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ORIGINAL ARTICLE

Erucylphosphohomocholine, the first intravenously applicable alkylphosphocholine, is cytotoxic to acute myelogenous leukemia cells through JNK- and PP2A-dependent mechanisms

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Alkylphospholipids and alkylphosphocholines (APCs) are promising antitumor agents, which target the plasma membrane and affect multiple signal transduction networks. We investigated the therapeutic potential of erucylphosphohomocholine (ErPC3), the first intravenously applicable APC, in human acute myelogenous leukemia (AML) cells. ErPC3 was tested on AML cell lines, as well as AML primary cells. At short (6–12 h) incubation times, the drug blocked cells in G2/M phase of the cell cycle, whereas, at longer incubation times, it decreased survival and induced cell death by apoptosis. ErPC3 caused JNK 1/2 activation as well as ERK 1/2 dephosphorylation. Pharmacological inhibition of caspase-3 or a JNK 1/2 inhibitor peptide markedly reduced ErPC3 cytotoxicity. Protein phosphatase 2A downregulation by siRNA opposed ERK 1/2 dephosphorylation and blunted the cytotoxic effect of ErPC3. ErPC3 was cytotoxic to AML primary cells and reduced the clonogenic activity of $CD34^+$ leukemic cells. ErPC3 induced a significant apoptosis in the compartment ($CD34^+$ $CD38^{Low/Neg}$ $CD123^+$) enriched in putative leukemia-initiating cells. This conclusion was supported by ErPC3 cytotoxicity on AML blasts showing high aldehyde dehydrogenase activity and on the side population of AML cell lines and blasts. These findings indicate that ErPC3 might be a promising therapeutic agent for the treatment of AML patients.

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Introduction

Two groups of antitumor drugs have been studied in the past, which act on cellular membranes. Group 1 is based on the structure of phospholipid molecules, the alkylphospholipids (APLs). They contain phosphate esters of glycerol. The study of APLs *in vitro* and the results on molecular mechanisms of cell regulation and growth inhibition have strongly contributed to the understanding of these molecules as possible antineoplastic drugs. Edelfosine is the lead compound for this class of drugs.[1]

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Group 2, however, are structurally less complex molecules. They are simply phosphocholine esters of long-chain alcohols, do not contain glycerol as structural element[2] and are referred to as alkylphosphocholines (APCs). APLs and APCs easily insert in the plasma membrane and by doing so they inhibit phospholipid turnover. The primary membrane domain targeted by APLs and APCs are lipid rafts.[3,4] APLs and APCs downregulate signaling pathways, which are important for survival such as phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK),[5,6] whereas they upregulate c-Jun N-terminal kinase (JNK) signaling, which then promotes apoptosis.[1] Edelfosine displayed considerable antitumor activity in preclinical models in both mouse and rat, but much less so in the clinic. Thus, the only clinical application of edelfosine was for purging bone marrow in autologous bone marrow transplantation in acute myelogenous leukemia (AML) patients.[7] This application was based on the observation that edelfosine selectively killed leukemic cells, while sparing normal bone marrow cells both *in vitro* and in a murine model *in vivo*.[8,9]

Miltefosine, the lead compound of APCs, has been approved for the topical treatment of skin metastases in breast cancer and the oral treatment of leishmaniasis.[10] Perifosine is a miltefosine analog that has been tested, either alone or in combination with other drugs, in several type I/II clinical trials against tumors, which included head and neck carcinoma, prostate cancer, melanoma, multiple myeloma (MM) and AML.[11] Some promising results have been observed in MM patients, who were administered perifosine in combination with dexamethasone and/or bortezomib.[12] Moreover, recent findings have highlighted that perifosine, at variance with chemotherapeutic drugs, could target putative cancer stem cells *in vivo* in breast tumor xenografts.[13] Perifosine has been tested in preclinical models of AML in which it displayed a cytotoxic affect and synergized with chemotherapeutic drugs and TRAIL.[14,15] A major disadvantage of perifosine is its poor gastrointestinal tolerability after oral administration, which leads to insufficient plasma levels for systemic cancer treatment. However, intravenous application of perifosine is impossible due to intravascular hemolysis and thrombophlebitis.[1] These important limitations for oral dosing could be overcome by compounds displaying longer alkyl chains with *cis*-monounsaturation. Erucylphosphohomocholine (ErPC3) is an APC displaying one *cis*-double bond in the

alkyl chain with 22 carbon atoms, which could be applicable intravenously in animals and displayed a high efficacy against tumor models *in vivo* and *in vitro*.[16,17] Recently, the *in vitro* cytotoxic effect of ErPC3 in AML cells has been documented.[18] In this study, we have further investigated the mechanisms that could underlay ErPC3 cytotoxicity in AML cells. We demonstrated that ErPC3 activated JNK 1/2. ErPC3 dephosphorylated ERK 1/2 through a mechanism involving protein phosphatase 2A (PP2A). Both JNK 1/2 inhibition by a synthetic peptide and PP2A downregulation by short interfering RNA (siRNA) blunted ErPC3 cytotoxicity. Moreover, we also documented ErPC3 cytotoxicity in primary AML populations, which are enriched in putative leukemia-initiating cells (LICs). Overall, our findings highlight several mechanisms that could explain ErPC3 cytotoxicity in AML cells, and suggest that ErPC3 might be a promising drug for treatment of AML patients.

Materials and methods

Chemicals and antibodies

The Cell Viability kit I (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, or MTT), Annexin V–fluorescein isothiocyanate (FITC) and Annexin V–phycoerythrin (PE) staining kits were from Roche Applied Science (Penzberg, Germany). The p110α PI3K inhibitor (compound 15e or [3-[4-(4-morpholinyl)thieno[3,2-d]pyrimidin-2-yl]-phenol) was from Alexis Biochemicals (Lausen, Switzerland). The MEK inhibitor CI-1040 and KO143 were from Axon Medchem BV (Groningen, The Netherlands). The Aldefluor kit was from StemCell Technologies (Vancouver, BC, Canada). The JNK 1/2 inhibitor peptide was from BioMol International (Plymouth Meeting, PA, USA). For western blot analysis, all the antibodies were from Cell Signaling Technology (Beverly, MA, USA), except for the antibodies to protein phosphatase 1 (PP1) and Ser 657 p-protein kinase C (PKC) α, which were from Millipore/Upstate (Billerica, MA, USA). For flow cytometric analysis, rabbit monoclonals to Ser 473 p-Akt, Thr 202/Tyr 204 p-ERK 1/2 (both AlexaFluor 647-conjugated), Thr183/Tyr 185 p-JNK and Ser p-BCL2 (AlexaFluor 488-conjugated) were from Cell Signaling Technology. R-PE-Cyanine-7 (PC7)-conjugated anti-CD34, FITC- or PE-conjugated anti-CD38, and phycocyanin-5 (PC5)-conjugated anti-CD123 (all mouse monoclonal IgGs) were from Beckman Coulter Immunology (Miami, FL, USA). PE-conjugated anti-rabbit IgG was from Sigma-Aldrich (St Louis, MO, USA). PE-conjugated antibody to ATP-binding cassette (ABC) G2 membrane transporter was from Millipore. For immunocytochemistry experiments, anti-phosphatidylinositol 3,4,5 trisphosphate (PIP3) monoclonal antibody from Echelon Biosciences (Logan, UT, USA) was used.

Cell culture

THP1, HL60 and NB4 AML cells were exponentially grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Mouse Ba/F3 cells expressing mutant (activated) p110α PI3K were cultured as previously described.[19]

Patients

Samples were obtained from patients at presentation of AML at the Policlinico S. Orsola-Malpighi Hospital, Bologna, Italy. Informed consent was obtained from all patients before receiving the samples, according to the Institutional guidelines. Bone marrow mononuclear cells were isolated by Ficoll-Paque (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) density-gradient centrifugation. The AML cases were defined according to the classification of the French–American–British committee. Percentage of blasts in the samples ranged between 75 and 91% and was determined by flow cytometry immunostaining.

Colony assay of CD34$^+$ cells from cord blood or AML patients

Healthy CD34$^+$ cells were obtained from cord blood. Healthy and leukemic CD34$^+$ cells were isolated using immunomagnetic cell separation (Miltenyi Biotec, Bergisch Gladbach, Germany) and clonogenic assays were performed as described elsewhere.[14]

Cell growth analysis by MTT assay

An MTT assay was used to analyze cell growth and viability, as previously described.[14]

Whole-cell lysate preparation and western blot analysis

This was performed according to standard techniques.[20] Briefly, cells were lysed and cell debris was removed by centrifugation at 13 000 r.p.m. for 15 min at 4 °C in a microfuge. Protein supernatants (40 μg of protein) were separated on SDS–polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with primary antibodies. They were then incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 in 5% non-fat dry milk in phosphate-buffered saline (PBS, pH 7.4)–Tween 20 for 1 h at room temperature. Antibody binding was detected by the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA).

Immunofluorescence staining

This was performed as previously described,[21–23] using an antibody to PIP3.[24]

Quantitive reverse transcriptase PCR for p110 PI3K isoforms

This was performed as described elsewhere,[25] using primers for p110 PI3K isoforms purchased from Applied Biosystems (Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase was used as a control (for example, a constitutively expressed housekeeping gene).

Transient protein downregulation by siRNA

siRNA targeting PP2A/C subunit mRNA (target sequence: 5′-GA ATCCAACGTTCAAGAGG-3′) and negative control (scrambled sequence) were purchased from Dharmacon Research Inc (Lafayette, CO, USA). The target sequence against PP2A/C was chosen by the web-based search program, and the lack of homology to any other gene was confirmed by a BLAST search (National Center for Biotechnology Information, National Institutes of Health, USA). Transfection of cells was performed using the Amaxa system (Amaxa, Cologne, Germany). Briefly, 10^6 cells in 100 μl of medium were mixed with 3 μg of siRNA and transferred to an Amaxa-certified cuvette. For transfection, the program V-01 was used for THP1 cells, whereas for AML primary cells, we used program U-15.[26] For both cell types, the Amaxa transfection kit V was used. Cells were examined for downregulation of the target gene and subsequent important effects, 48 h after transfection.

