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

Pier Luigi Tazzari,[1] Giovanna Tabellini,[5] Francesca Ricci,[1] Veronica Papa,[2] Roberta Bortul,[6] Francesca Chiarini,[2] Camilla Evangelisti,[2] Giovanni Martinelli,[3] Andrea Bontadini,[1] Lucio Cocco,[2] James A. McCubrey,[7] and Alberto M. Martelli[2,4]

[1]Servizio di Immunoematologia e Trasfusionale, Policlinico S. Orsola-Malpighi, [2]Dipartimento di Scienze Anatomiche Umane e Fisiopatologia dell'Apparato Locomotore and [3]Dipartimento di Ematologia ed Oncologia Medica "L. and A. Seràgnoli", Università di Bologna, [4]Istituto di Genetica Molecolare del Consiglio Nazionale delle Ricerche, Sezione di Bologna c/o Istituti Ortopedici Rizzoli, Bologna, Italy; [5]Dipartimento di Scienze Biomediche e Biotecnologie, Sezione di Istologia, Università di Brescia, Brescia, Italy; [6]Dipartimento Clinico di Biomedicina, Università di Trieste, Trieste, Italy; and [7]Department of Microbiology and Immunology, Brody School of Medicine at East Carolina University, Greenville, North Carolina

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 IC_{50}, 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 Cα/c-Jun-NH_2-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. [Cancer Res 2008;68(22):9394–403]

Introduction

The tumor necrosis factor (TNF) family member, TNF-related apoptosis-inducing ligand (TRAIL), was originally reported to induce apoptosis in many tumor cells but not in normal cells both *in vitro* and *in vivo* and thus represents a promising anticancer cytokine (1). TRAIL is expressed as a type II TNF trans-membrane protein, however, its extracellular domain can be proteolytically cleaved from the cell surface and acts as a soluble cytokine. TRAIL transmits the death signal via TRAIL-R1 and TRAIL-R2 (also referred to as DR4 and DR5, respectively) receptors, which, upon TRAIL binding, are oligomerized at the cell surface, thereby enabling the recruitment of the adaptor molecule Fas-associated death domain (FADD) and assembly of the death-inducing signaling complex (2). Two other TRAIL receptors, TRAIL-R3 and TRAIL-R4 (also referred to as DcR1 and DcR2) contain no or only a truncated death domain and do not induce apoptosis upon TRAIL binding. TRAIL-R3 and TRAIL-R4, therefore, act as decoy receptors (3). It has been suggested that preferential expression of decoy receptors on normal cells is one of the mechanisms underlying the proapoptotic action of TRAIL in neoplastic cells but not in healthy cells (4). Upon binding of TRAIL to R1 and R2 receptors, the extrinsic apoptosis pathway is activated (3). In recent years, TRAIL has stimulated hope for its therapeutic potential as an antineoplastic agent in different types of tumors, including hematologic malignancies such as acute myelogenous leukemia (AML; ref. 5). The *in vitro* cytotoxic response of AML cell lines to recombinant TRAIL varies from good to moderate (6, 7); however, a number of *in vitro* studies have convincingly shown that AML primary cells are resistant to the proapoptotic activity of TRAIL used as a single agent (e.g., ref. 8). TRAIL sensitivity of AML blasts could be increased by cotreatment with cytotoxic drugs such as daunorubicin (9) or histone deacetylase inhibitors (10). A recent report has highlighted that TRAIL sensitivity of human lung cancer cell lines could be considerably increased by cotreatment with the novel Akt inhibitor, perifosine (11). The phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway is activated in many patients with AML (12–14), and markedly influences AML sensitivity to various drugs, including TRAIL (6). Therefore, small molecules which inhibit this pathway are currently being developed for clinical use, one such inhibitor is perifosine (15). Perifosine is a phospholipid analogue which has shown promising preclinical activity and is also currently undergoing phase I/II clinical evaluation for AML treatment. Serum concentrations of up to 20 μmol/L of perifosine

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Alberto M. Martelli, Dipartimento di Scienze Anatomiche Umane e Fisiopatologia dell'Apparato Locomotore, Sezione di Anatomia Umana, Cell Signalling Laboratory, Università di Bologna, 40126 Bologna, Italy. Phone: 39-51209-1580; Fax: 39-51209-1695; E-mail: alberto.martelli@unibo.it.

