Synergistic Proapoptotic Activity of Recombinant TRAIL Plus the Akt Inhibitor Perifosine in Acute Myelogenous Leukemia Cells

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Abstract

To potentiate the response of acute myelogenous leukemia (AML) cells to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) cytotoxicity, we have examined the efficacy of a combination with perifosine, a novel phosphatidylinositol-3-kinase (PI3K)/Akt signaling inhibitor. The rationale for using such a combination is that perifosine was recently described to increase TRAIL-R2 receptor expression and decrease the cellular FLICE-inhibitory protein (cFLIP) in human lung cancer cell lines. Perifosine and TRAIL both induced cell death by apoptosis in the THP-1 AML cell line, which is characterized by constitutive PI3K/Akt activation, but lacks functional p53. Perifosine, at concentrations below IC₅₀, dephosphorylated Akt and increased TRAIL-R2 levels, as shown by Western blot, reverse transcription-PCR, and flow cytometric analysis. Perifosine also decreased the long isoform of cFLIP (cFLIP-L) and the X-linked inhibitor of apoptosis protein (XIAP) expression. Perifosine and TRAIL synergized to activate caspase-8 and induce apoptosis, which was blocked by a caspase-8–selective inhibitor. Up-regulation of TRAIL-R2 expression was dependent on a protein kinase Ccx/c-Jun-NH₂-kinase 2/c-Jun signaling pathway activated by perifosine through reactive oxygen species production. Perifosine also synergized with TRAIL in primary AML cells displaying constitutive activation of the Akt pathway by inducing apoptosis, Akt dephosphorylation, TRAIL-R2 up-regulation, cFLIP-L, and XIAP down-regulation, and c-Jun phosphorylation. The combined treatment negatively affected the clonogenic activity of CD34⁺ cells from patients with AML. In contrast, CD34⁺ cells from healthy donors were resistant to perifosine and TRAIL treatment. Our findings suggest that the combination of perifosine and TRAIL might offer a novel therapeutic strategy for AML.
have been reached during clinical evaluation (16, 17). We have recently shown the cytotoxic activity of perifosine, alone or in combination with chemotherapeutic drugs, in AML cells (18). Therefore, it was investigated whether perifosine could increase AML cell sensitivity to recombinant TRAIL. Here, we show in THP-1 AML cells that perifosine increased TRAIL-R2 expression and decreased levels of the long isoform of the cellular FLICE-inhibitory protein (cFLIP-L) and X-linked inhibitor of apoptosis protein (XIAP) at concentrations below the IC50. When perifosine was combined with TRAIL, there was a synergistic induction of apoptosis and increased levels of caspase-8 activation. Similar results were obtained using AML blasts with a constitutively active PI3K/Akt pathway. Perifosine and TRAIL combined treatment also decreased the clonogenic activity of CD34+ cells from patients with AML with active Akt, whereas it had no effect on CD34+ cells from healthy donors. Therefore, our findings suggest that perifosine, in combination with TRAIL, may represent an effective approach for the treatment of patients with AML.

Materials and Methods

Chemicals and antibodies. Perifosine was provided by AEterna Zentaris GmbH. For cell viability determination, Cell Viability Kit 1 (3,4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Roche Applied Science. Propidium iodide (PI; DNA-PREP kit) was from Beckman Coulter Immunology. The Annexin V-FITC staining kit was from Tau Technologies BV, whereas carboxyfluorescein fluorescent-labeled inhibitor of caspases (FLICA) apoptosis detection kit for caspase activity assay was from AbD Serotec. Recombinant human TRAIL, the c-Jun NH2-terminal kinase (JNK) inhibitor SP600125, and the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, were from EMD Biosciences. The protein kinase C (PKC) inhibitor G66976, phorbol 12-myristate 13-acetate (PMA), the reactive oxygen species (ROS) scavenger N-acetyl-l-cysteine (NAC), and dichlorodihydraflorescein diacetate were from Sigma-Aldrich. Antibodies to the following proteins were used for Western blot analysis: Akt, Ser473, p-Akt, XIAP, FADD, PKCα, caspase-8, Ser80, Ser416 p-PKCα/β, c-Jun, Ser63 p-c-Jun, Thr183/Tyr185 p-JNK 1/2, and β-tubulin were from Cell Signaling; PKCβ2 was from Santa Cruz Biotechnology; TRAIL receptor 1, R2, R3, R4, and cFLIP-S/L (which recognizes both the short and the long isoforms of the protein) were from ProSci, Inc. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology. For flow cytometric analysis, AlexaFlour 488-conjugated anti-Ser473 p-Akt was from Cell Signaling; mouse anti-human TRAIL receptor antibodies conjugated to phycoerythrin were from R&D Systems; mouse anti-human CD33 FITC-conjugated antibody was from BD Biosciences PharmaMing. Controls were performed with normal rabbit IgG conjugated to AlexaFlour 488 or normal mouse IgG conjugated to either phycoerythrin or FITC (Upstate).