Flow cytometric cell cycle and apoptosis analysis

Cell-cycle analysis was carried out on propidium iodide (PI)-stained samples,[27] whereas apoptosis was studied by the

binding of Annexin V–FITC to phosphatidylserine exposed on the cell membrane.[25] Samples were analyzed on a EPICS XL flow cytometer (Beckman Coulter Immunology).

Quadruple flow cytometric analysis with CD34, CD38, CD123 and Annexin V for apoptosis analysis

Cells were incubated with PC7-conjugated anti-CD34, FITC- or PE-conjugated anti-CD38, PC5-conjugated anti-CD123 and PE-conjugated Annexin V for 15 min at room temperature. In some cases, they were processed for intracellular staining with a rabbit monoclonal antibody to Thr183/Tyr 185 p-JNK 1/2, which was recognized by a PE-conjugated secondary antibody raised in goat, or an AlexaFluor 488-conjugated anti-Ser 70 p-BCL2, essentially as reported by others.[28] Samples were analyzed on dual-laser FC500 flow cytometer (Beckman Coulter Immunology). Appropriate anti-isotypic control antibodies were used to establish the specificity of quadruple staining.

Flow cytometric Aldefluor cell analysis for ALDH activity in AML patient cells

Briefly, AML cells were resuspended in the Aldefluor assay buffer, containing the aldehyde dehydrogenase (ALDH) substrate, Bodipy-aminoacetaldehyde (1 μM), according to the manufacturer. In each experiment, a sample of cells was stained under identical conditions with the specific ALDH inhibitor diethylaminobenzaldehyde (50 nM) as negative control.[29] Aldefluor fluorescence was excited at 488 nm, and fluorescence emission was detected using a standard FITC 530 nm band-pass filter[30]

Flow cytometric detection of AML SP cells

Cells were resuspended at 1×10^6 per ml in RPMI 1640/2% FCS. Hoechst 33342 dye was added at a final concentration of 5 μg/ml in the presence or the absence of verapamil (100 μM), or fumitremorgin c (10 μM), or KO143 (0.1 μM). Samples were incubated at 37 °C for 90 min, then washed with PBS/2% FCS, and resuspended in PBS/2% FCS. In some experiments, cells were prestained with PE-conjugated anti-ABCG2 monoclonal antibody. After a 20-min incubation, cells were washed with PBS/2% bovine serum albumin and then processed for Hoechst 33342 staining. Control samples for anti-ABCG2 were run with an irrelevant isotypic PE-conjugated antibody. Samples were analyzed with a Cell Lab Quanta SC (Beckman Coulter Immunology) flow cytometer equipped with ultraviolet lamp and 488 solid-state laser. The Hoechst 33342 dye was excited at 366 nm. Side population (SP) cells were gated by FL1/FL3 histogram, while ABCG2 staining was evaluated by FL2 channel.[31]

Statistical evaluation

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

Results

ErPC3 has cytotoxic proapoptotic activity on AML cell lines

As APLs and APCs are known for targeting both PI3K/Akt and MEK/ERK signaling, the activation states of these two networks in AML cell lines were first investigated. Although HL60 and NB4 displayed only activated ERK 1/2, THP1 cells had both activated Akt and ERK 1/2, as documented by western blot analysis (Figure 1a). Then, the cytotoxic effect of ErPC3 on AML cell lines was analyzed, using increasing concentrations of the drug. After 24 h, the rates of growth were measured using MTT assays. Cell lines displayed an IC_{50} for ErPC3 ranging from 4.9 μM (THP1) to 7.9 (HL60) to 13.1 μM (NB4) (Figure 1b). The effect of ErPC3 on cell-cycle progression was investigated next. Flow cytometric analysis of PI-stained THP1 cells treated with 10 μM ErPC3 documented an increase in G2/M phase cells and a decrease in G1 phase cells, which was already evident after 6 h of incubation with the drug and became more pronounced at 12 h (Figure 1c). It was next investigated whether ErPC3 cytotoxicity was related to apoptosis, using Annexin V–FITC/PI staining and flow cytometry. After treatment of THP1 cells with ErPC3 for 24 h, there was a concentration-dependent increase in early apoptotic (Annexin V–FITC positive only) and mid-late apoptotic (Annexin V–FITC/PI positive) cells. Both a caspase-9 and a caspase-3 inhibitor strongly reduced apoptosis (Figure 1d). Western blot analysis documented that cleavage of apical caspase-9 and of effector caspase-3 already occurred at 3 μM ErPC3 (not shown).

Overall, these findings demonstrated that ErPC3 reduced the growth of AML cell lines and that this effect was due to both cell-cycle arrest and apoptosis.

ErPC3 induces JNK 1/2 activation in THP1 cells

At 3 μM, ErPC3 rapidly and transiently activated JNK 1/2, as indicated by western blot analysis (Figure 2a). The use of a blocking peptide to JNK 1/2[32] documented that JNK 1/2 activation was important for ErPC3-mediated cytotoxicity, as the inhibitor peptide significantly increased THP1 cell survival in a concentration-dependent manner (Figure 2b). As a control, we demonstrated using western blot that the blocking peptide was able to prevent phosphorylation of ELK1, a JNK 1/2 substrate,[33] elicited by anisomycin, a well-established activator of JNK 1/2 in THP1 cells[14] (Figure 2c).

ErPC3 affects PI3K/Akt signaling in THP1 cells

Western blot analysis with antibodies to either Thr 308 or Ser 473 p-Akt, demonstrated a marked decrease in these p-Akt forms in response to ErPC3 after 24 h of treatment (Supplementary Figure 1a). Dephosphorylation on both Thr 308 and Ser 473 p-Akt residues was evident at 10 μM ErPC3, whereas at 20 μM ErPC3, no p-Akt was detectable. However, we also detected a reduction in the amount of Akt, which became apparent already at 10 μM, whereas at 20 μM, no Akt was visible. The loss of Akt following ErPC3 treatment was abolished by the inhibition of caspase-3 activity with Z-VAD-FMK, suggesting it was due to a caspase-3-dependent mechanism (Supplementary Figure 1b), as previously reported by our group in lymphoblastic leukemia cells.[20] ErPC3 also increased the expression levels of p110α PI3K in a concentration-dependent manner, but not those of either p110β or p110δ PI3K (Supplementary Figure 1a). Increased p110α PI3K expression was due to enhanced gene expression, as documented by quantitative reverse transcriptase PCR (Supplementary Figure 1c). This upregulation of p110α PI3K had functional consequences, because ErPC3 increased the amount of the end product of PI3K, PIP3, as demonstrated by immunofluorescence staining with a monoclonal antibody to PIP3.[34] Remarkably, compound 15e, a p110α PI3K-selective inhibitor,[35] prevented the increase in PIP3 levels in response to