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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 IC_{50}. 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 I 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 NH_2-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 Gö6976, phorbol 12-myristate 13-acetate (PMA), the reactive oxygen species (ROS) scavenger *N*-acetyl-L-cysteine (NAC), and dichlorodihydrofuorescein diacetate were from Sigma-Aldrich. Antibodies to the following proteins were used for Western blot analysis: Akt, Ser^{473} p-Akt, XIAP, FADD, PKCα, caspase-8, $Ser^{638}/^{641}$ p-PKCα/β, c-Jun, Ser^{63} p-c-Jun, Thr^{183}/Tyr^{185} p-JNK 1/2, and β-tubulin were from Cell Signaling; PKCβ2 was from Santa Cruz Biotechnology; TRAIL receptors R1, 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, AlexaFluor 488–conjugated anti-Ser^{473} 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 PharMingen. Controls were performed with normal rabbit IgG conjugated to AlexaFluor 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 Na_3VO_4. 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 Ser^{473} 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 (5×10^5) 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-RI 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-36243) 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, 10^6 cells in 100 μL of medium was mixed with 3 μg of siRNA and transferred 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. 1*A*). 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

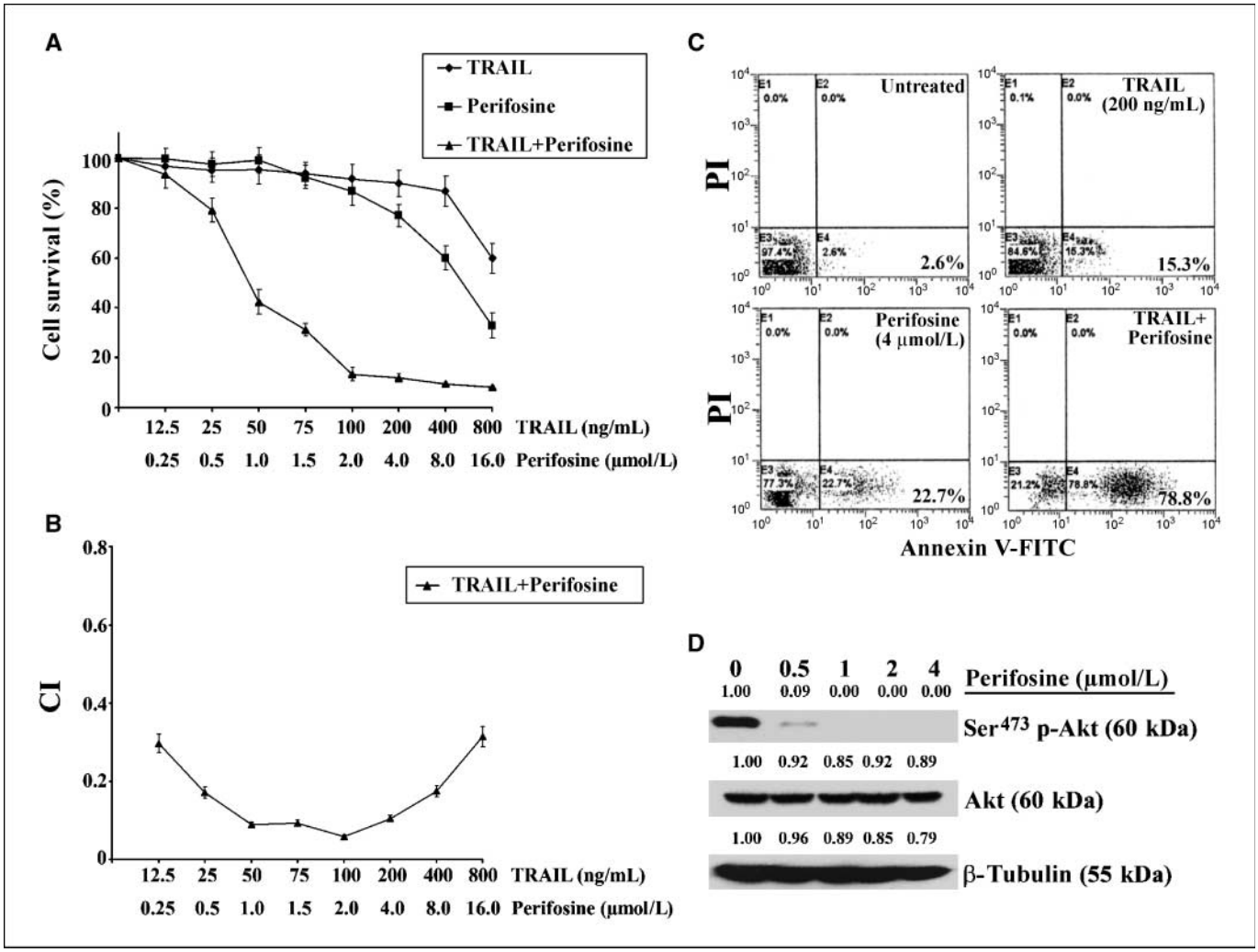

Figure 1. Perifosine synergistically enhances TRAIL-induced cell death in THP-1 cells. *A,* cells were treated for 24 h with either single agent alone or in combination at the indicated concentrations. Cell viability was then analyzed by MTT assays. *B,* CI as calculated from experiments reported in *A. C,* Annexin V-FITC/PI staining analysis of THP-1 cells treated with either perifosine or TRAIL alone and with two drugs together for 24 h. *Bottom right quadrants,* percentages of cells which are Annexin V–positive and PI-negative (early apoptotic cells). *D,* Western blot analysis demonstrating Ser^{473} p-Akt and total Akt levels in THP-1 cells treated with increasing concentrations of perifosine for 16 h. β-Tubulin served as loading control.

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. 1*B*). 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. 1*C*). Western blot analysis showed a marked decrease in Ser^{473} p-Akt phosphorylation at 0.5 μmol/L of perifosine. Akt dephosphorylation was complete at 1.0 μmol/L, whereas total Akt levels remained unchanged (Fig. 1*D*).

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. 2*A*). 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. 2*A*). Also, flow cytometric analysis highlighted selective enhanced TRAIL-R2 expression in response to perifosine treatment (Fig. 2*B*). 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. 2*C*). 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. 2*C*). 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. 2*D*).

