days, and the number of colonies was counted *in situ* by light microscopy at $\times 25$ magnification under an Olympus CKX 41 light microscope.

RT-PCR analysis for FasL mRNA

Primers were as follows: FasL forward, 5′-GCAGCCCTTCAATA CCCAT-3′; FasL reverse, 5′-CAGAGGTTGGACAGGGAAGAA-3′; glyceraldehyde-3-phosphate dehydrogenase forward 5′-TGAAG GTCGGAGTCAACGGATTTGGT-3′, reverse 5′-CATGTGGGCA TGAGGTCCACCAC-3′.[30] Thermal cycling conditions were an initial 2 min at 50 °C and 10 min at 95 °C. Cycle conditions were 15 s at 95 °C and 1 min at 60 °C. For FasL expression 50 cycles were performed, whereas for glyceraldehyde-3-phosphate dehydrogenase only 40 cycles of amplification were performed.

Statistical evaluation

The data are shown as mean values ± s.d. Data were statistically analyzed by a Dunnett test after one-way analysis of variance at a level of significance of $P<0.05$ vs control samples.

Results

Perifosine negatively affects cell survival and induces apoptosis in AML cell lines

To determine whether a treatment of AML cell lines with perifosine would result in diminished cell survival and increased apoptosis, THP-1 and MV 4–11 cells were incubated in the presence of increasing concentrations of the drug for either 24 or 48 h. To evaluate cell survival, MTT assays were employed. As shown in Figure 1a, perifosine caused a progressive decrease in THP-1 cell survival in a dose-dependent fashion. At 24 h, the IC_{50} was 15.8 µM. After a 48-h incubation, the cytotoxic effect was more evident also at much lower perifosine concentrations. As to MV 4–11 cells, they were more sensitive to the drug, as at 24 h the IC_{50} was 2.7 µM. Also in these cells, cytotoxicity markedly increased after 48 h of incubation with the drug.

It was next investigated whether decreased survival was related to apoptosis. To evaluate to what level PI staining represented the apoptotic cell population, a simultaneous detection of apoptosis by Annexin V-FITC and PI staining was performed, as described previously.[31] After treatment with 5 µM perifosine for 24 h, approximately 41% of THP-1 cells were positive for Annexin V (apoptotic cells), while 23% were positive for both Annexin V and PI and represented secondary necrotic cells (Figure 1b). Also MV 4–11 cells exhibited apoptotic features when stained with Annexin V-FITC/PI (data not shown).

Perifosine treatment results in downregulation of p-Akt and p-ERK 1/2 in THP-1 cells, as well as caspase activation and PARP cleavage

As perifosine is an Akt inhibitor, it was analyzed whether treatment with this chemical resulted in downregulation of Akt signaling. Western blot analysis with an antibody to Ser 473 p-Akt, demonstrated a marked decrease in this phosphorylated Akt form in response to a 24-h incubation with 0.1 µM perifosine (Figure 2a). At 1 µM perifosine, no Ser 473 p-Akt could be

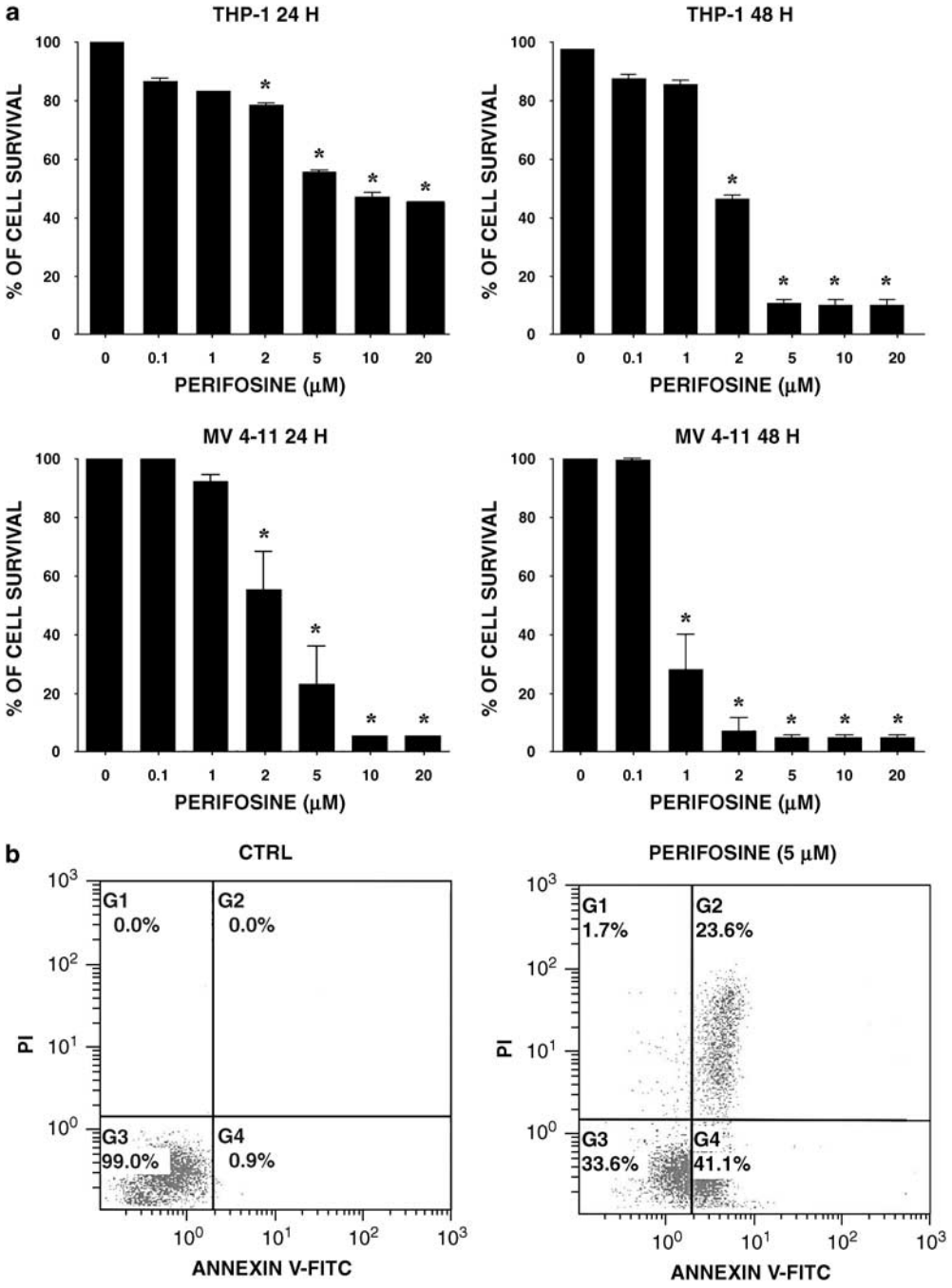

Figure 1 Perifosine cytotoxicity and apoptosis induction in AML cell lines. (**a**) MTT assays of THP-1 and MV 4–11 cells cultured with perifosine for 24 or 48 h. Results are the mean of three different experiments ± s.d. Asterisk indicates statistically significant difference ($P<0.005$) with respect to untreated cells. (**b**) Representative histograms showing flow cytometric analysis of Annexin V-FITC/PI-stained THP-1 cells. CTRL, untreated cells. Cells were cultured with perifosine for 24 h. AML, acute myelogenous leukemia; G1, necrotic cells; G2, secondary necrotic cells; G3, healthy cells; G4, apoptotic cells; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI, propidium iodide.

detected. Akt dephosphorylation on Ser 473 was a rapid event because it could be detected already after 30 min of incubation with 5 μM perifosine (data not shown).