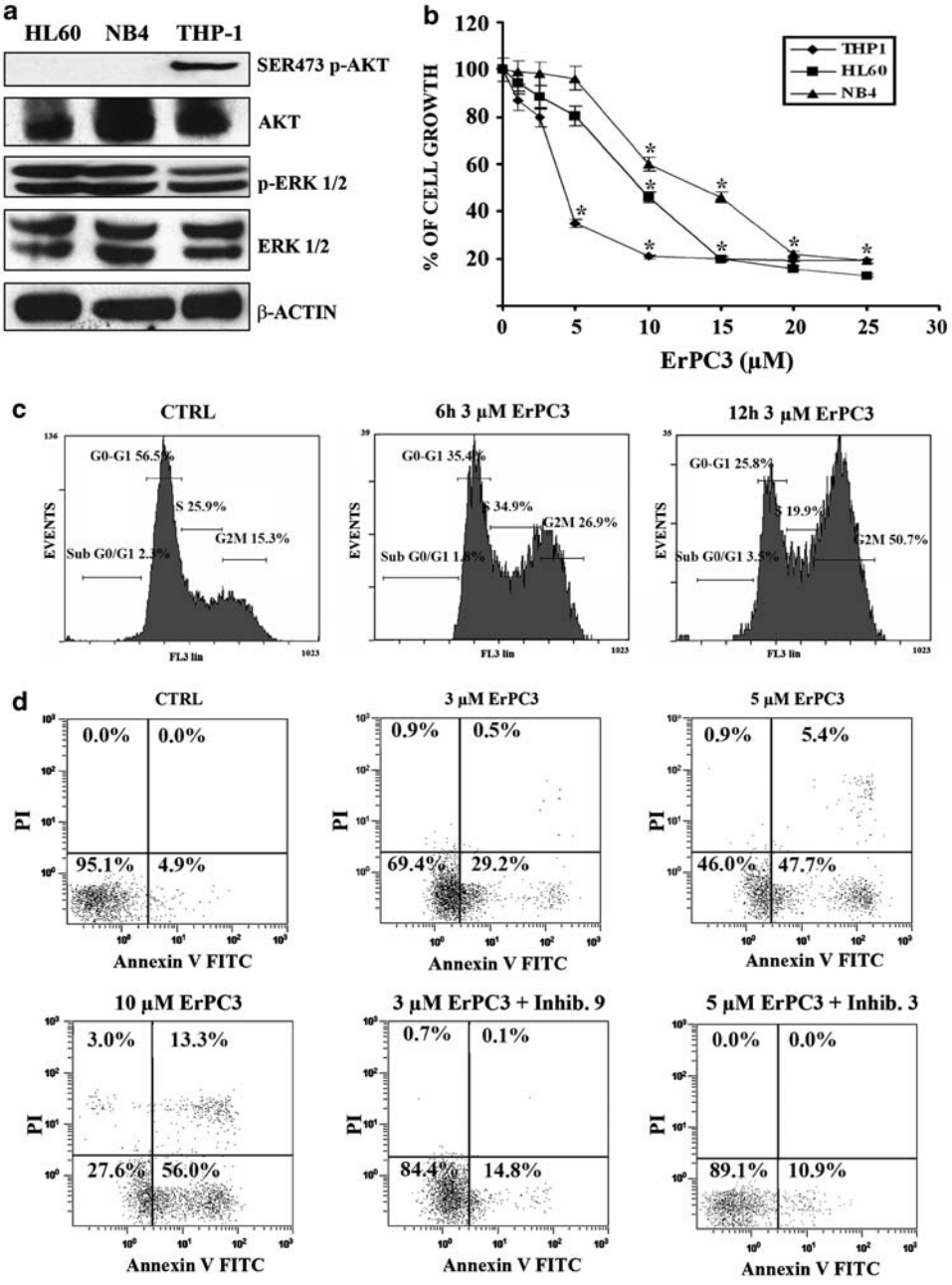

Figure 1 ErPC3 is cytotoxic to AML cell lines. (**a**) Western blot analysis documenting PI3K/Akt and/or MEK/ERK activation in AML cell lines. Protein (40 μg) from whole-cell extracts were separated by SDS–polyacrylamide gel electrophoresis, blotted to nitrocellulose membranes and probed with antibodies. β-Actin served as a loading control. (**b**) MTT assays of AML cell lines treated with ErPC3 for 24 h. Results are the mean of at least three different experiments ± s.d. The asterisks indicate statistically significant difference ($P<0.01$) with respect to untreated cells. (**c**) Cell-cycle analysis of PI-stained THP1 cells. The histograms are representative of three separate experiments that were performed in triplicate. (**d**) Flow cytometric analysis of Annexin V–FITC/PI-stained THP1 cells treated with ErPC3 for 24 h. The percentages of early apoptotic cells (Annexin V–FITC$^+$/PI$^-$; lower right quadrant) and late apoptotic/necrotic cells (Annexin V–FITC$^+$/PI$^+$; upper right quadrant) are indicated. The histograms are representative of three separate experiments that were performed in triplicate. For experiments with caspase inhibitors (Z-DEVD-FMK for caspase-3 and AC-LEHD-CHO for caspase-9), the cells were preincubated with the chemicals (20 μM) for 2 h before ErPC3 treatment.

ErPC3 treatment, but not under basal conditions (Supplementary Figure 1d). These findings were consistent with the notion that, in THP1 cells, p110δ PI3K is the most important isoform for regulating downstream signaling events under basal conditions.[36]

Akt dephosphorylation/cleavage in THP1 cells was detected at a concentration (10 μM ErPC3), which was well above the IC_{50} for this drug in this cell line (around 5 μM, see Figure 1a). This observation suggested that Akt downmodulation could not be a major factor in determining cell sensitivity to ErPC3. However, previous results have documented that PI3K/Akt activation rendered neoplastic cells more sensitive to perifosine.[37] To establish if PI3K/Akt activation would indeed sensitize hematopoietic cells to ErPC3, we took advantage of mouse Ba/F3 cells overexpressing mutant (activated) forms of p110α PI3K. These cells display elevated, constitutive Akt activation, are interleukin-3 independent and are leukemogenic when transplanted in sublethally irradiated Balb/C mice.[19] However, as presented

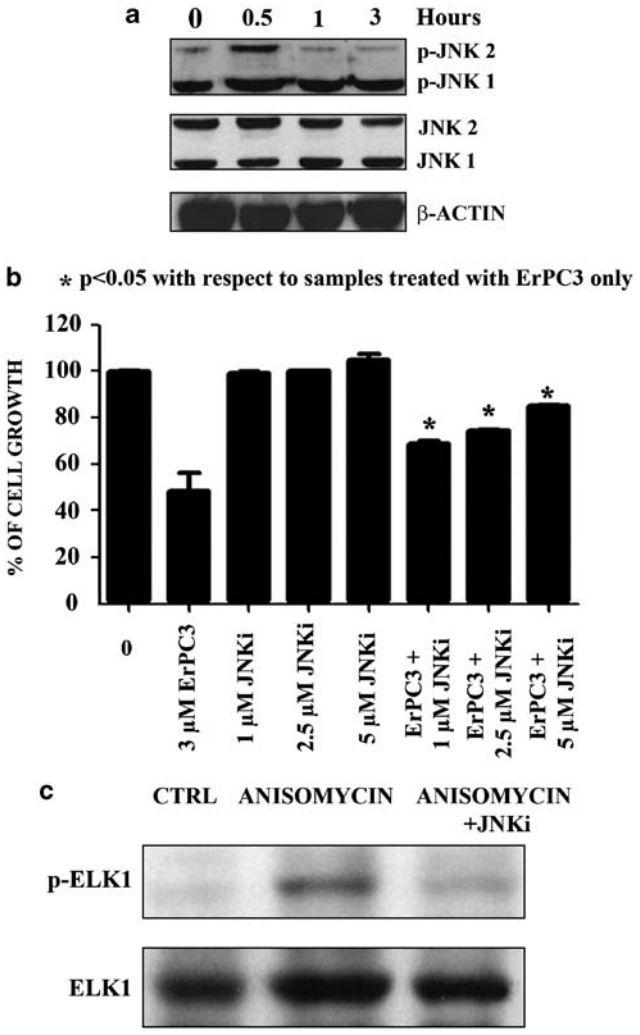

Figure 2 ErPC3 activates JNK 1/2 in THP1 cells. (**a**) Western blot analysis for p-JNK 1/2 and JNK 1/2 in THP1 cells treated with 3 μM ErPC3 for increasing periods of time. β-Actin served as a loading control. (**b**) MTT assays of AML cell lines treated with ErPC3 (3 μM for 24 h) in the absence or in the presence of a JNK 1/2 inhibitor peptide (JNKi). Results are the mean of three different experiments ± s.d. The asterisks indicate statistically significant difference ($P<0.05$) with respect to untreated cells. (**c**) Western blot analysis for p-ELK1 and ELK1 in THP1 cells challenged with anisomycin. Cells were treated with the chemical (10 μg/ml) for 1 h. In case of JNK 1/2 peptide inhibitor (JNKi) treatment, cells had been preincubated with the peptide (1 μM) for 2 h before incubation with anisomycin. An antibody specific to Ser 383 p-ELK1 was used. CTRL: untreated cells.

in Supplementary Figure 2a, MTT assays documented that Ba/F3 cells overexpressing mutant p110α PI3K were not more sensitive to ErPC3 than control (transfected with the empty vector only) cells. Western blot analysis demonstrated Akt phosphorylation in the former, but not in the latter Ba/F3 cells (Supplementary Figure 2b). Overall, these findings suggested that Akt activation could not be considered a major factor that influenced hematopoietic cell sensitivity to ErPC3.

ErPC3 affects MEK/ERK signaling in THP1 and HL60 cells

In THP1 cells, ERK 1/2 was already completely dephosphorylated at 3 μM ErPC3, whereas no MEK1/2 dephosphorylation was detectable even at the highest drug concentration tested (20 μM). Similar results were observed with HL60 cells, even if in this case MEK dephosphorylation was detected at 10 μM ErPC3 (Figure 3a). This observation prompted us to investigate mechanisms, other than MEK 1/2 inhibition, which could explain the decreased levels of p-ERK 1/2 in response to ErPC3. If THP1 cells, in addition to ErPC3, were treated with okadaic acid (300 nM), an inhibitor of both PP1 and PP2A, the decrease in ERK 1/2 phosphorylation was completely reversed (Figure 3b). This suggested that ErPC3 activated protein phosphatases, which could then be involved in dephosphorylating p-ERK 1/2. As there are reports indicating that PP2A is sometimes involved in ERK 1/2 dephosphorylation,[38–40] we downregulated the catalytic subunit of PP2A (PP2A/C) by siRNA,[41] then we analyzed whether downmodulation affected THP1 cell sensitivity to ErPC3. Western blot analysis documented that specific siRNA to PP2A/C reduced its expression by ~75%, whereas a control (scrambled) siRNA had no effect (Figure 3c). In addition, both siRNAs did not affect PP1 expression levels. MTT assays demonstrated that in cells with downregulated PP2A/C expression, the cytotoxic effect of ErPC3 was significantly blunted, whereas in cells treated with scrambled siRNA, ErPC3 cytotoxicity was comparable with control cells (Figure 3c). Western blot analysis demonstrated that PP2A/C downmodulation indeed prevented ERK 1/2 dephosphorylation by ErPC3 (Figure 3d).

A downstream target of MEK/ERK signaling, which is important for cell survival, is BCL2. BCL2 phosphorylation on Ser 70 blocks its antiapoptotic function.[42]. As it is established that BCL2 phosphorylation on Ser 70 is regulated not only by ERK 1/2 and PP2A[41,43] but also by the stress-activated JNK,[42] it was important to investigate whether ErPC3 could indeed modulate the levels of p-BCL2. As documented by western blotting, ErPC3 decreased p-BCL2 phosphorylation levels on Ser 70 (Figure 3e). However, in cells exposed to PP2A/C-specific siRNA, but not in those treated with control siRNA, ErPC3 was ineffective in decreasing p-BCL2 levels. A decrease in p-BCL2 was also observed in both THP1 and HL60 cells treated with a specific MEK inhibitor (CI-1040, 0.5 μM), but not in those exposed to the JNK 1/2 inhibitor peptide (Figure 3f). Overall, these findings indicated important roles for PP2A and ERK 1/2 signaling in the regulation of p-BCL2 levels in AML cells treated with ErPC3, and suggested that ERK 1/2 is upstream of BCL2. We also investigated the expression of other proteins whose expression is under the control of MEK/ERK signaling and which could be important for cell survival, including BCL-XL, survivin and MCL1.[44–46] However, in THP1 cells none of these proteins decreased in response to ErPC3 treatment. In contrast, ErPC3 upregulated proapoptotic Bim levels, consistently with ERK 1/2 signaling inhibition[47] (Figure 3f). Ba/F3 cell clones displayed p-ERK 1/2; however, ErPC3 treatment resulted in slight or no dephosphorylation (Supplementary Figure 2c). This finding strengthened the contention that ERK 1/2 dephosphorylation is critical for the cytotoxic activity of ErPC3. As PKCα can activate Raf,[48] it might be that ErPC3 promoted PP2A dephosphorylation of PKCα to suppress Raf-mediated activation of MEK, as documented by others.[49] Therefore, PKCα phosphorylation was investigated in AML cell lines after treatment with increasing concentrations of ErPC3. However, as shown in Supplementary Figure 2d, neither in THP1 nor in HL60 cells, ErPC3 exposure resulted in PKCα dephosphorylation on Ser 657. Actually, PKCα phosphorylation levels increased, in agreement with our own previous findings obtained with perifosine.[15] We did not detect p-PKCα in NB4 cells.