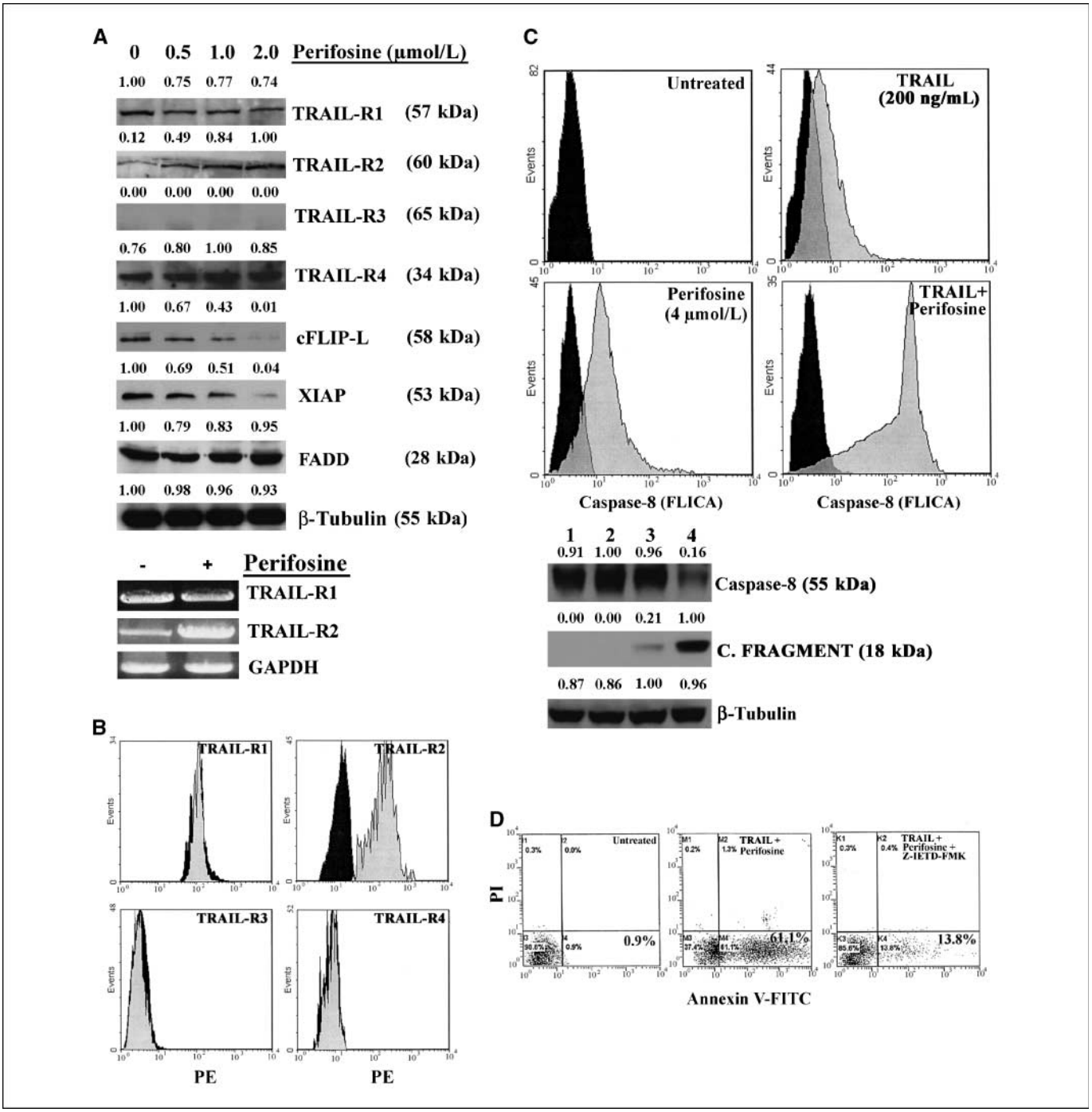

Figure 2. Perifosine decreases cFLIP-L and XIAP expression, and down-regulates TRAIL-R2 expression in THP-1 cells. *A,* Western blot and RT-PCR analysis performed on THP-1 cells treated for 16 h with perifosine. In case of RT-PCR analysis for TRAIL-R1 and TRAIL-R2 mRNA, perifosine concentration was 0.5 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 Gö6976, 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 Gö6976 and perifosine treatment, whereas cFLIP-L and XIAP levels did not significantly change in cells treated with Gö6976 and perifosine when compared with perifosine alone (Fig. 3*A*). 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. 3*B*). 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. 3*C*). 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. 3*D*). The efficacy of PKCα down-regulation by specific siRNA was evaluated by Western blot analysis (Fig. 3*D*). 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. S1*A*). Cell fractionation experiments showed that in response to perifosine treatment, the amount of membrane-bound PKCα increased,