Cell culture, patients, and clonogenic assays. THP-1 human acute monocytic leukemia cells were grown as previously reported (18). Samples were obtained from patients upon presentation of AML, before chemotherapy treatment. Informed consent was obtained from all patients prior to obtaining the samples, according to institutional guidelines. Bone marrow or peripheral blood mononuclear cells were isolated by Ficoll-Paque (Amersham Biosciences) density gradient centrifugation. The percentage of blasts in the samples ranged between 75% and 91% and was checked by flow cytometry staining, depending on the phenotype of the leukemia (usually CD13, CD33, CD34, CD45, alone or in combination). Blast cells were cultured in RPMI 1640 supplemented with 20% FCS. CD34+ progenitor cells from patients with AML or from cord blood were isolated using immunomagnetic cell separation (Miltenyi Biotec) and cultured as reported previously (14).

Cell viability analysis by MTT assay. An MTT assay was used to analyze cell growth and viability, as reported elsewhere (19).

Flow cytometric detection of apoptosis and ROS generation. This was performed as previously reported (18, 20).

Whole cell lysate preparation, cell fractionation, Western blot, and densitometric analysis of blots. This was performed as previously described (14). For analysis of THP-1 cells, 40 μg of protein per lane was loaded, whereas for AML blasts, 80 μg of protein per lane was loaded. Densitometric analysis was as reported by Nyakern and colleagues (21). For each blot, the band with the highest intensity was normalized to 1, whereas other bands were expressed as a fraction. Values from densitometric scanning are indicated above each protein band. All the blots shown are representative of at least three separate experiments.

Immunoprecipitation. Cells were lysed in 50 mmol/L of Tris (pH 8.0), 50 mmol/L of KCl, 10 mmol/L of EDTA, 1% Nonidet P-40, protease inhibitor cocktail, and 2 mmol/L of Na3VO4. Immunoprecipitation was performed overnight using polyclonal antibodies to either PKCα or β2, according to standard procedures. Antibodies were captured using protein A/G-agarose and immunoprecipitates washed with lysis buffer.

Caspase activity assay. Flow cytometric assays were performed to determine caspase activity, using the FLICA Apoptosis Detection kit according to the manufacturer’s instructions, as reported elsewhere (18).

Flow cytometric detection of Ser73 p-Akt, TRAIL receptors, and CD33. P-Akt detection was performed essentially as previously reported (13). Anti-TRAIL, TRAIL receptors, and CD33 antibodies (final concentration, 10 μg/mL) were used on fresh, unfixed cells (5 × 104) according to the manufacturer’s procedure. Then, cells were washed thrice with PBS, fixed with 0.5% paraformaldehyde in PBS, again washed thrice with PBS, and immunostained for p-Akt. At least 5,000 events were analyzed for each sample in all flow cytometric analyses. All the flow cytometric data are representative of three different experiments.

Reverse transcription-PCR analysis for TRAIL-R1 and TRAIL-R2 mRNA. This was performed exactly according to Zhang and colleagues (22). PCR-amplified products were electrophoresed on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and were visualized under UV light.