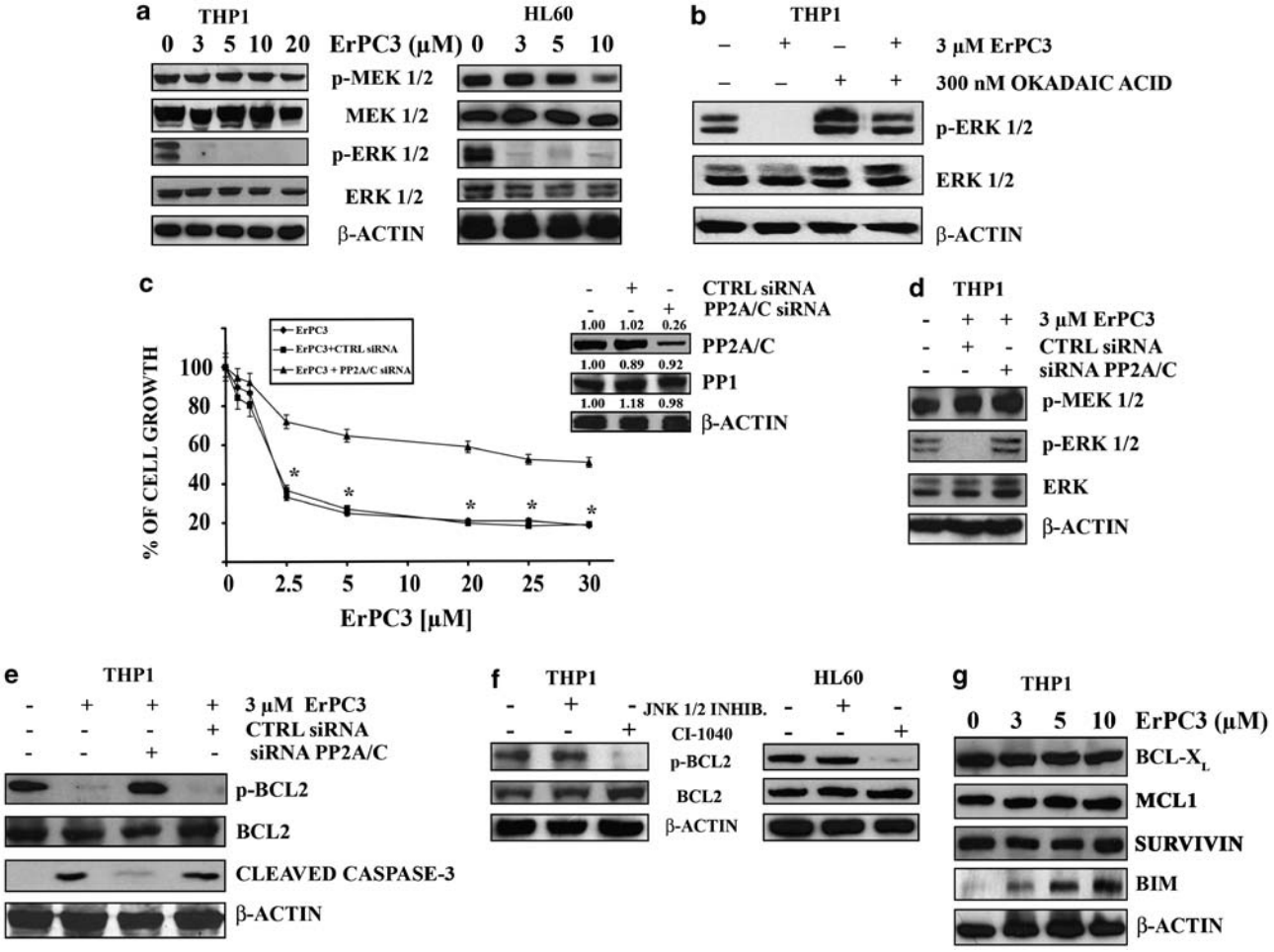

Figure 3 ErPC3 affects MEK/ERK signaling in THP1 and HL60 cells through PP2A. (**a**) Western blot analysis for p-MEK 1/2, MEK 1/2, p-ERK 1/2 and ERK 1/2 expression levels in response to increasing concentrations of ErPC3 administered for 24 h. (**b**) Western blot analysis documenting that the protein phosphatase inhibitor, okadaic acid, counteracted the ErPC3-elicited p-ERK 1/2 dephosphorylation. THP1 cells were incubated with ErPC3 for 24 h. (**c**) PP2A/C downregulation by means of siRNA blunts the cytotoxic effect of ErPC3. MTT assays of THP1 cells treated with ErPC3 for 24 h. Results are the mean of at least three different experiments ± s.d. The asterisks indicate statistically significant difference ($P<0.01$) with respect to untreated cells. CTRL siRNA: control (scrambled) siRNA. The blot in panel c documents decreased PP2A/C expression in cells treated for 48 h with siRNA specific to PP2A/C. Protein levels were quantified using the Image J software (http://rsbweb.nih.gov/ij/). Band intensities of control were normalized to 1, and treated samples were expressed as fraction of control.[20] (**d**) Western blot analysis demonstrating that siRNA to PP2A/C reverses p-ERK 1/2 dephosphorylation caused by ErPC3 (24 h). CTRL siRNA: scrambled siRNA. (**e**) Western blot analysis documenting that, in THP1 cells with downmodulated PP2A/C expression, p-BCL2 was not dephosphorylated and caspase-3 was cleaved less in response to ErPC3 treatment (24 h) when compared with control cells or cells incubated with control (CTRL) siRNA. (**f**) Western blot analysis showing that, in both THP1 and HL60 cells, p-BCL2 levels were under the control of MEK/ERK, but not JNK signaling. Cells were treated for 24 h with CI-1040 (0.5 μM, a MEK inhibitor) or the JNK 1/2 inhibitor peptide (1 μM). (**g**) Western blot analysis for antiapoptotic proteins in THP1 cells incubated for 24 h in the presence of increasing concentrations of ErPC3.

ErPC3 is cytotoxic to primary AML cells and inhibits clonogenic activity of leukemic CD34$^+$ cells

The efficacy of ErPC3 was then analyzed on bone marrow samples obtained from 13 patients with AML. In these samples, activation of PI3K/Akt and MEK/ERK signaling pathways was analyzed using quantitative flow cytometry. A ratio of fluorescence intensity >2.0 was considered positive for pathway activation.[50] In these samples, MTT assays (72 h) documented an IC_{50} for ErPC3 ranging from 7.5 to 14.5 μM (Table 1).

To formally prove that a PP2A-dependent mechanism was causal to the cytotoxic effect of ErPC3 also in primary leukemic cells, we silenced the PP2A/C expression in AML cells by transfection of specific siRNA and assayed transfected cells for apoptosis. Supplementary Figure 3a shows that AML cells could be efficiently transfected by PP2A/C siRNA. The downmodulation of PP2A/C significantly reduced AML blast sensitivity to 15 μM ErPC3 after a 72-h incubation with the drug (Supplementary Figure 3b). ErPC3 did not influence clonogenic activity of healthy CD34$^+$ cells from cord blood; however, in leukemic CD34$^+$ cells, ErPC3 exhibited a statistically significant inhibitory effect (Figure 4a).

ErPC3 induces apoptosis in the CD34$^+$ CD38$^{Low/Neg}$ CD123$^+$ AML blast subset in which it activates JNK 1/2 and dephosphorylates p-BCL2

A key issue in AML therapy is to target the compartment of LICs, which are thought to be involved in AML chemoresistance and relapse.[51] The CD34$^+$ CD38$^{Low/Neg}$ CD123$^+$ population has been previously reported as being a cell compartment enriched in LICs[28]. AML samples were treated with 15 μM ErPC3, and apoptosis was analyzed by quadruple color flow

Table 1 AML patients, activation status of signaling pathways and efficacy of ErPC3

Patient	*FAB*	*RFI p-AKT*	*RFI p-ERK1/2*	*IC_{50} ErPC3 (μM)*
1	M1	3.4	2.6	10.6 ± 1.8
2	M0	3.8	3.9	9.8 ± 1.5
3	M1	1.4	1.6	13.8 ± 2.3
4	M2	1.5	3.7	9.1 ± 1.9
5	M1	1.9	2.8	10.9 ± 2.1
6	M4	5.8	2.2	9.6 ± 1.5
7	M5	2.1	2.2	14.5 ± 2.6
8	M4	1.5	4.4	8.5 ± 2.8
9	M4	2.3	2.6	10.5 ± 1.9
10	M5	1.2	3.3	8.5 ± 1.4
11	M2	1.1	4.1	7.5 ± 1.7
12	M0	2.8	1.5	13.2 ± 2.7
13	M0	2.5	1.7	13.4 ± 3.1

Abbreviations: AML, acute myelogenous leukemia; ErPC3, erucylphosphohomocholine; FAB, French–American–British; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; RFI, ratio of fluorescence intensity.
AML blasts were isolated from patient bone marrow. They were then stained for Ser 473 p-Akt or p-ERK 1/2 using antibodies conjugated to AlexaFluor 647. We analyzed RFI between the mean fluorescence intensity (MFI) of the stained cells and the MFI of the isotypic control. Cells were considered as positive if the RFI was >2. For calculation of the IC_{50}, cells were incubated in triplicate with ErPC3 for 72 h, then MTT assays were performed.

cytometry.[28] An antibody to p-JNK 1/2 documented that ErPC3 activated JNK 1/2 also in this AML cell subset (Figure 4b). Using Annexin V-PE staining in this specific subpopulation, it was demonstrated that ErPC3 induced significant concentration-dependent apoptosis in all the six samples tested. Apoptosis ranged between 6 and 9% in control samples vs 25 and 31% in treated (15 μM ErPC3) samples. It can be noted that the JNK 1/2 peptide inhibitor significantly reduced apoptosis in this immature leukemic cell subpopulation (Figure 4c). Moreover, the same technique highlighted that ErPC3 (15 μM) dephosphorylated Ser 70 p-BCL2 in this AML cell subset (Figure 4d). These results indicated that ErPC3 may target a cell compartment enriched in putative LICs and that JNK activation is important for ErPC3 cytotoxicity also in AML primary cells.

