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Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) Promotes Mitochondrial Dysfunction and Apoptosis Induced by 7-Hydroxystaurosporine and Mitogen-Activated Protein Kinase Kinase Inhibitors in Human Leukemia Cells That Ectopically Express Bcl-2 and Bcl-xL

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ABSTRACT

Previous studies have demonstrated that cotreatment with mitogen activated-protein kinase kinase (MEK) 1/2 inhibitors (e.g., PD184352) and the checkpoint abrogator 7-hydroxystaurosporine (UCN-01) dramatically induces apoptosis in a variety of human leukemia and multiple myeloma cell types. The purpose of this study was to evaluate the roles of Bcl-2 family members and the relative contribution of the intrinsic mitochondrial versus the extrinsic receptor-related apoptotic pathways to MEK inhibitors/UCN-01–induced leukemic cell death. Cotreatment of U937 cells with PD184352 and UCN-01 resulted in the activation of procaspase-3, -9, and -8 as well as Bid cleavage. PD184352/UCN-01–induced mitochondrial dysfunction and apoptosis were both substantially attenuated in cells ectopically expressing Bcl-2, an N-terminal phosphorylation loop-deleted mutant Bcl-2, or Bcl-xL, but not in cells expressing dominant-negative (DN) caspase-8, cytokine response modifier A (cowpox virus-encoded antiapoptotic protein), or DN Fas-associated death domain. Coadministration of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or TNF-α substantially increased MEK inhibitors (e.g., PD184352 or U0126)/UCN-01–induced mitochondrial dysfunction, activation of procaspase-8 and Bid, and apoptosis in Bcl-2– and Bcl-xL–overexpressing cells but not in those in which the extrinsic pathway was interrupted. Together, these findings suggest that the MEK inhibitors/UCN-01 regimen primarily induces leukemic cell apoptosis by engaging the intrinsic, mitochondrial apoptotic pathway and that resistance to these events conferred by increased expression of certain antiapoptotic Bcl-2 family members can be overcome, at least in part, by coadministration of TRAIL and other agents that activate the extrinsic apoptotic cascade.

Apoptosis represents a tightly regulated and evolutionarily conserved program of cell suicide involving caspase activation, which is involved in normal cellular homeostasis (Rathmell and Thompson, 2002). It has also been implicated in the response of tumor cells to a wide variety of cytotoxic agents (Johnstone et al., 2002; Schmitt, 2003). Apoptosis proceeds through two major pathways: the intrinsic, mitochondrial pathway, and the extrinsic, receptor-related pathway (Igney and Krammer, 2002). In the former, diverse environmental stresses trigger the release of proapoptogenic proteins (e.g., cytochrome *c*, Smac/DIABLO, apoptosis-inducing factor) from mitochondria (Igney and Krammer, 2002). Release of cytochrome *c* into the cytoplasm facilitates its association with dATP, apoptosis-activating factor-1 and procaspase-9, thus forming a complex referred to as the apoptosome, in which procaspase-9 is activated and in turn recruits and activates executioner caspases (e.g., caspase-3) (Igney and Krammer, 2002; Shi, 2002). Activated caspase-3 is responsible for the degradation of a large number of cellular constit-

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ABBREVIATIONS: Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI; DN, dominant-negative; MEK, mitogen activated-protein kinase kinase; UCN-01, 7-hydroxystaurosporine; ΔBcl-2, phosphorylation loop (amino acids 32–80)-deleted Bcl-2/pSFFV; chk1, checkpoint kinase 1; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FADD, Fas-associated death domain; CrmA, cytokine response modifier A (cowpox virus-encoded antiapoptotic protein); PARP, poly(ADP-ribose) polymerase; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; $\Delta\Psi$m, mitochondrial membrane potential; FITC, fluorescein isothiocyanate; PI, propidium iodide; PD184352, 2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3,4-difluorobenzamide; STI571, Gleevec; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene; pNA, *p*-nitroanilide.