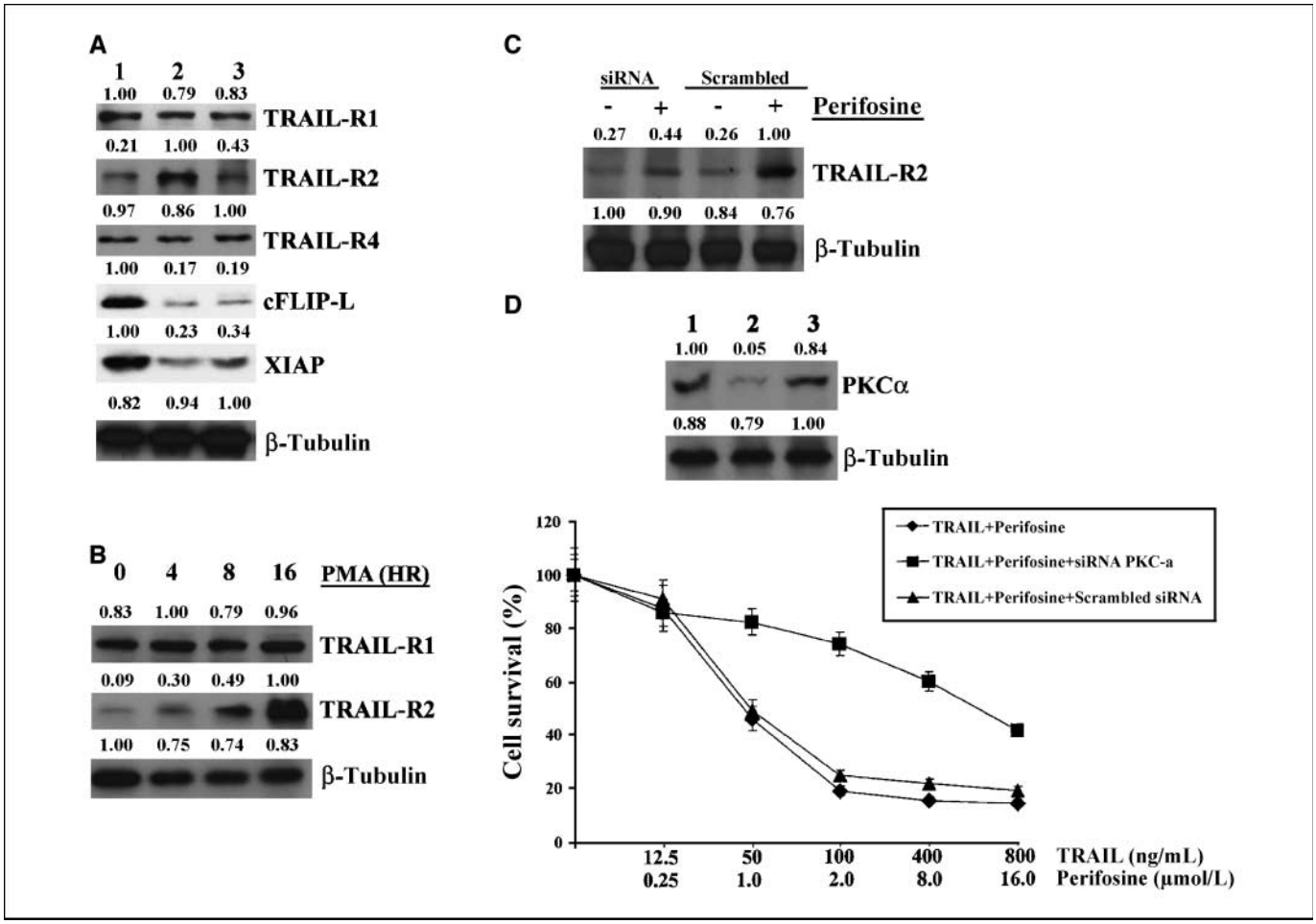

Figure 3. Increased TRAIL-R2 expression in THP-1 cells is dependent on PKCα. *A,* Western blot analysis of THP-1 cell extracts. *Lane 1,* untreated cells; *lane 2,* cells treated with perifosine (2 μmol/L); *lane 3,* cells treated with perifosine (2 μmol/L) + Gö6976 (0.5 μmol/L). Treatments were for 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.