ErPC3 targets both primary AML cells with high levels of ALDH and the SP of AML cell lines and patients

Cells with high levels of ALDH are also considered to be enriched in LICs.[29,52,53] It was therefore investigated whether ErPC3 could target this subpopulation using Aldefluor-stained samples from six AML patients. Diethylaminobenzaldehyde, an inhibitor of ALDH, was used to demonstrate the specificity of the staining. The number of $ALDH^+$ cells varied greatly in each of these AMLs (Figure 5a), but in all cases a very pronounced and statistically significant decrease in the number of these cells was observed after 24 h of incubation with 15 μM ErPC3 (Figures 5a–b). Moreover, as a further control, we repeated the experiments using samples that were double stained for CD34 and ALDH, as it has been reported that $CD34^-/ALDH^+$ cells are normal erythroid progenitors.[54] Thus, we could assess that ErPC3 indeed targeted a $CD34^+/ALDH^+$ leukemic subpopulation (not shown).

Finally, we sought to demonstrate whether ErPC3 could target the SP of THP1 and HL60 cells, as this subpopulation, which overexpresses ABCG2 (also referred to as Breast Cancer Resistance Protein or BRCP),[55,56] is thought to share some properties of tumor-initiating cells.[57,58] The SP of THP1 and HL60 cells was identified by the live-cell DNA-binding dye, Hoechst 33342. As a control, Hoechst 33342 staining could be inhibited after incubation with the broad-specificity ABC membrane transporter inhibitor, verapamil, or the selective ABCG2 transporter inhibitor, fumitremorgin c. A concentration-dependent decrease in the amount of SP cells was evident in samples treated with ErPC3 for 24 h (Figures 6a,b). Expression of ABCG2 on THP1 SP cells was documented by double staining with Hoechst 33342 and a PE-conjugated antibody, which recognizes an extracellular epitope of the transporter (Figure 6c). The bulk of the THP1 and HL60 cell population did not display any positivity for ABCG2 (data not shown). It was next investigated whether ErPC3 was also effective in reducing the SP of eight AML patients. In this case, to identify the SP we used the fumitremorgin c analog, KO143 (0.1 μM), which is more potent than fumitremorgin c and is nontoxic at effective *in vitro* and *in vivo* concentrations.[59] The proportion of SP cells in bone marrow samples varied widely among patients, from 3.0 to 12.1% (average 6.85 ± 3.49), in agreement with others.[57] After ErPC3 treatment (15 μM for 24 h), the SP of AML primary cells was not detectable anymore. Staining for ABCG2 confirmed the expression of this transporter also in the SP of AML patients (Figure 6d).

Discussion

ErPC3 is an APC, a class of antitumor agents that differs from APLs, as APCs do not contain glycerol as structural element. In this study, we have addressed the efficacy of ErPC3 in decreasing cell survival of AML cell lines and blasts. We have demonstrated that ErPC3, in a dose-dependent manner, was cytotoxic to AML cell lines. In THP1 cells, ErPC3 induced a G2/M block and then apoptosis. An inhibitor of caspase-9 was less effective than an inhibitor of caspase-3 in reducing apoptosis. This observation is consistent with the fact that ErPC3 activated other apical caspases, including caspase-8 and -10 (not shown). ErPC3 also induced a transient activation of JNK 1/2. The relevance of JNK 1/2 upregulation for ErPC3 cytotoxicity was demonstrated by the use of a JNK 1/2 inhibitor peptide, which increased cell survival, and this was consistent with the notion that JNK signaling was very important for apoptosis induced by ErPC3 in glioblastoma cells.[60] The peptide we used in our study is one of the most selective JNK 1/2 inhibitors, which are currently available, as it inhibits interactions between JNK 1/2 and downstream targets.[33] It is worth mentioning here that a transient JNK 1/2 activation has been previously observed with a different APC, perifosine.[25]

In THP1 cells, we observed a dephosphorylation of Akt on both Ser 473 and Thr 308, which became apparent at 10 μM ErPC3. However, Akt dephosphorylation was accompanied by a decrease in total Akt, which was due to Akt cleavage. A novel finding that emerged from our studies is that ErPC3 upregulated the levels of p110α PI3K and its end product, PIP3. This could be interpreted as a mechanism of defence implemented by the cells against a proapoptotic insult. Nevertheless, increased PIP3 production did not result in increased phosphorylation levels of Akt, and p110α PI3K inhibition with a selective inhibitor did not increase ErPC3 cytotoxicity (data not shown), so that the relevance of PIP3 upmodulation in response to ErPC3 treatment needs to be further addressed.

ErPC3 (3 μM) completely dephosphorylated ERK 1/2 in both THP1 and HL60 cells, whereas p-MEK 1/2 dephosphorylation was undetectable in THP1 cells or detected at a much higher

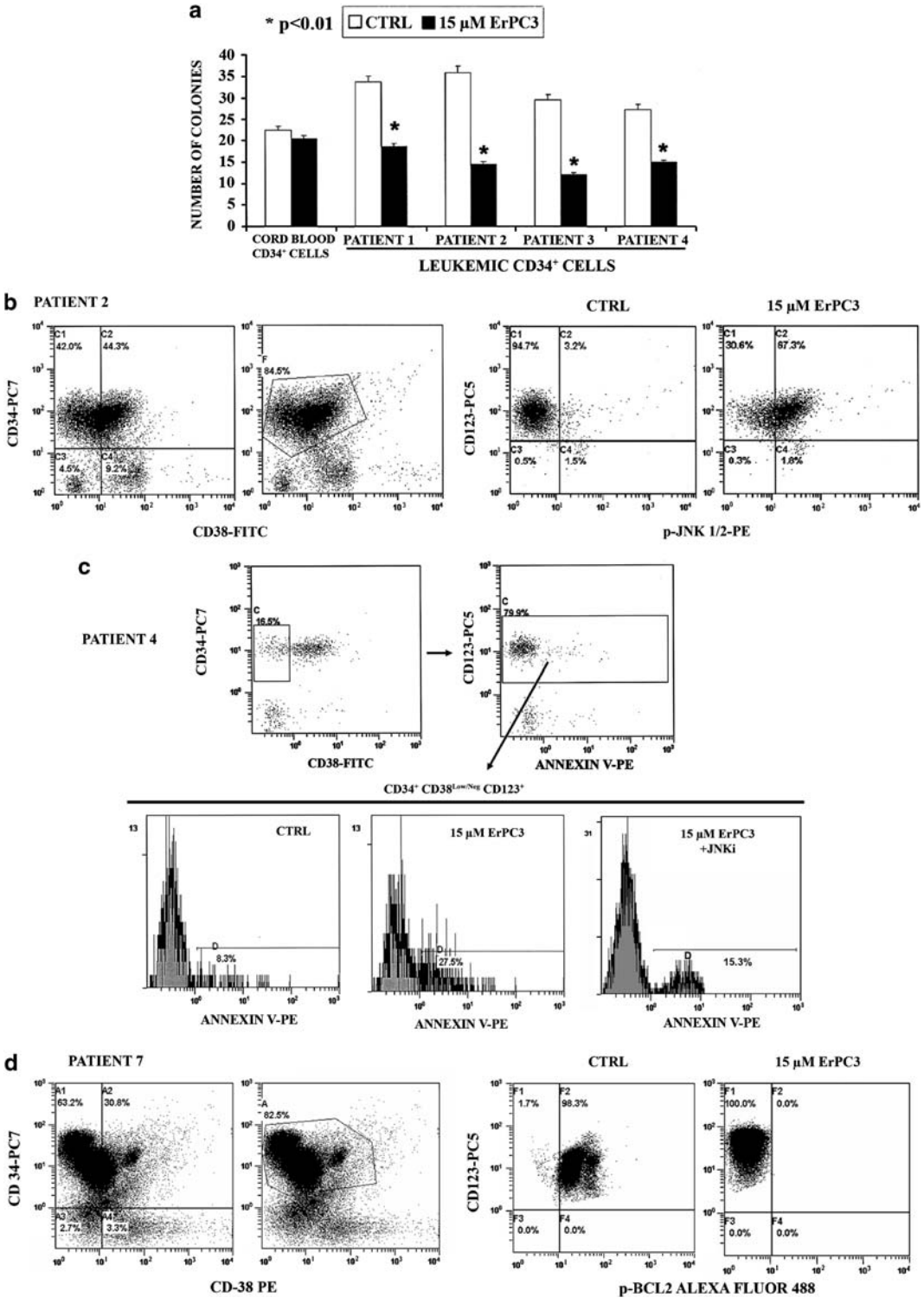

Figure 4 ErPC3 reduces the clonogenic activity of leukemic $CD34^+$ cells and induces apoptosis in the $CD34^+$, $CD38^{Low/Neg}$, $CD123^+$ leukemic cell compartment. (**a**) Clonogenic assays. Cord blood or leukemic patient's $CD34^+$ cells were seeded in a semisolid methylcellulose medium containing GM-CSF, interleukin-3 and -6 for 14 days. The *x* axis indicates the number of colonies per view (observation at $\times 25$ under an Olympus light microscope, Olympus, Tokyo, Japan). Results are the mean ± s.d. from three different experiments. (**b**) Blast cells from AML patients were incubated for 48 h in the presence of ErPC3, and then subjected to flow cytometric analysis with antibodies to CD38-FITC, CD34-PC7, CD123-PC5, and an unconjugated primary antibody to JNK 1/2, which was followed by a PE-conjugated secondary antibody. Results are shown for patient 2. (**c**) The $CD34^+$ $CD38^{Low/Neg}$ $CD123^+$ subpopulation was then analyzed for Annexin V–PE staining. Annexin V–PE-positive cells in the selected $CD34^+$ $CD38^{Low/Neg}$ $CD123^+$ compartment are shown for patient 4. Note how a JNK 1/2 inhibitor peptide (1 μM) decreased ErPC3-dependent apoptosis. (**d**) ErPC3 dephosphorylates Ser 70 p-BCL2 in the $CD34^+$ $CD38^{Low/Neg}$ $CD123^+$ leukemic subpopulation. Experimental details are as in b, except that PE-conjugated anti-CD38 and AlexaFluor 488-conjugated anti-Ser 70 p-BCL2 antibodies were used. Results are shown for patient 7.