Combined drug effects analysis. To characterize the interactions between TRAIL and perifosine, the combination effect and a potential synergy was evaluated from quantitative analysis of dose-effect relationships described by Chou and Talalay (23). CalcuSyn software (Biosoft) was used to calculate combination indices (CI).

Transient protein down-regulation by short interfering RNA. Scrambled (sc-44230) and specific short interfering RNAs (siRNA) to either PKCα (sc-563243) or c-Jun (sc-29223) were from Santa Cruz Biotechnology. Transfection of THP-1 cells was performed using the Amaxa system (Amaxa) following their specifications (24). Briefly, 106 cells in 100 μL of medium was mixed with 3 μg of siRNA, and transfected to an Amaxa-certified cuvette. For transfection, we used the program V-01. Transfection efficiency was between 75% and 85% (data not shown), as checked by flow cytometry, using a fluorescein-labeled nontargeted siRNA control (Cell Signaling). Cells were examined for gene down-regulation and other properties 48 h after transfection.

Statistical evaluation. The data are presented as mean values from three separate experiments ± SD. Data were statistically analyzed by a Dunnet test after one-way ANOVA at a level of significance of P < 0.05 versus control samples.

Results

Effect of TRAIL and perifosine on THP-1 cell survival. Exposure for 24 hours to increasing concentrations of recombinant TRAIL or perifosine induced a dose-dependent decrease in cell survival, as evaluated by MTT assays (Fig. 1A). At the highest tested concentration of TRAIL (800 ng/mL) or perifosine (16 μmol/L), survival was 60% or 32%, respectively. To establish whether a combined treatment consisting of TRAIL and perifosine was synergistic, THP-1 cells were cultured with serial concentrations of TRAIL (range, 12.5–800 ng/mL) and perifosine (range, 0.25–16.0 μmol/L) at a constant ratio for 24 hours and data were analyzed by the method of Chou and Talalay (23). The combined
treatment was much more cytotoxic than either of the single treatments. All the combinations gave an effect which ranged from synergistic (CI < 0.6) to highly synergistic (CI < 0.3; Fig. 1B). To determine whether decreased cell survival was related to apoptosis, an Annexin V-FITC/PI analysis was performed. When samples were analyzed by flow cytometry, it became evident that the combined TRAIL and perifosine treatment induced apoptotic cell death of THP-1 cells, whereas when the single drugs were used alone, much lower effects were observed (Fig. 1C). Western blot analysis showed a marked decrease in Ser\(^{473}\) p-Akt phosphorylation at 0.5 \(\mu\)mol/L of perifosine. Akt dephosphorylation was complete at 1.0 \(\mu\)mol/L, whereas total Akt levels remained unchanged (Fig. 1D).

Perifosine increases TRAIL-R2 expression in THP-1 cells and down-regulates cFLIP-L and XIAP levels. Given that perifosine up-regulates TRAIL-R2 expression in human lung carcinoma cells (11), we investigated if this was also true for THP-1 cells. The expression of TRAIL receptors in untreated THP-1 cells was examined by flow cytometry. Under basal conditions, it was observed that these cells expressed TRAIL-R1, TRAIL-R2, and TRAIL-R4, but no TRAIL-R3 (data not shown), in agreement with others (25). Western blot analysis showed that perifosine increased the levels of TRAIL-R2 in a dose-dependent manner. The amount of the other TRAIL receptors expressed by THP-1 cells was almost unchanged (Fig. 2A). A dose-dependent decrease in c-FLIP-L and XIAP expression was also observed in THP-1 cells treated with perifosine, whereas FADD levels were not affected. Increased expression of TRAIL-R2 protein by Western blot was corroborated by reverse transcription-PCR (RT-PCR) analysis, which showed an increase in TRAIL-R2, but not in TRAIL-R1, mRNA (Fig. 2A). Also, flow cytometric analysis highlighted selective enhanced TRAIL-R2 expression in response to perifosine treatment (Fig. 2B). Moreover, this technique showed no changes in surface TRAIL expression by perifosine (data not shown).