uents, including actin, gelsolin, and the inhibitor of caspase-activated DNase, among many others, which collectively induce the characteristic features of apoptosis. In contrast, the extrinsic apoptotic pathway is engaged by the ligation of ligands to receptors of the TNF-related pathway, of which Fas/APO is a prototypical example (Igney and Krammer, 2002). Engagement of this pathway involves formation of the death-inducing signaling complex containing activated Fas-associated death domain (FADD) which recruits and cleaves the initiator caspase procaspase-8 (Medema et al., 1997). Activated caspase-8 can directly activate downstream executioner caspases in the absence of activated caspase-9 or, alternatively, can cleave the BH3-only domain Bcl-2 family member Bid, which translocates to the mitochondria and induces cytochrome *c* release (Luo et al., 1998). This leads to caspase-3 activation, which results in further caspase-8 cleavage, and amplification of the apoptotic process. In type I cells, activation of caspase-8 by ligation of TNF receptors is sufficient to permit apoptosis to proceed to completion. In type II cells, initial caspase-8 activation by itself is insufficient to trigger apoptosis, and recruitment of the mitochondrial pathway (i.e., by Bid cleavage and translocation) is required for the full engagement of the cell-death program (Scaffidi et al., 1998).

The observation that activation of the extrinsic pathway can amplify apoptotic signals initiated by cytotoxic drugs acting primarily through the induction of mitochondrial pathway activation (von Haefen et al., 2003) has prompted the search for strategies capable of triggering the receptor-related cascade. This has led to the identification of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its death receptors DR4 and DR5 (Ashkenazi, 2002). Activation of DR4 and DR5, like that of Fas/APO, leads to activation of the initiator caspase, caspase-8, and its downstream targets (Kischkel et al., 2000). Interestingly, TRAIL seems to exert selective toxicity toward neoplastic cells, possibly because of the presence in normal cells of high levels of decoy receptors or the caspase-8 antagonist FLICE-inhibitory protein (Pan et al., 1997; Kim et al., 2000). In this context, coadministration of TRAIL has been shown to increase the lethal effects of conventional cytotoxic drugs in several systems (Tomek et al., 2003).

Recently, attention has focused on the ability of novel inhibitors of signal transduction pathways to induce apoptosis in neoplastic cells, the most prominent example of which is the Bcr/Abl kinase inhibitor STI571 (Capdeville et al., 2002). In addition, evidence has accumulated that tumor cells may be particularly susceptible to simultaneous interruption of separate signaling pathways. For example, our group has reported that coadministration of mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitors in conjunction with the protein kinase C and Chk1 inhibitor UCN-01 leads to a very dramatic increase in apoptosis in both hematopoietic and nonhematopoietic tumor cells (Dai et al., 2001, 2002; McKinstry et al., 2002; Yu et al., 2002). Furthermore, this regimen produces very early and extensive mitochondrial dysfunction in such cells, including the release of proapoptotic proteins (i.e., cytochrome *c* and Smac/DIABLO) into the cytosol. Currently, however, no information exists concerning the extent to which antiapoptotic proteins such as Bcl-2 and Bcl-xL might protect cells from such novel regimens. The goal of the present studies was to characterize the susceptibility of human leukemia cells ectopically expressing these proteins to the MEK1/2/Chk1 inhibitor regimen and to determine whether activation of the extrinsic pathway (i.e., by TRAIL) might attenuate resistance conferred by Bcl-2 overexpression. Our results indicate that ectopic expression of Bcl-2 or related proteins is highly effective in protecting leukemia cells from mitochondrial dysfunction and apoptosis induced by combined MEK1/2 and Chk1 inhibition. However, coadministration of TRAIL substantially increases the susceptibility of such cells to mitochondrial dysfunction and apoptosis induced by this regimen, suggesting that activation of the extrinsic cascade may effectively increase the antileukemic potential of novel regimens that act primarily through the mitochondrial pathway.

Materials and Methods

Cells and Reagents. The U937 cell line, derived from a patient with histiocytic leukemia, was purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium containing 10% fetal bovine serum, 200 U/ml penicillin, 200 μg/ml streptomycin, minimal essential vitamins, sodium pyruvate, and glutamine as reported previously (Dai et al., 2001). Bcl-2/U937 and pCEP4/U937 cells were obtained by stable transfection of cells with full-length Bcl-2 cDNA or an empty vector (pCEP4), after which clones were selected with hygromycin (Tang et al., 2000). U937 cells were also stably transfected with phosphorylation loop (amino acids 32–80)-deleted Bcl-2/pSFFV (ΔBcl-2) (Decker et al., 2002), Bcl-xL/3.1 (Wang et al., 2002), CrmA/3.1, dominant-negative (DN) caspase-8 (C→A mutation of amino acid 377, the catalytic cysteine)/3.1 and DN FADD (deletion of 80 N-terminal amino acids in the death effector domain)/3.1 cDNAs (Cartee et al., 2002), and empty vectors (pSFFV and pcDNA3.1), respectively, after which clones were selected using G418. CrmA, DN caspase-8, and DN FADD cDNAs were kindly provided by Dr. Kapil Bhalla (University of South Florida, Tampa, Florida).