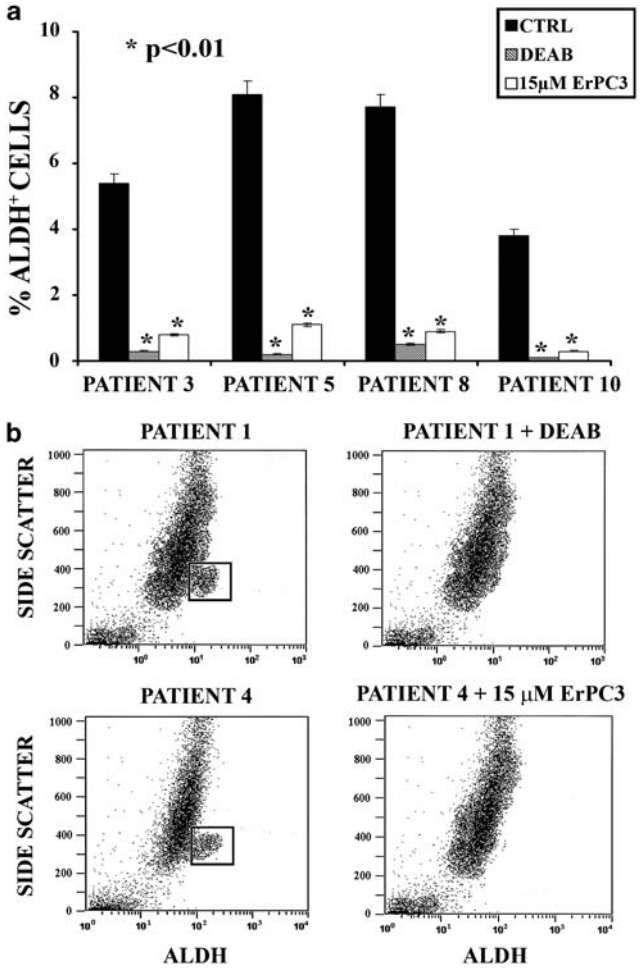

Figure 5 Effect of ErPC3 ALDH+ cells in AML patients. (**a**) ALDH+ cells in four different AML patients, after incubation for 48 h in the presence of 15 μM ErPC3. Bodipy-aminoacetaldehyde was used as the ALDH substrate. In each experiment, a sample of cells was stained under identical conditions with the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) (50 nM) as negative control. Results are the mean of three different experiments ± s.d. The asterisks indicate statistically significant difference ($P<0.01$) with respect to untreated cells. CTRL: untreated cells. (**b**) Flow cytometric analysis of $ALDH^+$ cells in two representative AMLs. Experimental conditions were as in panel a. $ALDH^+$ cells, which disappeared in the presence of DEAB, were gated. Square, portion of $ALDH^+$ cells that were gated for analysis.

ErPC3 concentration (10 μM) in HL60 cells. Our findings suggest that ERK 1/2 dephosphorylation was due to activation of PP2A, as both the protein phosphatase inhibitor okadaic acid and siRNA downregulation of PP2A/C blocked ErPC3-dependent ERK 1/2 dephosphorylation in THP1 cells. It is intriguing that a cholesterol-regulated dual-specificity ERK 1/2 phosphatase, composed of PP2A and the tyrosine phosphatase HePTP, has been demonstrated to localize to lipid rafts.[38] Considering that ALPs and APCs target the lipid rafts, we could hypothesize that ErPC3 activated this phosphatase complex, which would then dephosphorylate ERK 1/2. In the absence of PP2A/C, the complex could not form, so that ERK1/2 was not dephosphorylated. ERK 1/2 dephosphorylation was capable of impinging on BCL2, as also this antiapoptotic protein was dephosphorylated and inactivated in response to ErPC3 (or a MEK inhibitor) treatment in THP1 cells. The relevance of ERK 1/2 dephosphorylation to the ErPC3 cytotoxic effect was also demonstrated by experiments carried out in Ba/F3 cell clones, which were less sensitive to the drug than human AML cell lines and did not display ERK 1/2 dephosphorylation. It can be noted that ErPC3 did not cause caspase activation in Ba/F3 cells and there was no Akt cleavage in the clones (data not shown). Nevertheless, HL60 and NB4 cells, which displayed ERK 1/2, but not Akt phosphorylation, were less sensitive to ErPC3 than THP1 cells, which have both activated ERK 1/2 and activated Akt. This likely indicates that other factors are involved in determining AML cell sensitivity to ErPC3.

ErPC3 negatively affected cell survival of AML blast cells. Interestingly, also in primary AML cells, downmodulation of PP2A/C by siRNA significantly blunted ErPC3 cytotoxicity. This effect of ErPC3 on PP2A activity could be relevant for its therapeutic development, as recent findings have highlighted that PP2A expression levels are low in AML primary cells.[61] Thus, it could be important to develop drugs that increase PP2A activity.

ErPC3 did not negatively affect the clonogenic activity of $CD34^+$ cells from cord blood. In contrast, the clonogenic activity of $CD34^+$ cells from AML patients was significantly impaired by ErPC3. It is now well demonstrated that only a small subset of cells, referred to as LICs,[51] are able to maintain the leukemic cell pool in the long term. These cells are $CD34^+$, $CD38^{Low/Neg}$, $CD123^+$, although not all the cells showing this phenotype are probably true LICs. ErPC3 increased apoptosis in this AML cell subpopulation. Using flow cytometry we were also able to demonstrate that ErPC3 activated JNK 1/2 and dephosphorylated Ser 70 p-BCL2 in these cells and that JNK activation had an important role in the apoptosis induced by ErPC3. As a further proof that ErPC3 targets subpopulations that are enriched in putative LICs, we documented that the drug decreased the number of AML blasts with high levels of ALDH, and also markedly diminished the number of AML cells displaying the SP phenotype. Therefore, our results are fully consistent with two recent reports that have shown that perifosine was able to target SP cells in glioblastomas,[56] as well as cells with cancer stem-cell like properties in breast tumor xenografts.[13] These important properties of ErPC3 and perifosine are not always induced by traditional chemotherapeutic agents. It is at present unclear whether ErPC3 was cytotoxic to the SP cells or it simply blocked the activity of ABCG2, as it has been previously documented that membrane localization of ABCG2 in mouse bone marrow cells was dependent on Akt activity.[62] Nevertheless, ErPC3 decreased the SP of THP1 cells even at a concentration (3 μM) in which it did not affect Akt amount/phosphorylation. Moreover, ErPC3 targeted the SP of HL60 cells that do not display Akt activation. These observations suggest that ErPC3 could be directly cytotoxic to the SP cells. However, additional experiments are required to further address this issue.

Our findings suggest that Akt activation does not confer additional sensitivity to ErPC3 in mouse hematopoietic cells. The MTT assays performed in AML primary cells would indicate that ERK 1/2 activation is critical for ErPC3 sensitivity, as cells with low levels of p-ERK 1/2 generally displayed a higher IC_{50} for ErPC3; however, analysis of a larger cohort of patients is required. In addition, the levels and activation of JNK 1/2 in response to ErPC3 should be analyzed in a larger number of AML patients. JNK 1/2 could be a critical mediator of AML patient sensitivity to various drugs, as has been recently established for anthracyclines.[63] The role of ERK 1/2 in drug resistance is now firmly established.[64] In this connection, it is important to emphasize that ErPC3 synergizes *in vitro* with chemotherapeutic drugs commonly used for treating AML