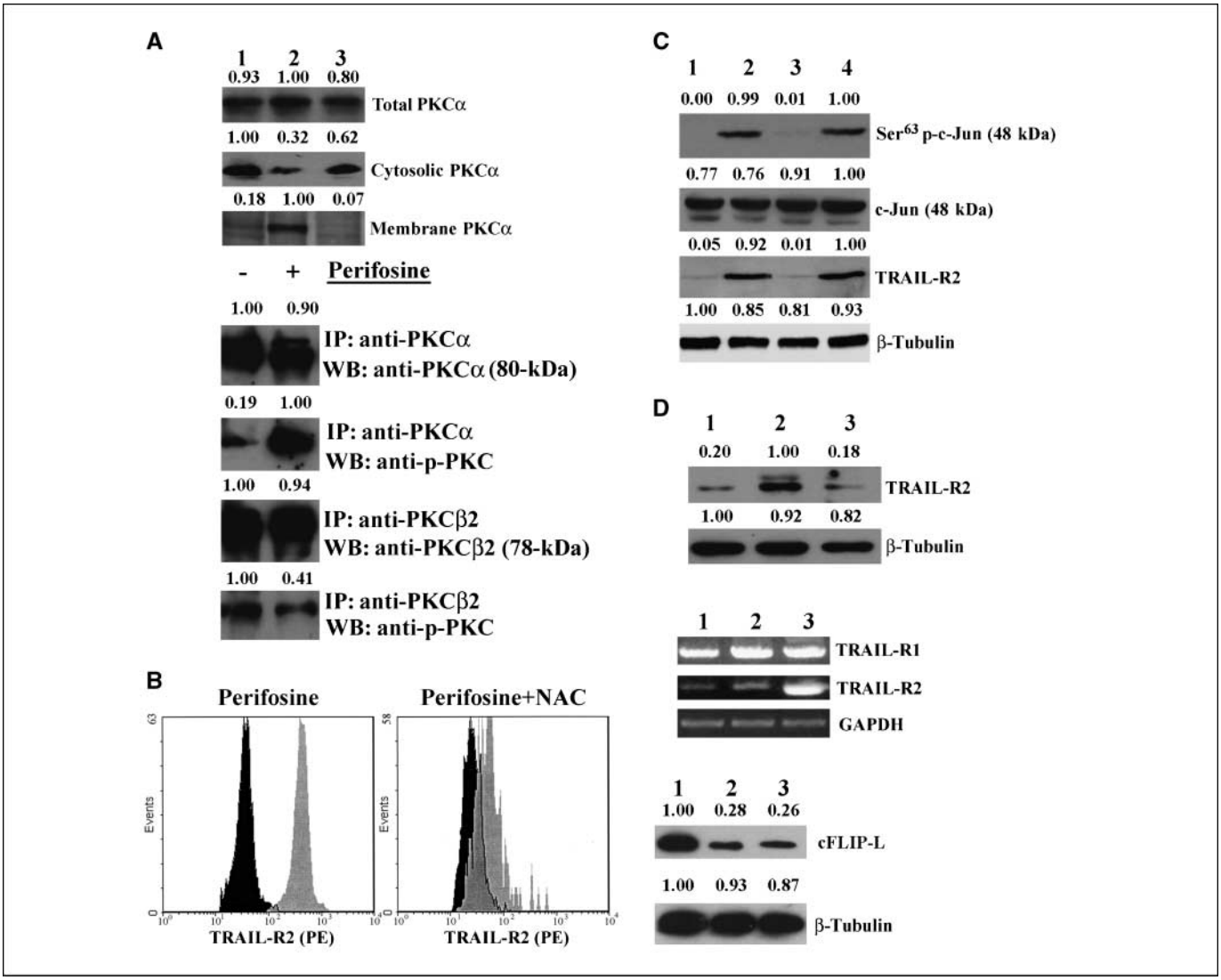

Figure 4. Perifosine induces PKCα and c-Jun activation in THP-1 cells. *A,* Western blot analysis for PKCα expression in different subcellular fractions. *Lane 1,* untreated cells; *lane 2,* cells treated with perifosine (2 μmol/L for 16 h); *lane 3,* cells treated with perifosine (2 μmol/L for 16 h) + NAC (15 mmol/L). Immunoprecipitation of cell extracts from untreated and perifosine-treated (2 μmol/L for 16 h) samples. *IP,* antibody used for immunoprecipitation; *WB,* antibody used for probing the blots. *B,* flow cytometric analysis, RT-PCR, and Western blot. Flow cytometric analysis shows surface expression of TRAIL-R2 receptor in untreated cells (*black-shaded histograms*) and cells treated with 2.0 μmol/L of perifosine for 16 h (*gray-shaded histograms*) with or without NAC (15 mmol/L). In the case of RT-PCR analysis for TRAIL-R1 and TRAIL-R2 mRNA, perifosine and NAC concentrations were similar to those used for flow cytometry. *Lane 1,* untreated cells; *lane 2,* NAC +perifosine; *lane 3,* perifosine alone. In the case of Western blot analysis for cFLIP-L expression, lanes were as for RT-PCR analysis. *C,* Western blot analysis for Ser^{63} p-c-Jun, c-Jun, and TRAIL-R2 levels in cells treated with perifosine (2 μmol/L for 16 h) in the presence or in the absence of JNK 1/2 or p38 MAPK inhibitors. *Lane 1,* untreated cells; *lane 2,* perifosine