Perifosine and TRAIL combined treatment results in enhanced caspase-8 activation. The combined treatment was associated with increased activation of caspase-8, as shown by FLICA assay (Fig. 2C). Western blot analysis corroborated flow cytometric findings, demonstrating a dramatic decrease in procaspase-8 levels and the appearance of the p18 cleaved fragment of caspase-8 in cells treated with perifosine and TRAIL.
combination (Fig. 2C). In contrast, no p18 fragment was detected in cells treated with TRAIL alone, whereas a very faint band was visible in samples exposed to perifosine alone. The relevance of caspase-8 activation for perifosine-induced apoptosis was shown by experiments in which cells were preincubated with a selective caspase-8 inhibitor (Z-IETD-FMK) prior to being treated with the combined drugs. This resulted in a much lower percentage of apoptotic cells (Fig. 2D).

Figure 2. Perifosine decreases cFLIP-L and XIAP expression, and down-regulates TRAIL-R2 expression in THP-1 cells. A, Western blot and RT-PCR analysis performed on THP-1 cells treated for 16 h with perifosine. In case of RT-PCR analysis for TRAIL-R1 and TRAIL-R2 mRNA, perifosine concentration was 0.5 μmol/L for 16 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. B, flow cytometric analysis demonstrating surface expression of TRAIL receptors in untreated cells (black-shaded histograms) and cells treated with 2.0 μmol/L of perifosine (gray-shaded histograms) for 16 h. C, FLICA and Western blot analysis of THP-1 cells treated with either perifosine or TRAIL alone and with two drugs together for 24 h. For FLICA analysis: black-shaded histograms, untreated cells; gray-shaded histograms, drug-treated cells. For Western blot analysis: lane 1, untreated cells; lane 2, TRAIL-treated cells; lane 3, perifosine-treated cells; lane 4, TRAIL- and perifosine-treated cells. For Western blot analysis, TRAIL and perifosine concentrations were similar to those used for FLICA analysis. Drug treatment was for 24 h. D, Annexin V-FITC/PI staining analysis of THP-1 cells treated with the two drugs together for 24 h. Samples had been pretreated for 1 h with the caspase-8 inhibitor, Z-IETD-FMK (20 μmol/L). Drug concentrations were as in C. Bottom right quadrants, percentages of cells which are Annexin V-positive and PI-negative (early apoptotic cells).
Up-regulation of TRAIL-R2 requires PKC activity. A recent report has highlighted that TRAIL-R2 up-regulation in non–small cell lung cancer cells required PKC activity (26). Therefore, we investigated whether this was true also in THP-1 cells. Preliminary experiments indicated that THP-1 cells expressed only two of the conventional PKC isoforms, α and β2, whereas β1 and γ were not expressed (data not shown). When cells were treated with perifosine and G6976, a well-established inhibitor of PKC conventional isoforms (27), the increase in TRAIL-R2 expression was lower. No changes in the expression of other TRAIL receptors (R1 and R4) were observed in response to G6976 and perifosine treatment, whereas cFLIP-L and XIAP levels did not significantly change in cells treated with G6976 and perifosine when compared with perifosine alone (Fig. 3A). A time-dependent increase in TRAIL-R2, but not TRAIL-R1, expression levels was detected when THP-1 cells were treated with PMA, an activator of PKC conventional isoforms (ref. 28; Fig. 3B). Down-regulation of PKCα levels by specific siRNA, but not treatment with scrambled siRNA, resulted in a much lower induction of TRAIL-R2 by perifosine (Fig. 3C). MTT assays showed a lower cytotoxic effect of the perifosine and TRAIL combined treatment in cells with down-regulated PKCα expression, but not in those treated with scrambled siRNA (Fig. 3D). The efficacy of PKCα down-regulation by specific siRNA was evaluated by Western blot analysis (Fig. 3D). Overall, these findings indicated that up-regulation of TRAIL-R2 by perifosine was dependent on PKCα and was required to maximally potentiate the proapoptotic effect of TRAIL.