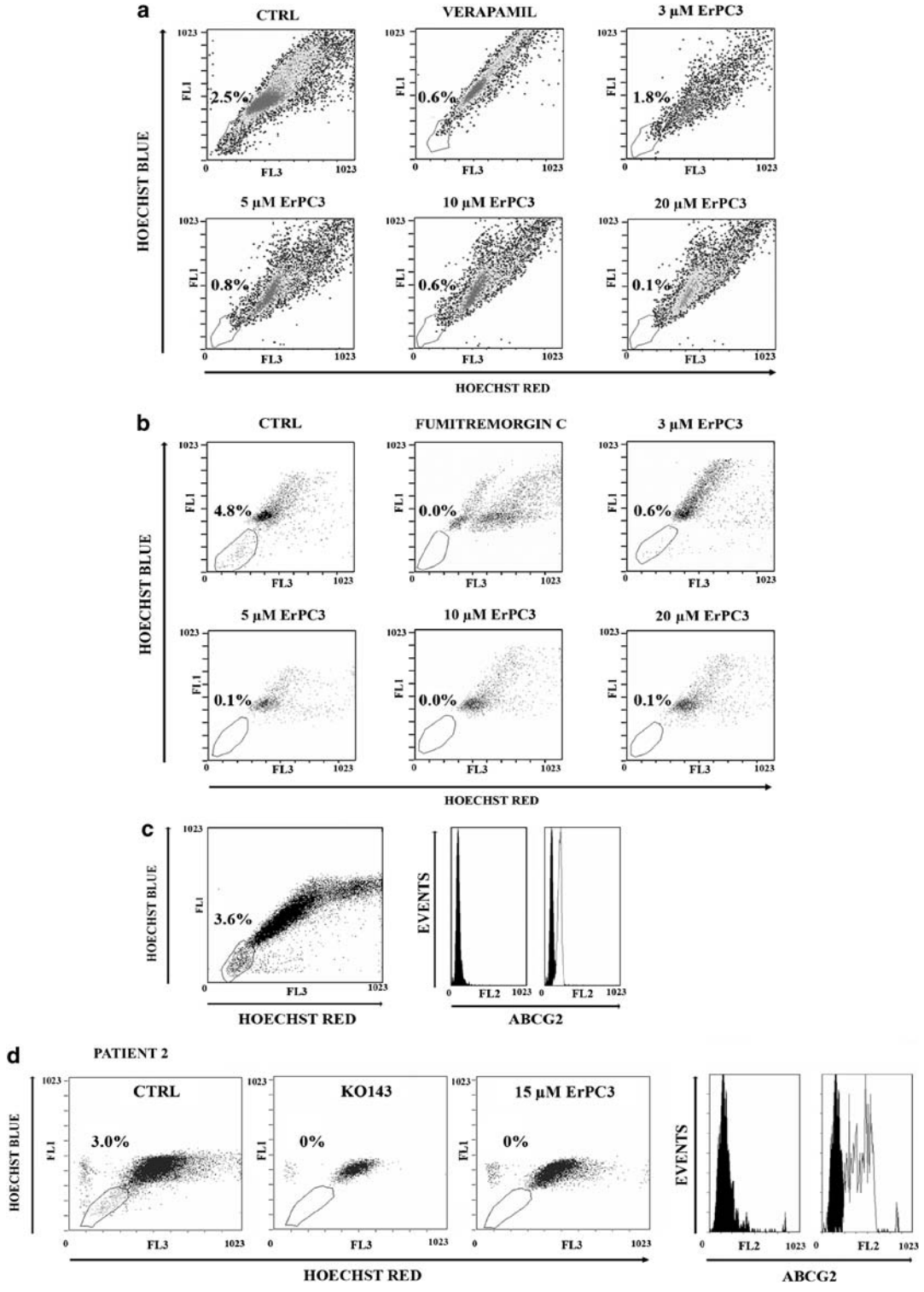

Figure 6 ErPC3 reduces the amount of SP cells. (**a** and **b**) THP1 (**a**) and HL60 (**b**) cells were stained with Hoechst 33342 dye (5 μg/ml) in the presence or absence of either verapamil (100 μM) or fumitremorgin c (10 μM), then analyzed by flow cytometry. The SP, which disappeared in the presence of either verapamil or fumitremorgin c, was gated and shown as a percentage of the whole viable cell population. Incubation in the presence of increasing concentrations of ErPC3 was for 24 h. (**c**) Flow cytometric analysis of THP1 cells double stained with Hoechst 33342 and a PE-conjugated antibody to ABCG2. Black histograms: negative control (irrelevant antibody); white histograms: cells positive for ABCG2. (**d**) Flow cytometric analysis of the SP and ABCG2 expression in patient 2. Bone marrow mononuclear cells were incubated with ErPC3 (15 μM for 24 h). For experimental details, see panels **a**–**c** in this figure. KO143 was used at 0.1 μM. In **a**–**d**, one representative of three different separate experiments is shown. CTRL: cells stained with Hoechst 33342 only.

patients.[18] ErPC3 has been studied in a phase I dose escalation trial at the University of Munich. Thereby, plasma steady state levels of 10–20 μM ErPC3 after repeated intravenous application have been reached without showing toxicity (data not shown). These promising clinical data and our preclinical findings showing significant apoptosis in AML cells at dose levels of 15 μM ErPC3 strongly suggest that ErPC3 could be a valuable drug for improving our arsenal for the treatment of AML patients who still face a poor prognosis, especially those aged $\geqslant$60 years.[65]

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

1 van Blitterswijk WJ, Verheij M. Anticancer alkylphospholipids: mechanisms of action, cellular sensitivity and resistance, and clinical prospects. *Curr Pharm Design* 2008; **14**: 2061–2074.
2 Berger MR, Muschiol C, Schmahl D, Eibl HJ. New cytostatics with experimentally different toxic profiles. *Cancer Treat Rev* 1987; **14**: 307–317.
3 van der Luit AH, Vink SR, Klarenbeek JB, Perrissoud D, Solary E, Verheij M *et al.* A new class of anticancer alkylphospholipids uses lipid rafts as membrane gateways to induce apoptosis in lymphoma cells. *Mol Cancer Ther* 2007; **6**: 2337–2345.
4 Gajate C, Mollinedo F. Edelfosine and perifosine induce selective apoptosis in multiple myeloma by recruitment of death receptors and downstream signaling molecules into lipid rafts. *Blood* 2007; **109**: 711–719.
5 Steelman LS, Abrams SL, Whelan J, Bertrand FE, Ludwig DE, Basecke J *et al.* Contributions of the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways to leukemia. *Leukemia* 2008; **22**: 686–707.
6 McCubrey JA, Steelman LS, Abrams SL, Bertrand FE, Ludwig DE, Basecke J *et al.* Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways for effective leukemia therapy. *Leukemia* 2008; **22**: 708–722.
7 Vogler WR, Berdel WE, Olson AC, Winton EF, Heffner LT, Gordon DS. Autologous bone marrow transplantation in acute leukemia with marrow purged with alkyl-lysophospholipid. *Blood* 1992; **80**: 1423–1429.
8 Andreesen R, Modolell M, Weltzien HU, Eibl H, Common HH, Lohr GW *et al.* Selective destruction of human leukemic cells by alkyl-lysophospholipids. *Cancer Res* 1978; **38**: 3894–3899.
9 Vogler WR, Olson AC, Okamoto S, Somberg LB, Glasser L. Experimental studies on the role of alkyl lysophospholipids in autologous bone marrow transplantation. *Lipids* 1987; **22**: 919–924.
10 Eibl H. Miltefosine for visceral leishmaniasis. *New Engl J Med* 2000; **342**: 894–895.
11 Gills JJ, Dennis PA. Perifosine: update on a novel Akt inhibitor. *Curr Oncol Rep* 2009; **11**: 102–110.
12 Mitsiades CS, Hideshima T, Chauhan D, McMillin DW, Klippel S, Laubach JP *et al.* Emerging treatments for multiple myeloma: beyond immunomodulatory drugs and bortezomib. *Semin Hematol* 2009; **46**: 166–175.
13 Korkaya H, Paulson A, Charafe-Jauffret E, Ginestier C, Brown M, Dutcher J *et al.* Regulation of mammary stem/progenitor cells by PTEN/Akt/β-catenin signaling. *PLoS Biol* 2009; **7**: e1000121.
14 Papa V, Tazzari PL, Chiarini F, Cappellini A, Ricci F, Billi AM *et al.* Proapoptotic activity and chemosensitizing effect of the novel Akt inhibitor perifosine in acute myelogenous leukemia cells. *Leukemia* 2008; **22**: 147–160.
15 Tazzari PL, Tabellini G, Ricci F, Papa V, Bortul R, Chiarini F *et al.* Synergistic proapoptotic activity of recombinant TRAIL plus the Akt inhibitor Perifosine in acute myelogenous leukemia cells. *Cancer Res* 2008; **68**: 9394–9403.
16 Georgieva MC, Konstantinov SM, Topashka-Ancheva M, Berger MR. Combination effects of alkylphosphocholines and gemcitabine in malignant and normal hematopoietic cells. *Cancer Lett* 2002; **182**: 163–174.
17 Jendrossek V, Erdlenbruch B, Hunold A, Kugler W, Eibl H, Lakomek M. Erucylphosphocholine, a novel antineoplastic ether lipid, blocks growth and induces apoptosis in brain tumor cell lines *in vitro*. *Int J Oncol* 1999; **14**: 15–22.
18 Fiegl M, Lindner LH, Juergens M, Eibl H, Hiddemann W, Braess J. Erufosine, a novel alkylphosphocholine, in acute myeloid leukemia: single activity and combination with other antileukemic drugs. *Cancer Chemother Pharmacol* 2008; **62**: 321–329.
19 Horn S, Bergholz U, Jucker M, McCubrey JA, Trumper L, Stocking C *et al.* Mutations in the catalytic subunit of class IA PI3K confer leukemogenic potential to hematopoietic cells. *Oncogene* 2008; **27**: 4096–4106.
20 Fala F, Blalock WL, Tazzari PL, Cappellini A, Chiarini F, Martinelli G *et al.* Proapoptotic activity and chemosensitizing effect of the novel Akt inhibitor (2S)-1-(1H-Indol-3-yl)-3-[5-(3-methyl-2H-indazol-5-yl)pyridin-3-yl]oxyprop an2-amine (A443654) in T-cell acute lymphoblastic leukemia. *Mol Pharmacol* 2008; **74**: 884–895.
21 Nyakern M, Tazzari PL, Finelli C, Bosi C, Follo MY, Grafone T *et al.* Frequent elevation of Akt kinase phosphorylation in blood marrow and peripheral blood mononuclear cells from high-risk myelodysplastic syndrome patients. *Leukemia* 2006; **20**: 230–238.
22 Follo MY, Finelli C, Bosi C, Martinelli G, Mongiorgi S, Baccarani M *et al.* PI-PLCβ-1 and activated Akt levels are linked to azacitidine responsiveness in high-risk myelodysplastic syndromes. *Leukemia* 2008; **22**: 198–200.
23 Follo MY, Finelli C, Mongiorgi S, Clissa C, Bosi C, Martinelli G *et al.* PKR is activated in MDS patients and its subcellular localization depends on disease severity. *Leukemia* 2008; **22**: 2267–2269.
24 Silva A, Yunes JA, Cardoso BA, Martins LR, Jotta PY, Abecasis M *et al.* PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest* 2008; **118**: 3762–3774.
25 Chiarini F, Del Sole M, Mongiorgi S, Gaboardi GC, Cappellini A, Mantovani I *et al.* 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. *Leukemia* 2008; **22**: 1106–1116.
26 Renner AG, Dos Santos C, Recher C, Bailly C, Creancier L, Kruczynski A *et al.* Polo-like kinase 1 is overexpressed in acute myeloid leukemia and its inhibition preferentially targets the proliferation of leukemic cells. *Blood* 2009; **114**: 659–662.
27 Chiarini F, Fala F, Tazzari PL, Ricci F, Astolfi A, Pession A *et al.* Dual inhibition of class IA phosphatidylinositol 3-kinase and mammalian target of rapamycin as a new therapeutic option for T-cell acute lymphoblastic leukemia. *Cancer Res* 2009; **69**: 3520–3528.
28 Park S, Chapuis N, Bardet V, Tamburini J, Gallay N, Willems L *et al.* PI-103, a dual inhibitor of class I A phosphatidylinositide 3-kinase and mTOR, has antileukemic activity in AML. *Leukemia* 2008; **22**: 1698–1706.
29 Cheung AM, Wan TS, Leung JC, Chan LY, Huang H, Kwong YL *et al.* Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engrafting potential. *Leukemia* 2007; **21**: 1423–1430.
30 Riccioni R, Senese M, Diverio D, Riti V, Buffolino S, Mariani G *et al.* M4 and M5 acute myeloid leukaemias display a high sensitivity to Bortezomib-mediated apoptosis. *Br J Haematol* 2007; **139**: 194–205.
31 Cabana R, Frolova EG, Kapoor V, Thomas RA, Krishan A, Telford WG. The minimal instrumentation requirements for Hoechst side population analysis: stem cell analysis on low-cost flow cytometry platforms. *Stem Cells* 2006; **24**: 2573–2581.