UCN-01 was kindly provided by Dr. Edward Sausville (Developmental Therapeutics Program/Cancer Therapy Evaluation Program, National Cancer Institute, Bethesda, Maryland). It was dissolved in DMSO at a stock concentration of 10^{-3} M, stored at −80°C, and subsequently diluted with serum-free RPMI 1640 medium before use. MEK1/2 inhibitor PD184352 (Upstate Biotechnology, Lake Placid, NY) and U0126 (BIOMOL Research Laboratories, Plymouth Meeting, PA), and the protein synthesis inhibitor cycloheximide (Sigma Chemical, St. Louis, MO) were supplied as powders, dissolved in sterile DMSO, and stored frozen under light-protected conditions at −20°C. Recombinant human soluble TRAIL/Apo2 was purchased from Apotech (Lausen, Switzerland). Recombinant human TNF-α (Calbiochem, San Diego, CA) was rehydrated in PBS containing 0.5% bovine serum albumin, aliquoted, and stored at −80°C. In all experiments, the final concentration of DMSO did not exceed 0.1%.

Experimental Format. All experiments were performed using logarithmically growing cells (3–5 × 10^5 cells/ml). Cell suspensions were placed in sterile 25-cm^2 T-flasks (Corning Glassworks, Corning, NY) and treated simultaneously with PD184352 and UCN-01 alone or in combination in a 37°C/5% CO_2 atmosphere, generally for 24 h. In some studies, PD184352 (or U0126) and UCN-01 were coadministered in the presence of either TRAIL/Apo2 or TNF-α. After drug treatment, cells were harvested and subjected to further analysis as described below.

Analysis of Apoptosis and Mitochondrial Membrane Potential. The extent of apoptosis was evaluated by the assessment of Wright-Giemsa–stained preparation under light microscopy and scoring the number of cells exhibiting classic morphological features of apoptosis. For each condition, 5 to 10 randomly selected fields per condition were evaluated, encompassing at least 800 cells. To con-

firm the results of morphologic analysis, cells were also evaluated by Annexin V-FITC/propidium iodide (PI) staining and flow cytometry as described previously (Dai et al., 2002). Results of flow-cytometric analysis were found to parallel closely those obtained by morphologic assessment ($R > .90$). For analysis of mitochondrial membrane potential ($\Delta\Psi m$), 2×10^5 cells were incubated with 40 nM $DiOC_6$ (Molecular Probes, Eugene, OR) in PBS at 37°C for 20 min and then analyzed by flow cytometry as described previously (Dai et al., 2001). The percentage of cells exhibiting low levels of $DiOC_6$ uptake, which reflects a loss of mitochondrial membrane potential, was determined with the use of FACScan (BD Biosciences, San Jose, CA).

Western Blot Analysis. Whole-cell pellets were lysed in 1× SDS sample buffer, and 30 μg of protein for each condition was subjected to Western blot analysis by following procedures described previously (Dai et al., 2001, 2002). Where indicated, the blots were reprobed with antibodies against actin (Transduction Laboratories, Lexington, KY) to ensure equal loading and transfer of proteins. The following antibodies were used as primary antibodies: anti-caspase-9 (1:2000, rabbit polyclonal; BD PharMingen, San Diego, CA), anti-caspase-3 (1:1000, rabbit polyclonal; BD PharMingen), cleaved caspase-3 (Asp175) antibody (1:1000, rabbit polyclonal; Cell Signaling Technology Inc., Beverly, MA), anti-caspase-8 (1:2000, mouse monoclonal; Alexis Corporation, San Diego, CA), anti-PARP (1:2500, mouse monoclonal; BIOMOL), anti-human Bcl-2 oncoprotein (1:2000, mouse monoclonal; DAKO, Carpinteria, CA), Bcl-xS/L antibody (S-18) (1:500, rabbit polyclonal; Santa Cruz Biochemicals, Santa Cruz, CA), and Bid antibody (1:1000, rabbit polyclonal; R&D Systems, Minneapolis, MN).