Also Thr 308 p-Akt was dramatically downregulated by perifosine already at 0.1 μM. However, increasing the concentration of the drug did not result in further dephosphorylation of this p-Akt form. In contrast, the amount of total Akt protein was not affected by the inhibitor. Akt inhibition had functional consequences, because the well-established Akt downstream target p70S6K, showed a decreased phosphorylation level on a residue (Thr 389), which requires Akt activation, whereas the expression of total p70S6K was unaffected by the drug. Western blot analysis also demonstrated a decrease in the levels of Thr 202/Tyr 204 p-ERK 1/2 in response to perifosine treatment, which became evident at 1 μM and then slightly further diminished, whereas total levels of ERK 1/2 were unchanged. This was associated with MEK 1/2 dephosphorylation (Figure 2a).

It is well known that a distinguishing feature of apoptotic cell death is the activation of a family of proteases referred to as caspases,[32] which are responsible for most of the biochemical

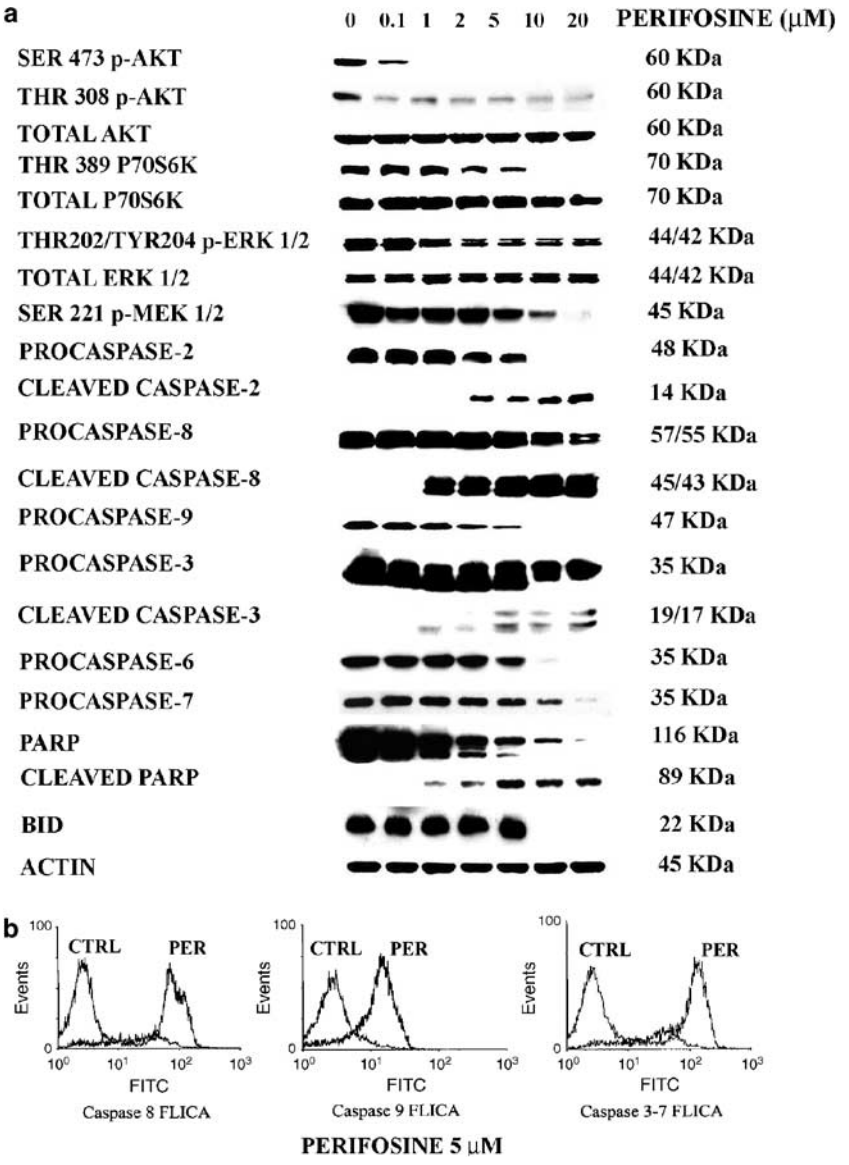

Figure 2 Perifosine induces Akt and ERK 1/2 dephosphorylation as well as caspase activation and PARP cleavage in THP-1 cells. (**a**) Western blot analysis. Cells were cultured for 24 h in the presence of increasing concentrations of perifosine. Whole cell lysates were subjected to SDS-PAGE, and electrotransferred to nitrocellulose membranes, which were then probed with the indicated antibodies. The molecular weight of the antigens is indicated at right. (**b**) Caspase activity analyzed by the FLICA assay. Cells were cultured for 24 h in the presence of 5 μM perifosine (PER). CTRL, untreated cells. One representative of three different experiments is shown. ERK, extracellular signal-regulated kinase; FLICA, fluorescent-labeled inhibitor of caspases; PARP, poly(ADP-ribose) polymerase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

and morphological changes which characterize this type of cell death. Western blotting analysis revealed that cleavage of apical caspase-8 was already evident at 1 μM perifosine, whereas cleavage of caspase-2 and decreased level of procaspase-9 became evident at 2 μM perifosine. As to executioner caspase-3, -6 and -7, they were all activated (Figure 2a). Cleavage of procaspase-3 into the characteristic 17/19-kDa fragments was already detectable at 1 μM perifosine, whereas decrease in both procaspase-6 and -7 was seen at higher concentrations of the drug (that is, 5 μM). Caspase activation was accompanied by the cleavage of the caspase-3 substrate PARP and by Bid cleavage.

Caspase activation was also examined by flow cytometric analysis of FLICA-stained samples. This technique confirmed activation of caspase-8, -9 and -3/7 at 5 μM perifosine for 24 h (Figure 2b). Pretreatment of THP-1 cells with selective caspase inhibitors strongly diminished the cytotoxic effects of perifosine (data not shown).