Up-regulation of TRAIL-R2 by perifosine is dependent on a ROS/PKCα/JNK 2/c-Jun pathway. The mechanism of PKCα activation by perifosine was next investigated. It has been shown that PKCα could be activated (phosphorylated) by ROS (29, 30), which also induced its membrane binding. Moreover, perifosine caused ROS production in U937 AML cells (31). Therefore, it was investigated if perifosine also caused ROS production in THP-1 cells. ROS generation was analyzed by flow cytometry after labeling of cells with the ROS-selective probe, DCFH-DA. Perifosine (2 μmol/L) caused an increase in ROS levels, which was blocked by the ROS scavenger, NAC (Supplementary Fig. S1A). Cell fractionation experiments showed that in response to perifosine treatment, the amount of membrane-bound PKCα increased,

Figure 3. Increased TRAIL-R2 expression in THP-1 cells is dependent on PKCα. A, Western blot analysis of THP-1 cell extracts. Lane 1, untreated cells; lane 2, cells treated with perifosine (2 μmol/L); lane 3, cells treated with perifosine (2 μmol/L) + G6976 (0.5 μmol/L). Treatments were for 16 h. B, Western blot analysis for TRAIL-R1 and TRAIL-R2 expression in THP-1 cells treated with PMA (100 ng/mL) for increasing periods of time. C, Western blot analysis for TRAIL-R2 expression levels in cells incubated with perifosine (2 μmol/L for 16 h). Cells treated for 48 h with PKCα-specific siRNA or scrambled siRNA. D, results from MTT assays in cells treated with TRAIL and perifosine for 24 h at the indicated concentrations. Western blot analyses for PKCα levels in THP-1 cell extracts also shown. Lane 1, untreated cells; lane 2, cells treated with siRNA specific for PKCα; lane 3, cells treated with scrambled siRNA. Cells were analyzed 48 h after transfection. β-Tubulin served as loading control.
whereas cytosolic PKCa decreased (Fig. 4A). These changes in PKCa subcellular localization were largely prevented by NAC, suggesting that they were dependent on ROS production. Moreover, perifosine treatment resulted in increased Thr638/641 p-PKCa levels but not in PKCβ2 isoforms, as shown by immunoprecipitation experiments with antibodies selective for total (unphosphorylated and phosphorylated) PKCa and PKCβ2 isoforms, followed by Western blotting with an antibody which recognizes both PKCa and PKCβ1/2 phosphorylated on Thr638/641 (Fig. 4A).

Flow cytometric analysis also showed that the perifosine-induced increase in TRAIL-R2 expression was markedly reduced if cells were treated with NAC in addition to perifosine, whereas RT-PCR documented that NAC was indeed capable of almost completely blocking the perifosine-dependent increase in TRAIL-R2 mRNA levels. However, NAC did not affect the perifosine-evoked decrease in cFLIP-L levels (Fig. 4B). Recent findings have highlighted that TRAIL-R2 gene expression could be under the control of a JNK/c-Jun pathway (32), and that in some cell models, PKCa could be upstream of JNK (33, 34). Perifosine (2 μmol/L) upregulated p-JNK2 (54 kDa) but not p-JNK1 (46 kDa) in THP-1 cells, and this event could be blocked by NAC (Supplementary Fig. S1B). Perifosine treatment resulted in c-Jun phosphorylation on Ser63, and this phosphorylation was inhibited by SP600125 (a JNK 1/2–selective inhibitor) but not by SB203580 (a p38 MAPK–selective inhibitor). Moreover, SP600125, but not SB203580, suppressed the perifosine-dependent increase in TRAIL-R2 expression (Fig. 4C).
Finally, when c-Jun levels were down-regulated by siRNA specific for c-Jun (Supplementary Fig. S1C), the perifosine-evoked increase in TRAIL-R2 expression was reduced significantly (Fig. 4D). Taken together, these findings strongly suggested that perifosine could up-regulate TRAIL-R2 expression through a ROS → PKCα → JNK 2 → c-Jun signaling pathway.