only; *lane 3,* perifosine + SP600125 (10 μmol/L); *lane 4,* perifosine + SB203580 (5 μmol/L). *D,* Western blot analysis for TRAIL-R2 in cells with down-regulated c-Jun levels. *Lane 1,* untreated cells; *lane 2,* cells treated with perifosine (2 μmol/L for 16 h); *lane 3,* perifosine-treated cells with c-Jun down-regulated by siRNA specific for c-Jun. β-Tubulin served as loading control.

whereas cytosolic PKCα decreased (Fig. 4*A*). These changes in PKCα subcellular localization were largely prevented by NAC, suggesting that they were dependent on ROS production. Moreover, perifosine treatment resulted in increased Thr$^{638/641}$ p-PKCα levels but not in PKCβ2 isoforms, as shown by immunoprecipitation experiments with antibodies selective for total (unphosphorylated and phosphorylated) PKCα and PKCβ2 isoforms, followed by Western blotting with an antibody which recognizes both PKCα and PKCβ1/2 phosphorylated on Thr$^{638/641}$ (Fig. 4*A*).

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. 4*B*). 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, PKCα could be upstream of JNK (33, 34). Perifosine (2 μmol/L) up-regulated p-JNK 2 (54 kDa) but not p-JNK 1 (46 kDa) in THP-1 cells, and this event could be blocked by NAC (Supplementary Fig. S1*B*). Perifosine treatment resulted in c-Jun phosphorylation on Ser^{63}, 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. 4*C*).