32 Borsello T, Clarke PG, Hirt L, Vercelli A, Repici M, Schorderet DF *et al.* A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat Med* 2003; **9**: 1180–1186.
33 Barr RK, Kendrick TS, Bogoyevitch MA. Identification of the critical features of a small peptide inhibitor of JNK activity. *J Biol Chem* 2002; **277**: 10987–10997.
34 Chen R, Kang VH, Chen J, Shope JC, Torabinejad J, DeWald DB *et al.* A monoclonal antibody to visualize PtdIns(3,4,5)P(3) in cells. *J Histochem Cytochem* 2002; **50**: 697–708.
35 Hayakawa M, Kaizawa H, Moritomo H, Koizumi T, Ohishi T, Okada M *et al.* Synthesis and biological evaluation of 4-morpholino-2-phenylquinazolines and related derivatives as novel PI3 kinase p110α inhibitors. *Bioorgan Med Chem* 2006; **14**: 6847–6858.
36 Billottet C, Grandage VL, Gale RE, Quattropani A, Rommel C, Vanhaesebroeck B *et al.* A selective inhibitor of the p110δ isoform of PI 3-kinase inhibits AML cell proliferation and survival and increases the cytotoxic effects of VP16. *Oncogene* 2006; **25**: 6648–6659.
37 Kondapaka SB, Singh SS, Dasmahapatra GP, Sausville EA, Roy KK. Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. *Mol Cancer Ther* 2003; **2**: 1093–1103.
38 Wang PY, Liu P, Weng J, Sontag E, Anderson RG. A cholesterol-regulated PP2A/HePTP complex with dual specificity ERK1/2 phosphatase activity. *EMBO J* 2003; **22**: 2658–2667.
39 Kim SH, Markham JA, Weiler IJ, Greenough WT. Aberrant early-phase ERK inactivation impedes neuronal function in fragile X syndrome. *Proc Natl Acad Sci USA* 2008; **105**: 4429–4434.
40 Chen L, Liu L, Yin J, Luo Y, Huang S. Hydrogen peroxide-induced neuronal apoptosis is associated with inhibition of protein phosphatase 2A and 5, leading to activation of MAPK pathway. *Int J Biochem Cell Biol* 2009; **41**: 1284–1295.
41 Deng X, Gao F, May WS. Protein phosphatase 2A inactivates Bcl2′s antiapoptotic function by dephosphorylation and up-regulation of Bcl2-p53 binding. *Blood* 2009; **113**: 422–428.
42 Deng X, Xiao L, Lang W, Gao F, Ruvolo P, May Jr WS. Novel role for JNK as a stress-activated Bcl2 kinase. *J Biol Chem* 2001; **276**: 23681–23688.
43 Deng X, Ruvolo P, Carr B, May Jr WS. Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. *Proc Natl Acad Sci USA* 2000; **97**: 1578–1583.
44 Nishioka C, Ikezoe T, Yang J, Yokoyama A. Inhibition of MEK signaling enhances the ability of cytarabine to induce growth arrest and apoptosis of acute myelogenous leukemia cells. *Apoptosis* 2009; **14**: 1108–1120.
45 Kumar P, Coltas IK, Kumar B, Chepeha DB, Bradford CR, Polverini PJ. Bcl-2 protects endothelial cells against gamma-radiation via a Raf-MEK-ERK-survivin signaling pathway that is independent of cytochrome c release. *Cancer Res* 2007; **67**: 1193–1202.
46 Zhang W, Konopleva M, Ruvolo VR, McQueen T, Evans RL, Bornmann WG *et al.* Sorafenib induces apoptosis of AML cells via Bim-mediated activation of the intrinsic apoptotic pathway. *Leukemia* 2008; **22**: 808–818.
47 Rambal AA, Panaguiton ZL, Kramer L, Grant S, Harada H. MEK inhibitors potentiate dexamethasone lethality in acute lymphoblastic leukemia cells through the pro-apoptotic molecule BIM. *Leukemia* 2009; **23**: 1744–1754.
48 Buitrago CG, Pardo VG, de Boland AR, Boland R. Activation of RAF-1 through Ras and protein kinase C alpha mediates 1α,25(OH)2-vitamin D3 regulation of the mitogen-activated protein kinase pathway in muscle cells. *J Biol Chem* 2003; **278**: 2199–2205.
49 Tsao CC, Nica AF, Kurinna SM, Jiffar T, Mumby M, Ruvolo PP. Mitochondrial protein phosphatase 2A regulates cell death induced by simulated ischemia in kidney NRK-52E cells. *Cell Cycle* 2007; **6**: 2377–2385.
50 Bardet V, Tamburini J, Ifrah N, Dreyfus F, Mayeux P, Bouscary D *et al.* Single cell analysis of phosphoinositide 3-kinase/Akt and ERK activation in acute myeloid leukemia by flow cytometry. *Haematologica* 2006; **91**: 757–764.
51 Misaghian N, Ligresti G, Steelman LS, Bertrand FE, Basecke J, Libra M *et al.* Targeting the leukemic stem cell: the Holy Grail of leukemia therapy. *Leukemia* 2009; **23**: 25–42.
52 Pearce DJ, Taussig D, Simpson C, Allen K, Rohatiner AZ, Lister TA *et al.* Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. *Stem Cells* 2005; **23**: 752–760.
53 Hess DA, Meyerrose TE, Wirthlin L, Craft TP, Herrbrich PE, Creer MH *et al.* Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. *Blood* 2004; **104**: 1648–1655.
54 Mirabelli P, Di Noto R, Lo Pardo C, Morabito P, Abate G, Gorrese M *et al.* Extended flow cytometry characterization of normal bone marrow progenitor cells by simultaneous detection of aldehyde dehydrogenase and early hematopoietic antigens: implication for erythroid differentiation studies. *BMC Physiol* 2008; **8**: 13.
55 Shukla S, Sauna ZE, Ambudkar SV. Evidence for the interaction of imatinib at the transport-substrate site(s) of the multidrug-resistance-linked ABC drug transporters ABCB1 (P-glycoprotein) and ABCG2. *Leukemia* 2008; **22**: 445–447.
56 Bleau AM, Hambardzumyan D, Ozawa T, Fomchenko EI, Huse JT, Brennan CW *et al.* PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell Stem Cell* 2009; **4**: 226–235.
57 Wulf GG, Wang RY, Kuehnle I, Weidner D, Marini F, Brenner MK *et al.* A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid leukemia. *Blood* 2001; **98**: 1166–1173.
58 Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res* 2007; **67**: 4827–4833.
59 Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G *et al.* Potent and specific inhibition of the breast cancer resistance protein multidrug transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* 2002; **1**: 417–425.
60 Kugler W, Erdlenbruch B, Otten K, Jendrossek V, Eibl H, Lakomek M. MAP kinase pathways involved in glioblastoma response to erucylphosphocholine. *Int J Oncol* 2004; **25**: 1721–1727.
61 Gallay N, Dos Santos C, Cuzin L, Bousquet M, Simmonet Gouy V, Chaussade C *et al.* The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* 2009; **23**: 1029–1038.
62 Mogi M, Yang J, Lambert JF, Colvin GA, Shiojima I, Skurk C *et al.* Akt signaling regulates side population cell phenotype via Bcrp1 translocation. *J Biol Chem* 2003; **278**: 39068–39075.
63 Lagadinou ED, Ziros PG, Tsopra OA, Dimas K, Kokkinou D, Thanopoulou E *et al.* c-Jun N-terminal kinase activation failure is a new mechanism of anthracycline resistance in acute myeloid leukemia. *Leukemia* 2008; **22**: 1899–1908.
64 McCubrey JA, Abrams SL, Ligresti G, Misaghian N, Wong EW, Steelman LS *et al.* Involvement of p53 and Raf/MEK/ERK pathways in hematopoietic drug resistance. *Leukemia* 2008; **22**: 2080–2090.
65 Brandwein JM, Leber BF, Howson-Jan K, Schimmer AD, Schuh AC, Gupta V *et al.* A phase I study of tipifarnib combined with conventional induction and consolidation therapy for previously untreated patients with acute myeloid leukemia aged 60 years and over. *Leukemia* 2009; **23**: 631–634.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)