Analysis of Cytosolic Cytochrome *c* and Smac/DIABLO. Cells (2×10^6) were washed in PBS and lysed by incubating for 30 s in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, and 350 μg/ml digitonin). The lysates were centrifuged at 12,000*g* for 1 min, and the supernatant was collected and added to an equal volume of 2× sample buffer. The protein samples were quantified, separated by 15% SDS- polyacrylamide gel electrophoresis, and subjected to Western blot analysis as described above. Anti-cytochrome *c* (mouse monoclonal, BD PharMingen) and Smac/DIABLO antibody (mouse monoclonal; Upstate Biotechnology) were used as primary antibodies at a dilution of 1:500.

Caspase Activity Assay. FLICE/Caspase-8, Caspase-3/CPP32, and Caspase-9/Mch6 Colorimetric Assay kits (BioVision, Mountain View, CA) were used to determine the activity of caspase-8, -3, and -9, respectively, according to the manufacturer's instructions. Briefly, 4×10^6 cells were washed with ice-cold PBS, resuspended in chilled cell lysis buffer, and incubated on ice for 10 min. The cell lysates were centrifuged at 10,000*g* for 1 min, and the supernatant (cytosolic fraction) was quantified for protein content before assay. Protein (200 μg per condition) was diluted in 50 μl of cell lysis buffer and mixed with 50 μl of 2× reaction buffer containing 10 mM dithiothreitol and 5 μl of 4 mM substrate (IETD-pNA for caspase-8, DEVD-pNA for caspase-3, or LEHD-pNA for caspase-9). After incubation at 37°C for 2 h, the absorbance at 405 nm was recorded using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). The fold increase in caspase activity was determined by comparing the readings with those for non–drug-treated controls.

Statistical Analysis. For the determination of apoptotic cells, analysis of $\Delta\Psi m$, and caspase activity assay, experiments were repeated at least three times. Values represent the means ± S.D. for at least three separate experiments performed in triplicate. The significance of differences between experimental variables was determined using the Student's *t* test.

Results

Previous studies had demonstrated that combined exposure of malignant hematopoietic cells to UCN-01 and MEK inhibitors resulted in a marked increase in mitochondrial dysfunction (e.g., cytochrome *c* release) (Dai et al., 2001, 2002). Effects of combined exposure (24 h) of U937 cells to UCN-01 and PD184352 were examined in relation to the activation of the initiator caspase-9 and -8, as well as the executioner caspase-3. Whereas the agents administered individually exerted little effect on caspase-9, -8, and -3 activation, combined treatment resulted in a marked increase in the activity of caspase-9 and -8 and an even greater increase in caspase-3 activity (Fig. 1). Western blot analysis revealed that combined, but not individual, treatment of cells with UCN-01 and PD184352 induced a pronounced cleavage of procaspase-8 as well as one of its major targets, Bid (Fig. 1, inset). These findings indicate that exposure of human leukemia cells to PD184352 plus UCN-01 leads to the activation of both the intrinsic mitochondrial as well as the extrinsic receptor-related pathways.

Bcl-2 and related proteins are believed to protect cells from noxious stimuli by preventing or attenuating mitochondrial dysfunction (Cory and Adams, 2002). To determine whether the ectopic expression of Bcl-2 could protect cells from PD184352/UCN-01–mediated toxicity, studies were performed using U937 cells ectopically expressing full-length Bcl-2, as well as an N-terminal phosphorylation loop-deleted protein (Fig. 2, A and B). The latter has been found to be more effective than its full-length counterpart in blocking apoptosis induced by diverse cytotoxic agents, including paclitaxel (Wang et al., 1999). As shown in Fig. 2, A and B, cells ectopically expressing full-length as well as the loop-deleted Bcl-2 Bcl-2 protein were essentially completely protected from UCN-01/PD184352-mediated loss of $\Delta\Psi m$ compared with their respective empty-vector control counterparts. Western analysis revealed parallel reductions in release of the proapoptotic proteins cytochrome *c* and Smac/DIABLO into the cytosolic S-100 fractions (Fig. 2, A and B). The ability of full-length and phosphorylation loop-deleted Bcl-2 to protect leukemic cells from UCN-01/PD184352-mediated mitochondrial dysfunction is consistent with the notion that this drug combination induces cell death by activating the intrinsic apoptotic pathway. These findings also argue against the