Synergistic cytotoxic effects of TRAIL/perifosine combined treatment on AML blasts with activated Akt. The efficacy of the perifosine and TRAIL combined treatment was then analyzed on samples obtained from patients with AML. Samples from 12 patients were studied (Table 1). Because levels of caspase-8 could influence the TRAIL sensitivity of AML blasts (35), we analyzed samples with comparable expression of caspase-8, as evaluated by Western blot analysis (data not shown). Activation of PKB/Akt signaling was studied by Western blot and/or flow cytometric analysis. Seven patients were positive for Ser473 p-Akt (Table 1). Cytotoxicity was due to apoptotic cell death, as shown by MTT assays (Fig. 5B), and was characterized by increased TRAIL-R2 expression which was documented by Annexin V-FITC/PI staining (Fig. 5)

Our findings point to PKCα as an important mediator of TRAIL-R2 up-regulation in THP-1 cells, as PKCα down-regulation by siRNA resulted in a much lower induction of TRAIL-R2 and XIAP expression. Moreover, c-Jun phosphorylation on Ser63 was not increased after treatment with perifosine (Supplementary Fig. S2B and C). FADD levels did not change in a significant manner. In Table 1, we summarize the results obtained by treating AML samples, showing the effects of perifosine on TRAIL-R2 expression and the average CI of the combined treatment.

Perifosine/TRAIL combined treatment negatively affects clonogenic activity of CD34+ cells from patients with AML. Finally, we investigated the cytotoxic effect of perifosine/TRAIL combined treatment on CD34+ cell clonogenic activity from cord blood and from patients with AML. As expected, neither TRAIL nor perifosine alone influenced the clonogenic activity of CD34+ cells from healthy donors, and the same was true of the combined treatment (Supplementary Fig. S2D). In contrast, in leukemic CD34+ cells, TRAIL moderately affected clonogenic activity and perifosine alone exhibited a statistically significant inhibitory effect in some samples (see for example, patient M2#7). However, in all patient samples with activated Akt, we consistently observed a strong inhibitory effect of the perifosine and TRAIL combined treatment on the CD34+ cell clonogenic activity.

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NOTE: Patients were classified according to the French-American-British (FAB) classification. The levels of Ser473 p-Akt and TRAIL-R2 were evaluated by flow cytometry and/or Western blotting. A CI < 0.9 was considered synergistic, whereas a CI between 0.9 and 1.1 was considered additive. Results are from three different experiments ± SD.

Abbreviations: P, perifosine; T, TRAIL; ED, effective dose.
Furthermore, down-regulation of c-Jun by siRNA also opposed the increase in TRAIL-R2. Given that in cells with down-regulated PKCα, perifosine was unable to increase JNK2 phosphorylation, we propose a mechanism whereby perifosine generates ROS, which in turn, activates a PKCα → JNK2 → c-Jun signaling pathway which leads to increased expression of TRAIL-R2.

XIAP and c-FLIP-L down-regulation caused by perifosine in THP-1 cells, could be due to an inhibition of the nuclear factor κB activity.

Figure 5. Perifosine and TRAIL combination is synergistic in AML primary cells with activated Akt. A, cells from patient M4#1 were treated for 48 h with either single agent alone or in combination at the indicated concentrations. Cell viability was analyzed by MTT assays. B, Annexin V-FITC/PI staining analysis of cells from patient M4#1 treated with either perifosine or TRAIL alone and with two drugs together for 48 h. Bottom right quadrants, percentages of cells which are Annexin V-positive and PI-negative (early apoptotic cells). C, flow cytometric analysis showing surface expression of TRAIL receptors and CD33 in cells from patient M4#1, either untreated or treated with 4.0 μmol/L of perifosine for 24 h. Anti-TRAIL receptor antibodies were conjugated to phycoerythrin, whereas anti-CD33 antibody was FITC-conjugated. D, Western blot analysis for Ser473 p-Akt, cFLIP-L, XIAP, FADD, and Ser63 p-c-Jun in extracts from AML patient primary cells. Perifosine treatment was for 24 h at 4.0 μmol/L concentration. β-Tubulin served as loading control.
which is under the control of the PI3K/Akt axis in AML cells (12). A recent investigation carried out on Waldenstrom macroglobulinemia cells has indeed shown that perifosine targets nuclear factor-κB (40). Future studies should address the mechanism(s) which underlie XIAP and cFLIP-L down-regulation by perifosine in AML cells. Nevertheless, our unpublished findings, obtained by siRNA technology, have indicated that down-regulation of these two proteins was not as critical as that of PKCζ for the potentiating effect of perifosine on TRAIL cytotoxicity on THP-1 cells.