Finally, when c-Jun levels were down-regulated by siRNA specific for c-Jun (Supplementary Fig. S1*C*), the perifosine-evoked increase in TRAIL-R2 expression was reduced significantly (Fig. 4*D*). 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 PI3K/Akt signaling was studied by Western blot and/or flow cytometric analysis. Seven patients were positive for Ser^{473} p-Akt (Table 1). The combination of perifosine and TRAIL was much more effective than either drug alone, as shown by MTT assays (Fig. 5*A*, patient M4#1). Cytotoxicity was due to apoptotic cell death, as documented by Annexin V-FITC/PI staining (Fig. 5*B*), and was characterized by increased TRAIL-R2 expression which was detected in $CD33^+$ AML cells by flow cytometric analysis of samples double-stained for TRAIL-R2 and CD33 (Fig. 5*C*). Combined treatment also resulted in higher levels of caspase-8 activation compared with single treatment, as shown by FLICA analysis (data not shown). Western blot analysis showed Ser^{473} p-Akt down-regulation by perifosine, in patient blasts with Akt activation, as well as a decrease of both cFLIP-L and XIAP, and up-regulation of Ser^{63} p-c-Jun (Fig. 5*D*). FADD levels were not affected by perifosine. In contrast, in patients with no activated Akt, we did not detect increased apoptosis in response to the combined treatment (Supplementary Fig. S2*A*, patient M4#2). In samples from these patients, there was neither increased TRAIL-R2 expression nor decreased cFLIP-L and XIAP expression. Moreover, c-Jun phosphorylation on Ser^{63} was not increased after treatment with perifosine (Supplementary Fig. S2*B* 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.

Table 1. Patient classification and response to combined treatment

FAB type	Ser^{473} p-Akt	TRAIL-R2 up-regulated	P + T synergism, average CI (ED_{50} + ED_{75} + ED_{90})
M1#1	++	++++	0.23 ± 0.03
M1#2	+	++	0.51 ± 0.05
M2#1	−	−	0.95 ± 0.10
M2#2	+++	++++	0.26 ± 0.04
M2#3	+++	++++	0.15 ± 0.02
M2#4	−	−	1.08 ± 0.15
M2#5	+	+/−	0.84 ± 0.09
M2#6	−	−	0.99 ± 0.12
M2#7	++	+++	0.39 ± 0.05
M4#1	+++	++++	0.18 ± 0.02

NOTE: Patients were classified according to the French-American-British (FAB) classification. The levels of Ser^{473} 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.

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. S2*D*). 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.

Discussion

In this study, we showed that perifosine sensitizes AML cells from both the THP-1 cell line and patients, to TRAIL-induced apoptosis, at least in part via a decrease in cFLIP-L and XIAP levels, and through a PKCα-mediated increase in TRAIL-R2 expression. cFLIP-L, which is structurally similar to caspase-8, can be recruited to the death-inducing signaling complex to inhibit the binding and activation of caspase-8 and acts as a powerful repressor of TRAIL-induced death signaling (3). Also XIAP, a potent cellular caspase inhibitor, is an important factor in TRAIL-induced cell death (36).

A recent report from Carter and colleagues (36) has shown that triptolide, an anticancer agent from a Chinese herb, sensitized AML cells to TRAIL by decreasing XIAP levels and increasing TRAIL-R2 expression through a p53-mediated mechanism. However, perifosine was able to up-regulate TRAIL-R2 in THP-1 cells which have a nonfunctional p53 (37). It has been established that TRAIL-R2 levels could be regulated through mechanisms which are p53-dependent or -independent (38). Among p53-independent mechanisms, it has been shown that the JNK/c-Jun axis could positively regulate TRAIL-R2 transcription (32). A functional activator protein-1 (AP-1) binding site has been shown in the promoter region of TRAIL-R2. JNK, by increasing c-Jun phosphorylation, leads to an increase in AP-1 activity (39). Interestingly, we have recently shown JNK-dependent increased AP-1 activity in T-lymphoblastic acute leukemia cells treated with perifosine (19).

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 a decrease in perifosine-dependent sensitization to TRAIL cytotoxic effect, whereas PMA, a conventional PKC isoform activator, increased TRAIL-R2 expression. Perifosine increased ROS production in THP-1 cells, and ROS could promote PKCα binding to the plasma membrane. Perifosine also induced increased levels of phosphorylation of JNK 2 (but not JNK 1) and c-Jun in THP-1 cells. NAC impaired the up-regulation of TRAIL-R2 by perifosine. The involvement of JNK 2/c-Jun signaling in the increased expression of TRAIL-R2 was shown by the fact that a JNK 1/2–selective inhibitor, but not a p38 MAPK inhibitor, blocked both c-Jun phosphorylation on Ser^{63} induced by perifosine and TRAIL-R2 up-regulation.