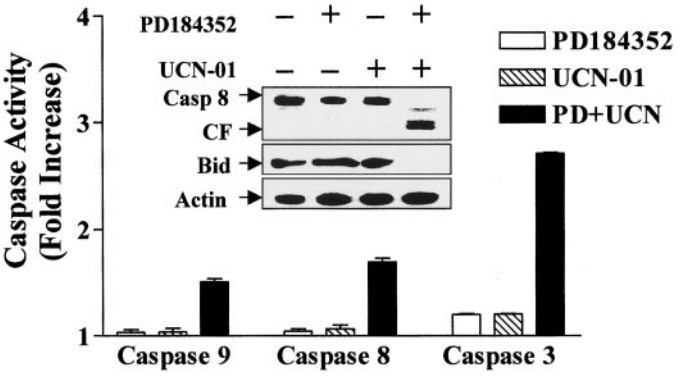

Fig. 1. Logarithmically growing U937 cells were incubated for 24 h in the presence of 10 μM PD184352 ± 100 nM UCN-01, after which cells were lysed and assayed for activity of caspase-9, -3, and -8, respectively, as described under *Materials and Methods*. Caspase activity is expressed as fold-increase relative to untreated control cells. Results represent the means ± S.D. for three separate experiments performed in triplicate. Alternatively, Western blot analysis was performed to monitor cleavage of caspase 8 and Bid (inset). Blots were subsequently stripped and reprobed with an antiactin antibody to ensure equivalent loading and transfer of proteins. Two additional studies yielded equivalent results.

possibility that Bcl-2 phosphorylation is involved in protection from the UCN-01/PD184352 regimen.

Consistent with these results, both the full-length Bcl-2 and the loop-deleted mutant protected UCN-01/PD184352-treated cells from procaspase-9, -8, and -3 processing, Bid cleavage, and PARP degradation (Fig. 3, A and B). These findings provide further support for the concept that these antiapoptotic proteins, by blocking UCN-01/PD184352-mediated mitochondrial dysfunction, prevent activation of both the intrinsic and extrinsic apoptotic caspase cascades.

Previous studies have demonstrated that Bcl-xL, like Bcl-2, acts by attenuating the ability of proapoptotic BH3-only domain Bcl-2 family members to induce mitochondrial dysfunction (Degterev et al., 2001). Consistent with such findings, U937 cells ectopically expressing the Bcl-xL protein were, like those overexpressing Bcl-2, highly resistant to UCN-01/PD184352-mediated mitochondrial dysfunction (i.e., diminished $\Delta\Psi$m) and apoptosis (empty-vector control: ~60%; Bcl-xL/U937: ~10%). They were also largely resistant to UCN-01/PD184352-mediated cytochrome *c* and Smac/DIABLO release; caspase-9, -8, and -3 processing; Bid cleavage; and PARP degradation (data not shown). Taken in conjunction with the preceding results, these findings suggest that the UCN-01/PD185352 regimen acts in large part by inducing mitochondrial dysfunction and that as a consequence, increased expression of the antiapoptotic proteins Bcl-2 and Bcl-xL afford a high degree of protection from the lethality of this drug combination.

It is well recognized that cross-talk exists between the mitochondria-dependent intrinsic pathway and death-receptor–mediated extrinsic pathway (Luo et al., 1998; Yin, 2000). To determine whether triggering the death-receptor pathway might help to overcome, at least in part, resistance to mitochondria-dependent stimuli conferred by overexpression of antiapoptotic Bcl-2 family members, TRAIL was coadministered with UCN-01/PD184352 in U937 cells ectopically expressing Bcl-2, ΔBcl-2, or Bcl-xL. Treatment with 100 ng/ml TRAIL alone only modestly induced cell death (i.e., ~20%) in empty-vector control cells (data not shown) and was essentially equally toxic to cells ectopically expressing Bcl-2, ΔBcl-2, or Bcl-xL (Fig. 4). Such findings are consistent with previous reports indicating that antiapoptotic members of the Bcl-2 family provide relatively little protection against death receptor-mediated apoptosis (Suliman et al., 2001). However, when TRAIL was combined with nontoxic concentrations of UCN-01/PD184352, a pronounced increase in the extent of apoptosis (i.e., ~50%) was observed in cells ectopically expressing Bcl-2, ΔBcl-2, or Bcl-xL (Fig. 4, top). These events were accompanied by marked increases in cleavage of caspase-8, Bid and PARP, and release of cytochrome c and Smac/DIABLO into the cytosolic S-100 fraction (Fig. 4, bottom). Consistent with these results, Annexin V-FITC/PI staining and flow cytometry demonstrated that the addition of TRAIL to UCN-01/PD184352-treated Bcl-2/U937 cells resulted in a particularly marked increase in the Annexin V^+/PI^+ fraction, corresponding to the late apoptotic cohort (Fig. 5), correlating closely with values obtained by morphologic analysis ($R > 0.90$). Identical results were obtained in Bcl-xL/U937 cells (data not shown).