Our results showed that a perifosine and TRAIL combination was also much more effective than either treatment alone in primary AML cells. Even though the analysis we performed was not as comprehensive as the one we did in THP-1 cells, due to the insufficient amount of cells recovered from most of the patients, perifosine dephosphorylated Akt, down-regulated XIAP and cFLIP-L expression, and up-regulated the levels of TRAIL-R2 and Ser63 p-c-Jun in some primary AML cells. AML blasts died by apoptosis and the combined treatment was much more effective in activating caspase-8 than either treatment alone. All the patient samples expressed TRAIL-R2 to some extent (data not shown); however, perifosine increased TRAIL-R2 expression only in samples with activated PI3K/Akt signaling. Accordingly, synergism was only observed in those AML samples which displayed activated Akt. The fact that despite the expression of TRAIL-R2 even under basal conditions, AML blasts were not sensitive to TRAIL alone, could be explained by the contemporaneous expression of TRAIL decoy receptors (8). After perifosine treatment, TRAIL-R2 was markedly up-regulated in AML blasts, and most likely this could overcome the antiapoptotic effect played by high levels of decoy receptors. However, it should not be ruled out that other proteins, which are critically important for TRAIL sensitivity (35, 41), are down-regulated by perifosine in AML primary cells.

At present, it is unclear why perifosine increased ROS generation only in primary AML cells with up-regulated PI3K/Akt signaling. Nevertheless, a recent report has highlighted that 7-ketochetosteryl acetate, which is incorporated into the lipid rafts of THP-1 cells (42), was able to increase ROS production by up-regulating the levels of NAD(P)H oxidase (NOX-4) in THP-1 cells. Interestingly, this was accompanied by a down-regulation of Akt (43). It might be that perifosine, by disrupting PI3K/Akt signaling at the lipid rafts, positively affects NOX-4 gene expression. Therefore, it would be interesting to investigate if NOX-4 gene expression is under the control of the PI3K/Akt axis in AML cells.

Our results point to the fact that a combination consisting of TRAIL and perifosine had no effect on the clonogenic activity of CD34+ cells from healthy donors, whereas it was markedly cytotoxic for CD34+ cells isolated from leukemic patients. Previous results have shown that TRAIL was not cytotoxic for normal CD34+ cells (44, 45), reflecting the lack of TRAIL receptors expressed in these cells (46). In contrast, TRAIL displayed proapoptotic activity in CD34+ cells from patients with AML (45), and we have confirmed these findings. Therefore, CD34+ AML cells express TRAIL receptors. Future investigations should be aimed towards investigating whether leukemic stem cells also express TRAIL receptors and whether they could be targeted by the combination of TRAIL and perifosine.

In conclusion, we have shown the in vitro efficacy of a TRAIL and perifosine combination treatment in AML cells. This combination was also synergistic in cells (THP-1) lacking functional p53. Even if p53 deletion and/or inactivating mutations are observed in only ~10% of patients with AML, p53 levels are frequently low in AML blasts due to overexpression of the negative regulator murine double minute (MDM2; ref. 47). Thus, the use of a drug which could up-regulate TRAIL-R2 levels independently of p53 could be extremely useful in leukemia therapy. Accordingly, it must be emphasized that the cytotoxic effect of triptolide and TRAIL combination was enhanced by the addition of the MDM2 antagonist, Nutlin-3a (36). In case of triptolide, another drug was required to maximize the effects of TRAIL, which could result in additional toxic side effects if administered to patients.

In summary, the combination of perifosine and TRAIL could represent a novel strategy for treating patients with AML by overcoming critical mechanisms of apoptosis resistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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