Perifosine does not affect PDK-1 phosphorylation and mTORC1/mTORC2 equilibrium in THP-1 cells

As perifosine interferes with the binding of the PH domain of Akt to PIP_3 and consequently impairs Akt phosphorylation on Thr 308,[17] it was investigated the phosphorylation state of the upstream kinase PDK-1, to rule out perifosine effects on targets other than Akt. PDK-1 phosphorylation on Ser 241 is thought to be essential for its activity.[33] However, perifosine did not affect the levels of Ser 241 p-PDK-1, in agreement with the observations of others[17,34] (Figure 3a). As a further proof that perifosine does not negatively affect PDK-1 activity, the phosphorylation state of Thr 423 PAK-1, a downstream target of

PDK-1,[35] was analyzed. Consistently, the drug did not change the levels of Thr 423 p-PAK-1 (Figure 3a). Because perifosine also caused dephosphorylation of Ser 473 p-Akt, we were interested in investigating if the drug could alter the equilibrium which exists between the mTORC1 complex (consisting of mTOR, Raptor, and mLST8) and the mTORC2 complex (consisting of mTOR, Rictor and mLST8). Indeed, there is evidence that the mTORC2 complex could be involved in Akt phosphorylation on Ser 473.[8] Perifosine, neither interrupted the mTORC1 and mTORC2 complex formation, which was unchanged, nor affected the overall expression levels of mTOR, Raptor or Rictor (Figure 3b). Moreover, to assess the relative contribution of PI3K–Akt and MEK–ERK 1/2 signaling to perifosine-induced apoptosis, p-Akt levels were downregulated by LY294002 (a selective PI3K inhibitor), while p-ERK 1/2 levels were downregulated by PD98059, a compound, which inhibits MEK. As demonstrated in Figure 3c, an almost complete downregulation of Thr 308 and Ser 473 p-Akt led to an approximately 40% decrease in THP-1 cell survival, whereas partial p-ERK 1/2 downregulation to a level similar to that obtained with perifosine, resulted in less than 20% survival decrease. Overall, these findings indicated that, in THP-1 cells an almost complete inhibition of the PI3K–Akt pathway by perifosine is more relevant to cell death than partial inhibition of MEK–ERK 1/2 signaling.

Perifosine treatment results in Bad dephosphorylation in THP-1 cells

A previous investigation has highlighted an important role played by Bad phosphorylation for AML cell survival.[36] It was therefore investigated by flow cytometric analysis if perifosine could dephosphorylate Bad on Ser 112, which is targeted by ERK 1/2, whereas PI3K–Akt signaling results in Bad phosphorylation on Ser 136.[37] As a positive control, we employed phorbol ester-stimulated Jurkat T-leukemia cells. Under normal growth conditions these cells are negative for p-ERK 1/2, whereas when stimulated with phorbol ester there is a robust ERK 1/2 phosphorylation.[29] Consistently, Ser 112 p-Bad was upregulated after phorbol ester treatment of Jurkat T-cells (Figure 3d). THP-1 cells were positive for Ser 112 p-Bad. A marked decrease in Ser 112 p-Bad phosphorylation levels was seen in response to

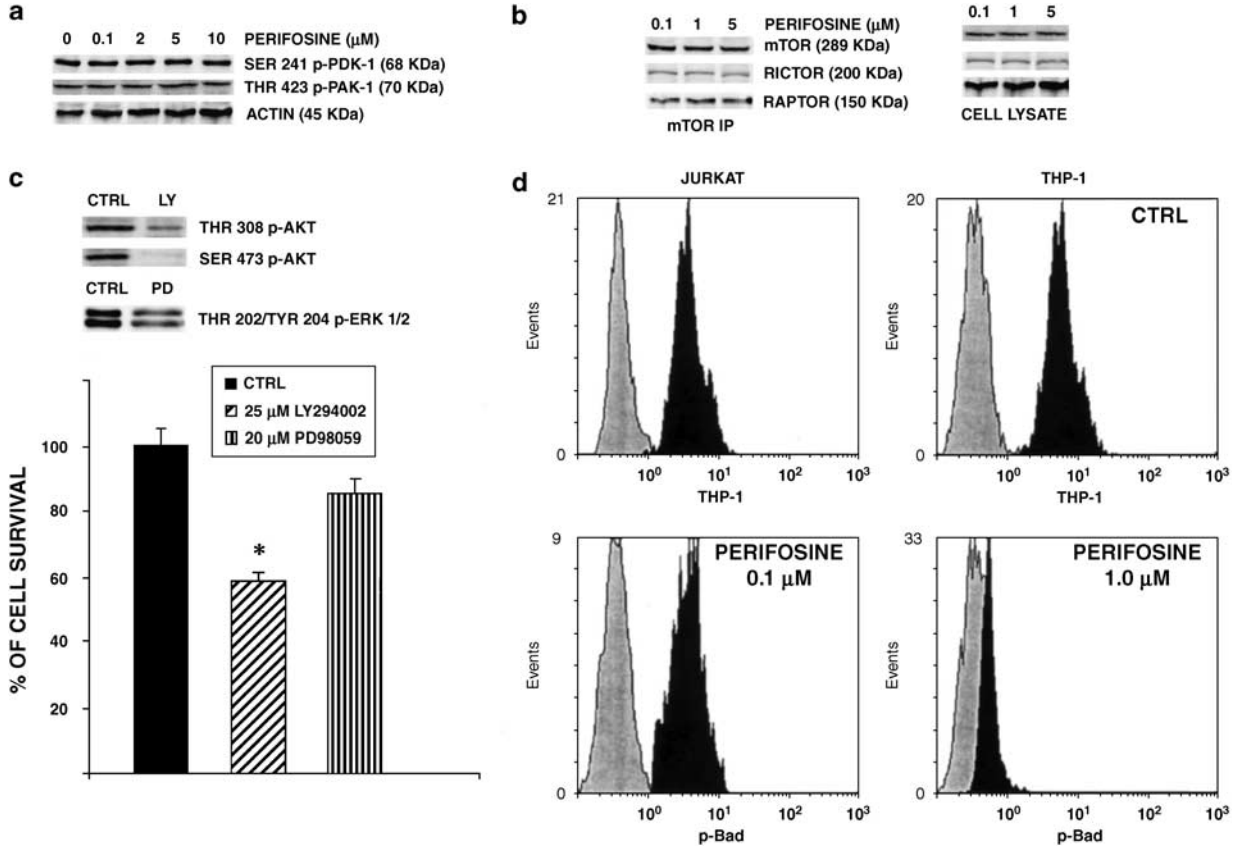

Figure 3 Perifosine does not affect PDK-1 phosphorylation and mTORC complex formation, but dephosphorylates Bad on Ser 112. (**a**) Western blot analysis. Cells were cultured for 24 h in the presence of increasing concentrations of perifosine Whole cell lysates were subjected to SDS-PAGE, and electrotransferred to nitrocellulose membranes which were then probed with the indicated antibodies. The molecular weight of the antigens is indicated at right. (**b**) mTOR immunoprecipitates (IP) and cell lysates prepared after perifosine treatment for 24 h were analyzed by western blot for the levels of mTOR, Rictor and Raptor. (**c**) Effect of LY294002 (LY, 25 μM) and PD98059 (PD, 20 μM) on p-Akt and p-ERK 1/2 levels (western blot), and on cell survival. CTRL: untreated cells. Cells were incubated with the inhibitors for 24 h. Cell survival was assessed by the MTT assay. The graph shows the results from three different experiments ± s.d. Asterisk indicates statistically significant difference ($P<0.005$) with respect to PD98059 treated cells. (**d**) Flow cytometric analysis of Ser 112 p-Bad phosphorylation. Jurkat cells were treated for 30 min with 80 nM phorbol ester to upregulate ERK 1/2 signaling.[29] Light-shaded histogram represents untreated cells, while dark-shade histogram represents stimulated cells. THP-1 cells were treated with the indicated concentrations of perifosine for 24 h. Light-shaded histograms represent cells stained with the secondary antibody only (FITC-conjugated anti-rabbit IgG), while dark-shade histogram represent cells stained with both the primary and the secondary antibody. CTRL, untreated cells; mTORC, mammalian target of rapamycin–rictor complex; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PDK-1, phosphoinositide dependent kinase 1; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

1.0 μM perifosine, whereas almost no decrease was detectable at 0.1 μM (Figure 3d). We were unable to detect Ser 136 p-Bad in THP-1 cells, also by western blot analysis (data not presented).