To confirm these findings, parallel studies were performed using TNF-α, a classic inducer of the death-receptor pathway. For these studies, U937 cells ectopically expressing Bcl-2, ΔBcl-2, or Bcl-xL were exposed to UCN-01 in conjunction with the MEK1/2 inhibitor U0126, which we have previously shown, like PD184352, to interact synergistically with UCN-01 to trigger apoptosis in human leukemia cells (Dai et al., 2001). As in the case of cells treated with TRAIL plus PD184352/UCN-01, coadministration of 5 ng/ml recombinant human TNF-α with U0126/UCN-01 significantly increased the percentage of apoptotic cells (Fig. 6A) as well as cleavage of caspase-8, Bid, and PARP and the release of cytochrome *c* and Smac/DIABLO (Fig. 6B). Similar results were obtained with PD184352 (data not shown). Taken to-

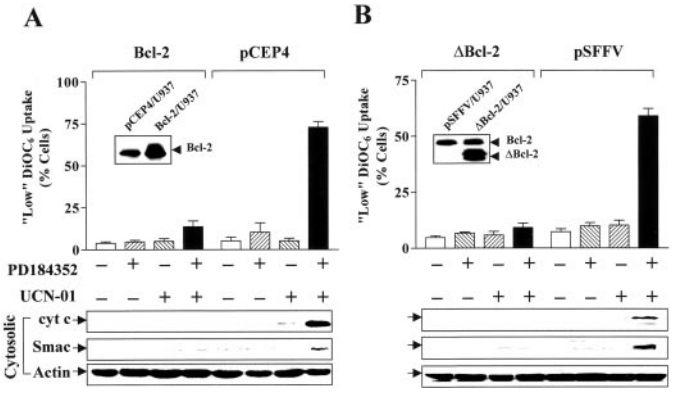

Fig. 2. U937 cells were stably transfected with full-length Bcl-2/pCEP4 (A), ΔBcl-2/pSFFV (B), and their corresponding empty vectors (pCEP4 and pSFFV). Ectopic expression of Bcl-2 and ΔBcl-2 was monitored by Western blot analysis (insets). Cells were treated with 10 μM PD184352 ± 100 nM UCN-01 for 24 h, after which the percentage of cells exhibiting reduced $\Delta\Psi$m was determined by monitoring $DiOC_6$ uptake. Results represent the means ± S.D. for three separate experiments performed in triplicate. Alternatively, cytosolic fractions were obtained as described under *Materials and Methods*, and the expression of cytochrome *c* and Smac/DIABLO were monitored by Western blot analysis. Blots were subsequently stripped and reprobed with an antiactin antibody for normalization. Two additional studies yielded equivalent results.