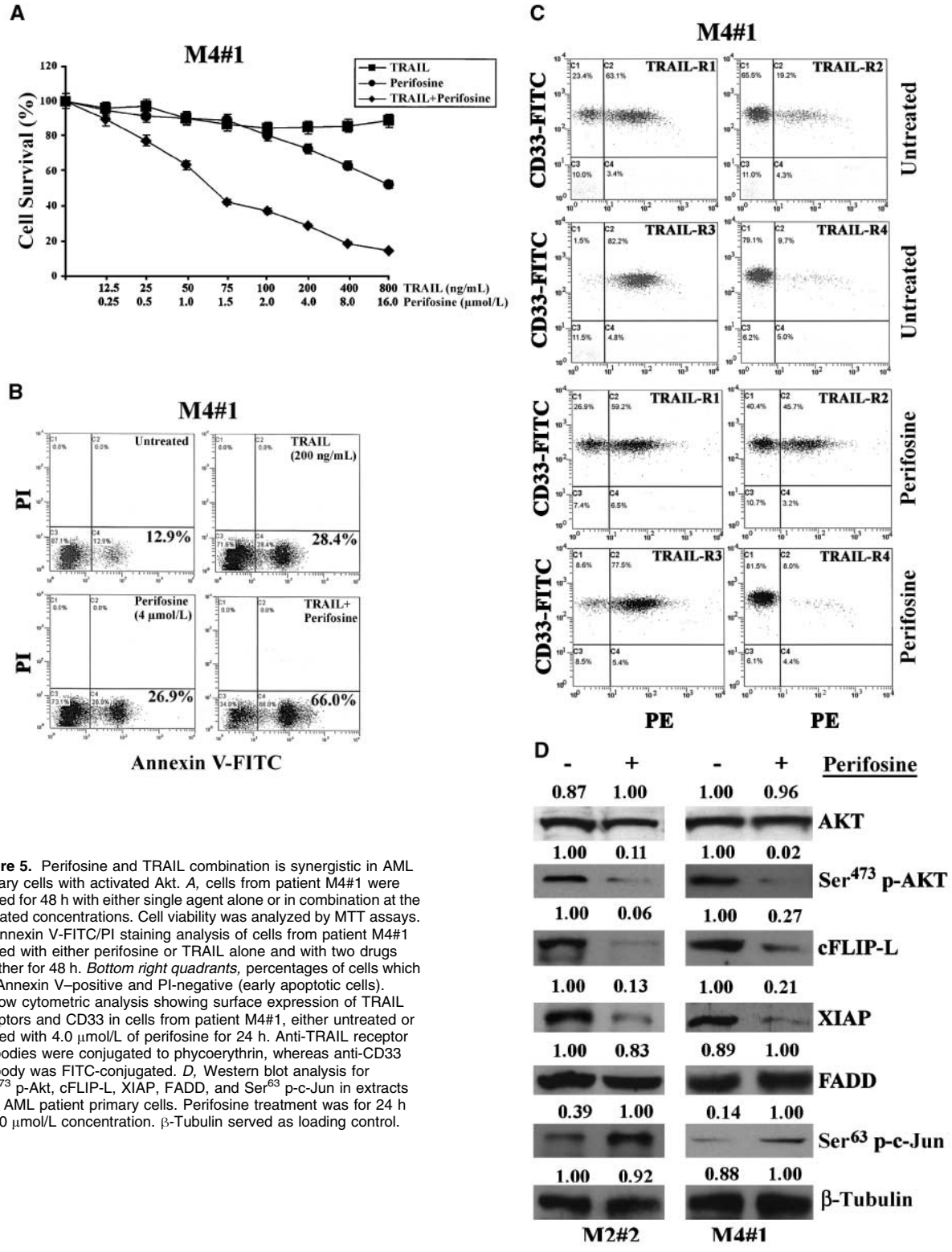

Figure 5. Perifosine and TRAIL combination is synergistic in AML primary cells with activated Akt. *A,* cells from patient M4#1 were treated for 48 h with either single agent alone or in combination at the indicated concentrations. Cell viability was analyzed by MTT assays. *B,* Annexin V-FITC/PI staining 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 Ser^{473} p-Akt, cFLIP-L, XIAP, FADD, and Ser^{63} 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.

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 JNK 2 phosphorylation, we propose a mechanism whereby perifosine generates ROS, which in turn, activates a PKCα → JNK 2 → 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,

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 PKCα 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 Ser^{63} 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-ketochetosterol, 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.

Acknowledgments

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