Cytotoxicity of perifosine is mediated by FAS–FasL and JNK in THP-1 cells

Previous results from our laboratory[34] have highlighted that, in Jurkat T leukemia cells, perifosine-elicited cytotoxicity was, at least in part, somehow dependent on the Fas–FasL system. Thus, it was investigated if this was also true for THP-1 cells. Exponentially growing THP-1 cells were treated with perifosine (5 μM), in the presence or absence of blocking monoclonal antibodies (2 μg ml^{-1}) to human Fas or FasL. After 24 h, cell survival was assessed by MTT. Both antibodies significantly reduced the cytotoxic effect of perifosine (Figure 4a). An isotype-matched control IgG1 mouse monoclonal antibody did not affect cell viability (data not shown). Flow cytometric analysis was employed to determine whether there was an increased expression of cell surface Fas and/or FasL in response to perifosine treatment. While the amount of Fas did not change, there was a dramatic increase in the amount of FasL after exposure of THP-1 cells to 5 μM perifosine for 24 h (Figure 4b). Moreover, reverse transcription (RT)-PCR analysis demonstrated an increase in the amount of FasL mRNA in response to perifosine treatment (Figure 4b). Since previous findings hinted that JNK could be another mediator of perifosine cytotoxicity in multiple myeloma (MM) cells,[15] we investigated whether this was true also for THP-1 cells. Perifosine (5 μM) increased phosphorylation of JNK at Thr 183/Tyr 185, as indicated by flow cytometric analysis. As a control, we treated THP-1 cells with anisomycin, which is known to increase JNK phosphorylation in THP-1 cells (Figure 4c).[38] When THP-1 cells were preincubated with the JNK selective inhibitor SP600125 (10 μM) and then treated for 24 h with 5 μM perifosine, there was a marked decrease in the cytotoxicity, as revealed by MTT analysis (Figure 4d). Blocking antibodies to Fas and FasL, as well as the JNK inhibitor, resulted in diminished levels of caspase activation (data not presented).

Perifosine downregulates p-Akt, but not p-FLT3 levels in MV 4–11 AML cells

Up to 30% of AML patients harbor internal tandem duplication (ITD) of the juxtamembrane domain of FLT3. This mutation results in ligand-independent dimerization of FLT3 and constitutive upregulation of its tyrosine kinase activity, ensuing in stimulation of downstream survival signaling pathways, including PI3K–Akt.[39] The MV 4–11 AML cell line, originally established from a patient with a AML FAB 5, carries FLT3 ITD.[40] To test the specificity of action of perifosine, we evaluated by flow cytometry its effects on both p-Akt and p-FLT3 levels in MV 4–11 AML cells. As a negative control for both antigens, we employed HL60 cells (Figure 5). Upon treatment with perifosine (2 μM for 24 h), there was a decrease in the levels of Ser 473 p-Akt, whereas the signal originating from Tyr 591 p-FLT3 was essentially unchanged in response to drug treatment.

Perifosine decreases cell survival of AML blasts from patients with activated p-Akt

The effects of perifosine have never been tested so far on AML primary cells. Seven AML patients were studied (Table 1), from whom we analyzed both p-Akt and p-ERK 1/2 levels by flow cytometry.[29] Three patients were negative for p-Akt; however, they displayed a robust activation of ERK 1/2. Four out of the seven patients were found to be positive for Ser 473 p-Akt. Two of these exhibited low levels of ERK 1/2 activation (Table 1 and data not shown). In patients without Akt upregulation, a highly significant difference in cell survival with respect to control ($P<0.01$) was observed at 5 μM perifosine (Figure 6a), whereas a highly significant survival decrease was observed at 2.0 μM perifosine in patients with Akt activation (Figure 5b). At 10 μM perifosine, cell survival was around 30% in cells from patients with Akt activation, whereas it was around 60% in patients without Akt activation. MTT assays revealed that the perifosine IC_{50} on AML blasts without Akt activation ranged from 16.7 to 18.9 μM (Figure 6a), whereas in case of AML primary cells displaying Akt activation, the IC_{50} was much lower, ranging from 5.6 to 7.4 μM (Figure 6b).

Effects of perifosine on clonogenic growth of CD34$^+$ hematopoietic progenitors from healthy donors and AML patients

The ability of perifosine to affect the colony growth of healthy and leukemic CD34$^+$ cells in semisolid culture was investigated. CD34$^+$ cells were separated from the bulk of leukemic mononuclear cells by immunomagnetic beads, and then they were cultured in standard medium, which contains GM-CSF, IL-3 and IL-6. Addition of perifosine (2 μM) to standard medium did not significantly affect the growth of colonies from healthy donors or patients without Akt activation (Figure 7a). In contrast, perifosine negatively affected the colony forming activity of CD34$^+$ cells from patients with activated Akt.

Perifosine sensitizes chemoresistant AML blasts to etoposide-induced cytotoxicity

Next, the ability of perifosine to sensitize AML cells to etoposide (0.5 μM) was examined. MTT assays showed that perifosine alone was capable of significantly reducing survival of AML blasts characterized by increased levels of p-Akt (cases 4–7). It strongly enhanced the effects of etoposide in causing a decrease of cell survival in all of these cases, which, on the other hand, were completely resistant to a 0.5 μM etoposide concentration (Figure 7b).