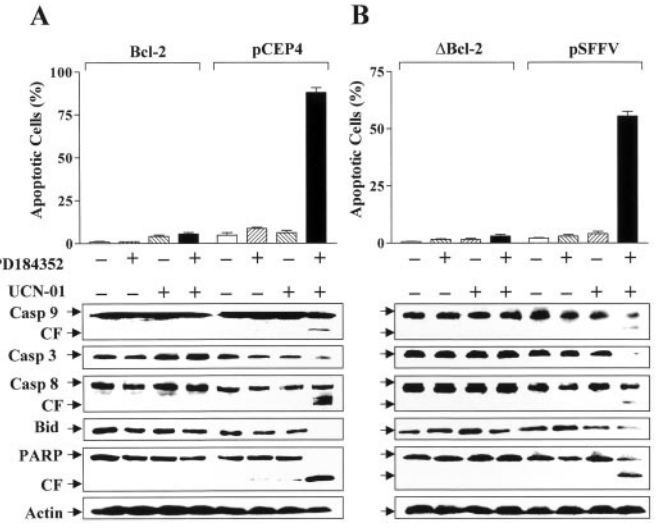

Fig. 3. U937 cells ectopically expressing Bcl-2 (A) and ΔBcl-2 (B) were treated with PD184352 ± UCN-01 as described in Fig. 2, after which the percentage of apoptotic cells was determined as described under *Materials and Methods*. Results represent the means ± S.D. for three separate experiments performed in triplicate. Alternatively, cells were lysed and subjected to Western blot analysis to monitor cleavage/degradation of caspase-9, -8, and -3, Bid, and PARP. Blots were subsequently stripped and reprobed with an antiactin antibody to ensure equivalent loading and transfer. Two additional studies yielded equivalent results. CF, cleavage fragment.

gether, these findings indicate that triggering the death-receptor–related extrinsic pathway significantly increases the sensitivity of leukemic cells overexpressing antiapoptotic Bcl-2 family members to the UCN-01/MEK inhibitor regimen. They also indicate that activation of the extrinsic pathway increases the antileukemic activity of regimens combining UCN-01 with MEK inhibitors other than PD184352.

To investigate the role of the extrinsic apoptotic pathway in PD184352/UCN-01-related lethality, U937 cells stably transfected with DN caspase-8, CrmA, or DN FADD cDNA were used (Cartee et al., 2002). These cells display significantly reduced cleavage/activation of casapse-8 and apoptosis when exposed to TNF-α plus cycloheximide (data not shown). As illustrated in Fig. 7, ectopic expression of DN caspase-8 or CrmA completely prevented cleavage of caspase-8 induced by the PD184352/UCN-01 regimen whereas only modestly decreasing the percentage of apoptotic cells (i.e., by ~20% compared with empty-vector controls) and failed to block the release of cytochrome *c* and the cleavage of caspase-3, caspase-9, and PARP. Moreover, ectopic expression of DN FADD exerted essentially no protective effects with respect to PD184352/UCN-01–induced caspase-8 cleavage, mitochondrial dysfunction, and apoptosis. Taken together, these findings indicate that in marked contrast to Bcl-2 and Bcl-xL, which substantially attenuate PD184352/UCN-01–induced mitochondrial dysfunction and cell death, ectopic expression of proteins that interfere with activation of the extrinsic pathway only minimally protect cells from the lethal effects of this regimen.

To evaluate the functional significance of the extrinsic pathway in potentiation of PD184252/UCN-01–mediated le-