Discussion

Perifosine is an alkylphospholipid, a novel class of antitumor agent structurally related to ether lipids. Alkylphospholipids have three main effects, which may 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 PI3K–Akt and ERK 1/2.[41] In phase I/II studies for advanced solid tumors, perifosine did not cause significant hematological toxicity.[14,42,43] Since both PI3K–Akt and ERK 1/2 signaling play a major role in AML cell survival and proliferation, in this study we have addressed the efficacy of perifosine, either used alone or in combination with etoposide, in decreasing cell survival of AML cell lines and blasts. We have demonstrated that perifosine, in a dose-dependent fashion, decreased survival of both THP-1 and MV 4–11 cell lines. In THP-1 cells, we observed a dephosphorylation of Akt on both Ser 473 and Thr 308, which became apparent at 0.1 μM perifosine. Perifosine did not affect phosphorylation and activity of the upstream kinase PDK-1, and did not alter the equilibrium between the mTORC1 and mTORC2 complexes. These findings strengthen the concept that perifosine

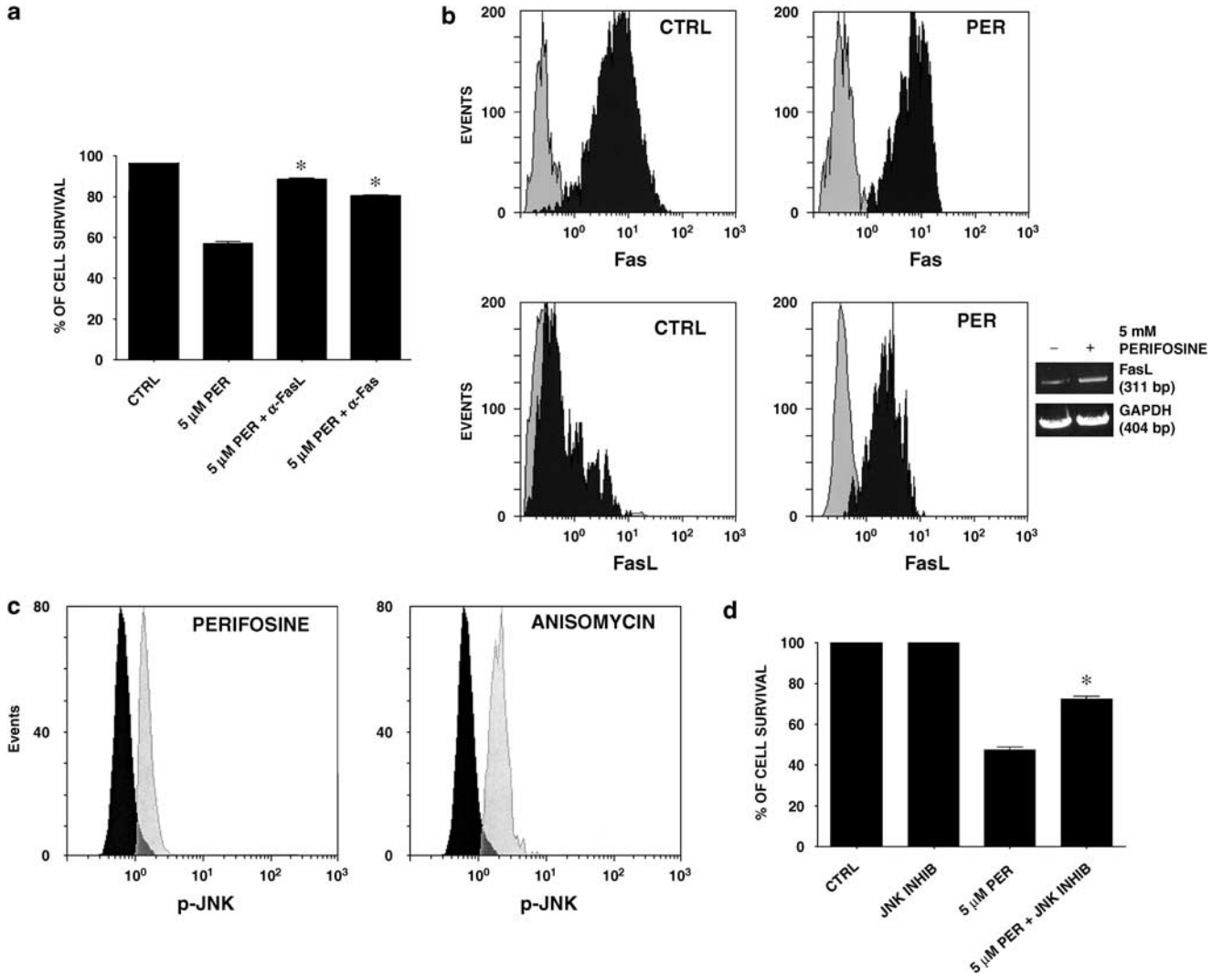

Figure 4 Perifosine cytotoxicity is dependent on Fas–FasL and JNK activation in THP-1 cells. (**a**) MTT assay. Cells were cultured for 24 h with perifosine (PER) in the absence or in the presence of blocking antibodies to either FasL (α-FasL) or Fas (α-Fas). Results are the mean of three different experiments ± s.d. Asterisk indicates statistically significant difference ($P<0.005$) with respect to cells incubated with perifosine only. CTRL, control (untreated) cells. (**b**) Flow cytometric analysis and RT-PCR. For flow cytometric analysis, control cells (CTRL) and cells incubated for 24 h with 5 μM perifosine (PER) were stained with FITC-conjugated anti-FAS and anti-FasL antibodies (black-shaded histogram). As a control, cells were stained with isotype-matched FITC-conjugated mouse IgG1 (gray-shaded histograms). (**c**) THP-1 cells were treated with 5 μM perifosine for 24 h or with 10 μg ml^{-1} anisomycin for 1 h, then analyzed for p-JNK levels by flow cytometry (gray-shaded histograms). Black shaded histograms represent samples stained with an Alexa Fluor 647 conjugate rabbit IgG (eBioscience, San Diego, CA, USA). (**d**) THP-1 cells were cultured for 24 h in the presence or in the absence of 10 μM SP600125 (JNK INHIB). Results are the mean of three different experiments ± s.d. Asterisk indicates statistically significant difference ($P<0.005$) with respect to cells incubated with perifosine (PER) only. CTRL, control (untreated) cells. FITC, fluorescein isothiocyanate; JNK, c-jun-N-kinase; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; RT, reverse transcription.

dephosphorylates Akt by impeding its binding to the plasma membrane, without affecting PI3K activity, as demonstrated by others.[17]

Akt dephosphorylation was accompanied by dephosphorylation of one of the Akt/mTORC1 pathway downstream targets, p70S6K. At a higher concentration (1 μM), perifosine also decreased the levels of Thr 202/Tyr 204 p-ERK 1/2. Therefore, this drug was capable of downmodulating two signaling pathways which are of fundamental importance for AML cell survival and drug resistance.[24,44,45] However, even at higher concentrations, perifosine was unable to completely dephosphorylate ERK 1/2 in THP-1 cells, even though the upstream kinase MEK 1/2 was almost completely dephosphorylated. In THP-1 cells, upregulation of the PI3K–Akt pathway is due to overexpression of p110δ PI3K which leads to higher levels of PIP_3,[46] whereas the reason for constitutive activation of ERK 1/2 is unknown. In some previous studies, perifosine treatment resulted in ERK 1/2 activation,[15,34] while in others, perifosine downmodulated ERK 1/2.[18,47] In the only report available dealing with AML cell lines, perifosine, when employed alone, did not inhibit ERK 1/2 phosphorylation.[27] Since we do not know the reason for ERK 1/2 activation in THP-1 cells, it is difficult to explain whether the perifosine effect is direct or mediated through other signaling pathways. It was previously thought that Akt inhibition should result in ERK 1/2 upregulation, given that active Akt dowregulates Raf-1 and, consequently, also ERK 1/2. However, it is known that PDK-1 can phosphorylate PAK-1 which in turn phosphorylates and activates Raf-1, ensuing in

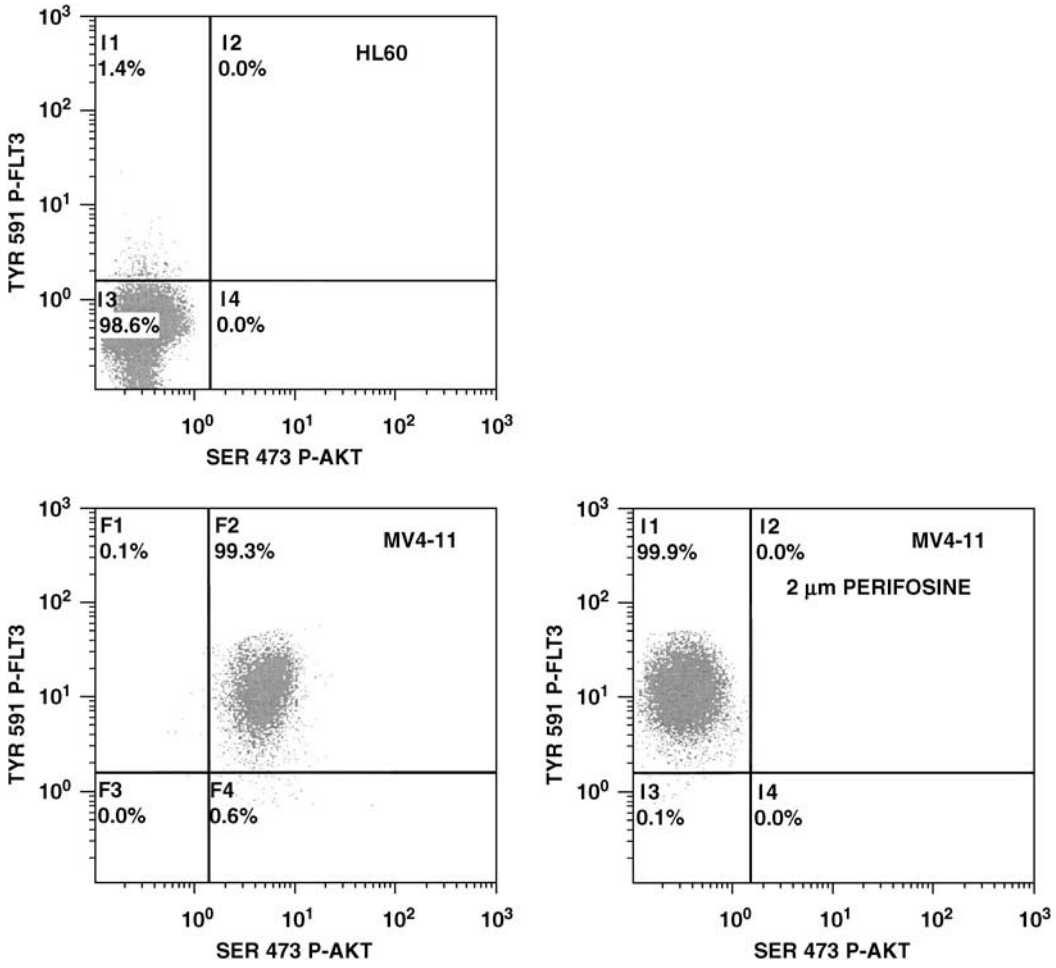

Figure 5 Perifosine dephosphorylates Ser 473 p-Akt, but not Tyr 591 p-FLT3, in MV 4-11 cells. Cells were stained with Alexa Fluor 488 conjugate anti-Tyr 591 p-FLT3, and Alexa Fluor 647 conjugate anti-Ser 473 p-Akt. Flow cytometric analysis of cells double stained for the two antigens were then performed as reported in Materials and methods section. HL60 cells served as negative control. Perifosine treatment was for 24 h. One representative of three different experiments is shown.

Table 1 Characteristics of AML patients and p-Akt and p-ERK 1/2 phosphorylation status

	Sex	*Age*	*FAB*	*Ser 473 p-Akt levels*	*Thr 202/Tyr 204 p-ERK 1/2 levels*	*Source of blasts*
1	F	66	M1	–	++++	PBL
2	M	58	M4	–	++++	PBL
3	F	69	M0	–	+++	BM
4	M	45	M2	++++	+	BM
5	F	63	M4	++	–	PBL
6	F	50	M0	+++	++	BM
7	M	50	M1	+++	–	BM

Abbreviations: BM, bone marrow; FAB, French–American–British classification for AML; PBL, peripheral blood.
Levels of phosphorylated proteins were analyzed by flow cytometry using Alexa Fluor 647 conjugate antibodies. For p-Akt: staining intensities were compared to that observed in unstimulated Jurkat T leukemia cells (set as ++++); for p-Erk: staining intensities were compared to that observed in phorbol ester-stimulated Jurkat T leukemia cells (set as ++++).[29] Controls were run with an Alexa Fluor 647 conjugate rabbit IgG (eBioscience, San Diego, CA, USA).

ERK 1/2 activation.[48] While we detected PAK-1 phosphorylation on a site (Thr 423) which is targeted by PDK-1, PAK-1 phosphorylation levels were not affected by perifosine, consistently with the lack of perifosine effect on PDK-1 activity. Therefore, elucidation of the inhibitory effect of perifosine on MEK–ERK signaling will require further investigations.

Perifosine also showed cytotoxic activity toward MV 4-11 cells, which contain FLT3 ITD. In this experimental model, perifosine was capable of dephosphorylating Akt without affecting the phosphorylation levels of FLT3. These findings suggest that perifosine could also be effective in patients with FLT3 ITD, which is common mutation in AML (approximately 25–30% of AML patients have FLT3 ITD) and is associated with a bad prognosis.[49]

Perifosine treatment resulted in activation of multiple caspases in THP-1 cells. In agreement with recent findings obtained

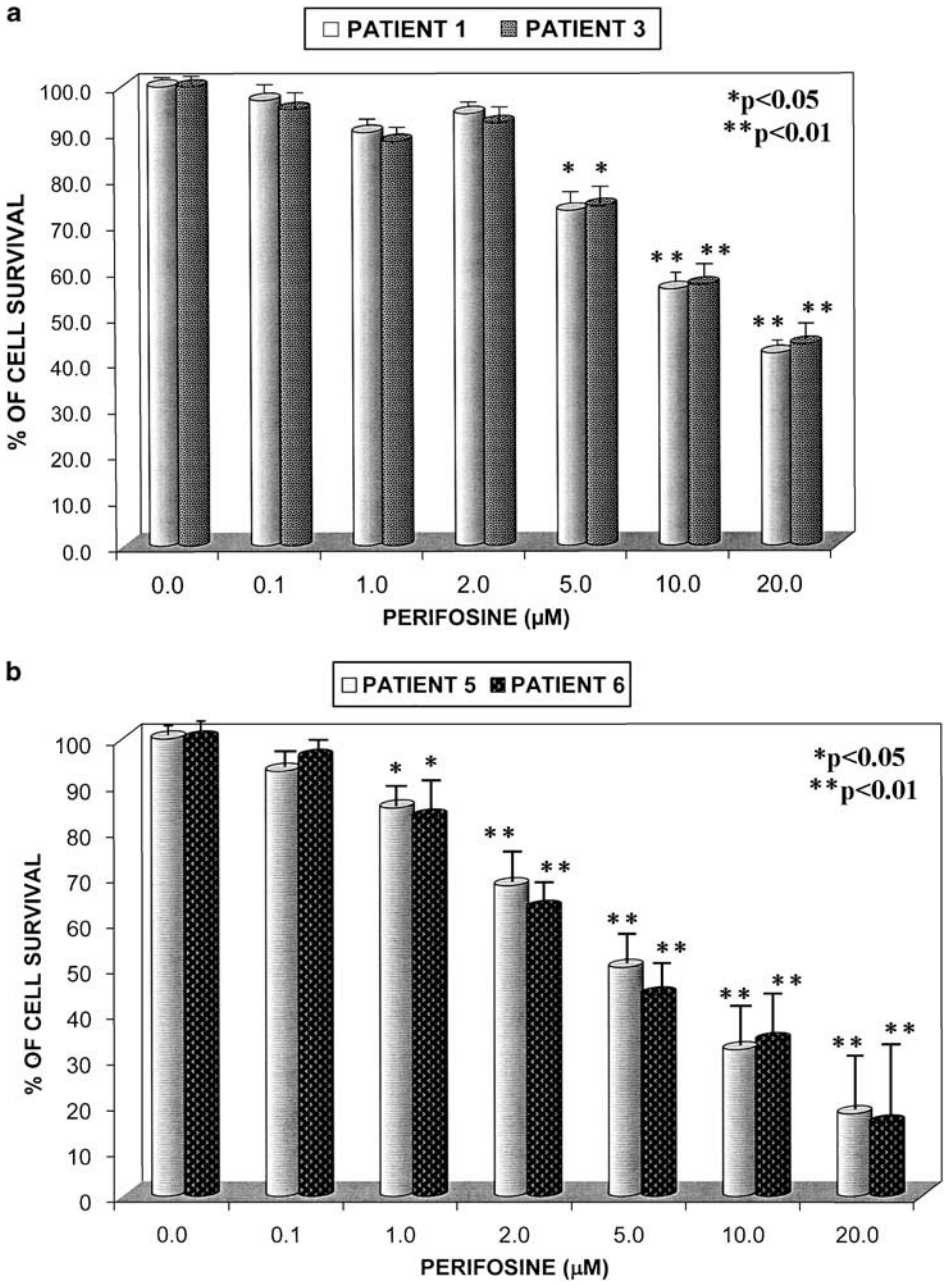

Figure 6 Perifosine decreases survival of AML blasts. (**a**) MTT assays for patients without Akt activation. (**b**) MTT assays for patients with Akt activation. Cells (3×10^4 per well) were incubated for 72 h in RPMI-1640 medium supplemented with 10% FCS and 20 ng ml^{-1} each of GM-CSF, IL-3 and IL-6. At the end of the incubation, MTT assays were performed. Results are the mean of three different experiments $\pm$ s.d. AML, acute myelogenous leukemia; FCS, fetal calf serum; GM-CSF, granulocyte–macrophage-colony stimulating factor; IL, interleukin; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

by analyzing MM cell lines,[50] the apical caspase which was activated at the lowest perifosine concentration was caspase-8. Indeed, it has been shown that perifosine was able to recruit to MM cell lipid rafts, Fas, Fas-associated death domain-containing protein and procaspase-8, leading to the formation of the death-inducing signaling complex. However, in MM cells, perifosine-mediated cytotoxicity was FasL independent,[50] whereas in THP-1 cells, the cytotoxic effect of perifosine was at least partly dependent on FasL, because a blocking antibody to either Fas or FasL significantly enhanced survival of cells treated with the drug. Both RT-PCR and flow cytometry confirmed that perifosine induced an increase in FasL mRNA and protein. The increase in FasL levels could depend on the fact that FasL expression is, at least partially, under the control of FoxO transcription factors. FoxO transcription factors, when phosphorylated by Akt, are no longer capable of positively influencing FasL expression, since they exit the nucleus.[51] However, when activation of Akt is blocked by perifosine, FoxO factors are once again capable of positively regulating FasL expression. In this connection, we have demonstrated FoxO factor dephosphorylation by perifosine in Jurkat T leukemia cells.[34] Moreover, we have also shown that in HL60 cells, FoxO factor nuclear localization is dependent on PI3K–Akt signaling.[52]

The activation of caspase-9 could depend on perifosine recruiting Bid to the lipid rafts.[50] Once cleaved by caspase-8, truncated Bid migrates to mitochondria where it releases

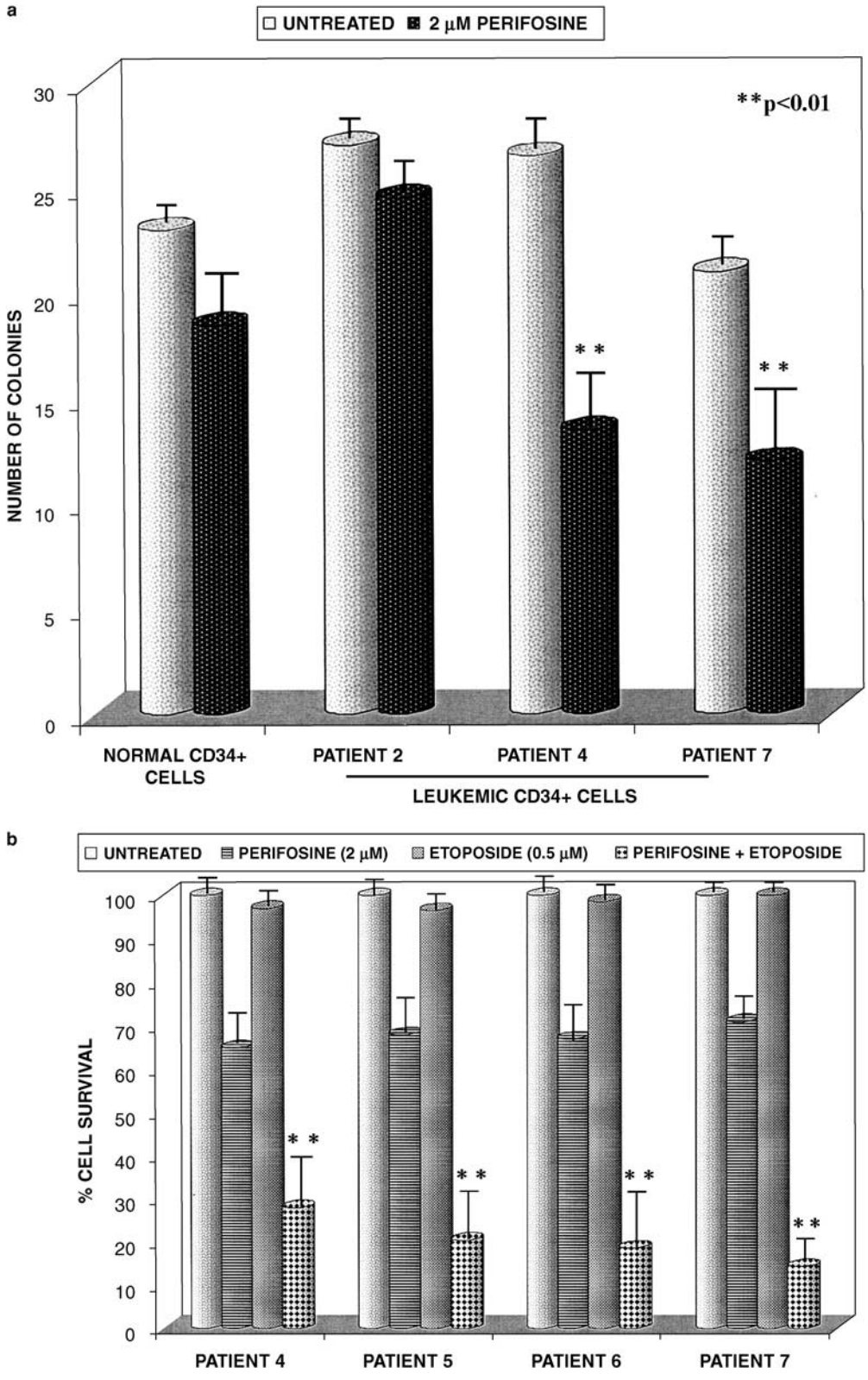

Figure 7 Perifosine negatively affects clonogenic growth of AML $CD34^+$ progenitor cells and enhances etoposide cytotoxicity of blasts from AML patients with Akt activation. (**a**) Colony forming assay of $CD34^+$ cells isolated from healthy donors, patients without Akt activation and patients with Akt activation. Cells were seeded in a semisolid methylcellulose medium containing GM-CSF, IL-3 and IL-6. The *x* axis indicates the number of colonies per view (observation at 25 × under an Olympus light microscope). (**b**) MTT assays, performed as described in Figure 5. The double asterisk indicates a statistically significant difference ($P<0.01$). In both (**a** and **b**), the results are the mean ± s.d. from three different experiments. AML, acute myelogenous leukemia; GM-CSF, granulocyte–macrophage-colony stimulating factor; IL, interleukin; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

cytochrome *c* and initiates the intrinsic apoptotic pathway.[53] Indeed, Bid is cleaved in THP-1 cells treated with perifosine. Even though activation of apical caspase-8 and -9 has been reported as a consequence of perifosine treatment,[15,34] this is the first report, to our knowledge, which demonstrates activation of apical caspase-2. Caspase-2 activation could be due to at least two reasons. First, it is known that caspase-2 could be activated by death receptors ligands such as FasL.[54,55] Second, it has been shown that reactive oxygen species are capable of activating caspase-2.[56] Interestingly, perifosine generates ROS in AML cells.[27] Experiments are currently underway in our laboratory to distinguish which of these two mechanisms is important in THP-1 cells. Nevertheless, our findings emphasize that JNK activation is also important for perifosine-mediated cytotoxicity in THP-1 cells, as perifosine increased JNK phosphorylation and a selective pharmacological inhibitor of JNK markedly enhanced survival in perifosine-treated cells. Therefore, our results are in agreement with the data of Hideshima *et al.*,[15] who demonstrated the fundamental role played by JNK activation in the induction of apoptotic cell death of perifosine-treated MM cells. In contrast, it has been shown that in U937 and HL60 AML cell lines, SP600125 did not significantly diminish perifosine-mediated apoptosis.[27] The reason for this discrepancy is at present unknown. In any case, there are several reports which have linked the Fas–FasL apoptotic pathway with JNK activation.[57] As to executioner caspase activation, caspase-3 was already activated at 1 μM perifosine, as was caspase-8. This suggests that, at this perifosine concentration, caspase-3 could be directly activated by cleaved caspase-8. It should also be emphasized that our findings indicate that a complete dephosphorylation of Akt on Ser 473 is linked with caspase activation, which is detected starting at 1 μM perifosine. The reason for this is unclear, but it could be related to downregulation of antiapoptotic proteins whose expression is regulated by Akt.[23]

We have also shown for the first time that perifosine negatively affected cell survival of AML blast cells with constitutive Akt activation. Some of these samples also exhibited concomitant weak ERK 1/2 activation. Therefore, partial inhibition of this pathway by perifosine could have contributed to decreased survival. It worth emphasizing here that the perifosine IC_{50} for patients with Akt activation was comprised in a range (5.6–7.4 μM), which should be easily reached *in vivo*. In contrast, in cells with no Akt activation, the IC_{50} for perifosine was much higher than in patients without activation. Since all of the patients with no Akt activation, showed robust ERK 1/2 phosphorylation, it might be that in these samples perifosine cytotoxicity was related to ERK 1/2 downregulation. In any case, the results with AML blasts are consistent with the fact that perifosine could only partially block ERK 1/2 activation in THP-1 cells, given that cell survival in patients without Akt upregulation was much higher than in those with Akt activation. Since simultaneous activation of multiple signaling networks, including PI3K–Akt and ERK 1/2, confers a negative prognosis to AML patients,[24] the use of an inhibitor which targets two of the pathways which are essential for AML blast survival, could be advantageous. Indeed, an emerging paradigm in anticancer therapy is that the use of pharmacological inhibitors targeting multiple signal transduction cascades could lead to better results than employing highly selective or specific blocking of only one of these pathways.[58] In any case, our results seem to indicate that AML patients with PI3K–Akt upregulation are likely to respond better to perifosine treatment.

Perifosine did not negatively affect the clonogenic activity of $CD34^+$ cells from healthy donors and this is consistent with the absence of hematological toxicity observed *in vivo* in mice and in patients during phase I/II trails for solid tumors.[15] In contrast, the clonogenic activity of $CD34^+$ cells from AML patients with active Akt pathway was significantly impaired by perifosine. Moreover, we have demonstrated that a concentration of perifosine which could be easily achieved during clinical trials (that is, 2 μM) and which is below the IC_{50} *in vitro*, strongly enhanced the cytotoxic effect of etoposide used at a low concentration (that is, 0.5 μM), suggesting that perifosine overcomes AML cell resistance to conventional chemotherapeutic drugs. Etoposide is a DNA-damaging agent that inhibits topoisomerase II, resulting in G2/M-phase arrest. A likely explanation to the increased apoptotic rate observed in cells treated with perifosine and etoposide, is provided by data implicating active Akt in the transition through G2/M phase of the cell cycle following DNA damage.[59] An increased Akt activity has been observed in cells entering G2 phase.[60] Thus, it is possible that drug-induced inactivation of Akt coupled to the action of etoposide, would lead to apoptotic cell death.[34]

In summary, our preclinical studies suggest that perifosine, either alone or on combination with existing drugs, could be useful for improving the outcome of AML patients with active Akt and ERK 1/2 pathways, including those with FLT3 ITD.

Acknowledgements

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