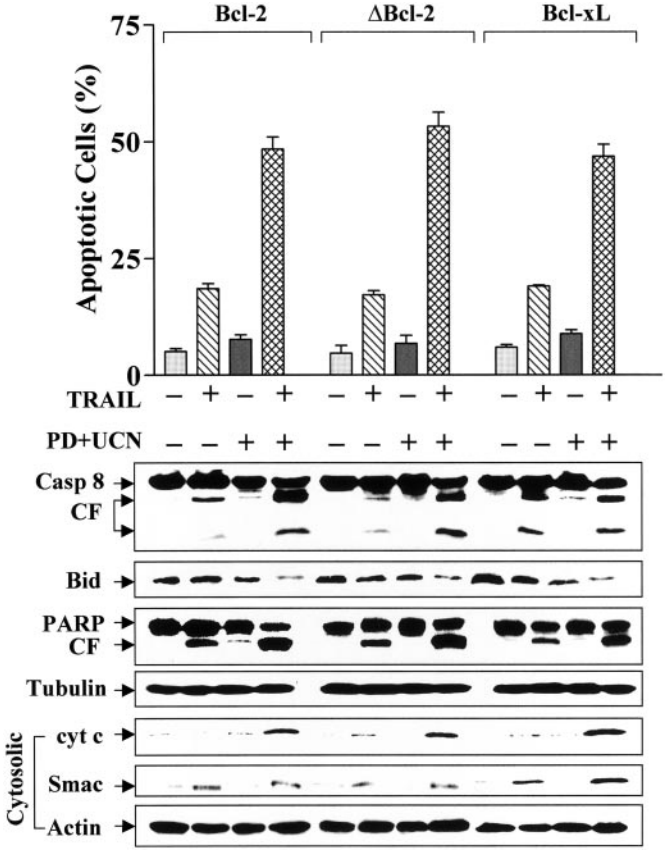

Fig. 4. U937 cells ectopically expressing Bcl-2, ΔBcl-2, or Bcl-xL were treated with 10 μM PD184352 + 100 nM UCN-01 for 24 h in the absence or presence of 100 ng/ml TRAIL, after which apoptosis was assessed as described under *Materials and Methods* (top). Results represent the means ± S.D. for three separate experiments performed in triplicate. Alternatively, cytosolic fractions were prepared as above to monitor the release of mitochondrial cytochrome *c* and Smac/DIABLO, and whole-cell lysates were subjected to Western blot analysis to monitor cleavage/degradation of caspase-8, Bid, and PARP (bottom). Blots were subsequently stripped and reprobed with antiactin or antitubulin antibody to ensure equivalent protein loading and transfer. Two additional studies yielded equivalent results. CF, cleavage fragment.

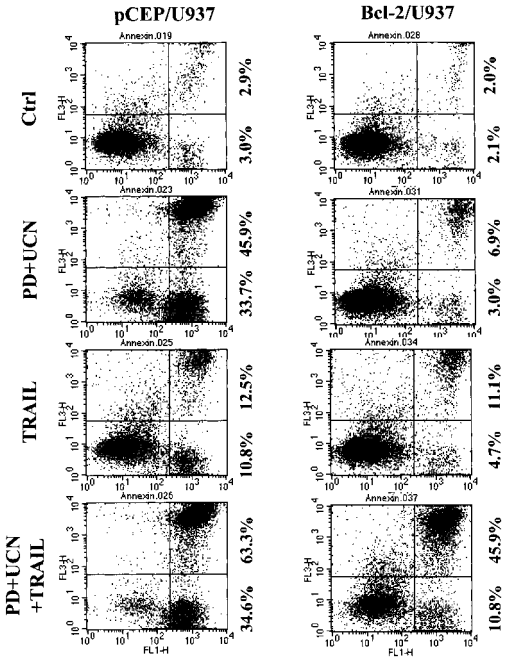

Fig. 5. U937 cells ectopically expressing Bcl-2 (Bcl-2/U937) or their empty vector controls (pCEP/U937) were treated with 10 μM PD184352 + 100 nM UCN-01 for 24 h in the absence or presence of 100 ng/ml TRAIL, after which the percentage of apoptotic cells was determined by Annexin V-FITC/PI staining and flow cytometry. The lower (Annexin V^+) and upper right (Annexin V^+/PI^+) quadrants correspond to early and late apoptotic cells. Values shown correspond to the percentage of cells in those quadrants. Two additional studies yielded equivalent results.

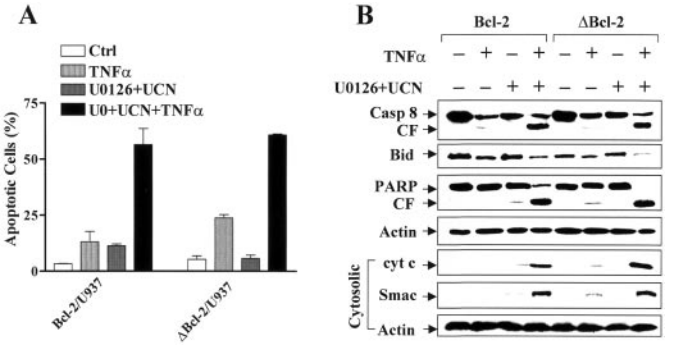

Fig. 6. A, U937 cells ectopically expressing Bcl-2 or ΔBcl-2 were treated with 20 μM U0126 + 100 nM UCN-01 for 24 h in the absence or presence of 5 ng/ml TNF-α, after which the percentage of apoptotic cells was determined as described under *Materials and Methods*. Results represent the means ± S.D. for three separate experiments performed in triplicate. B, in addition, cytosolic fractions were prepared as described above to monitor the release of mitochondrial cytochrome *c* and Smac/DIABLO. Alternatively, cells were lysed and subjected to Western blot analysis to monitor cleavage/degradation of caspase-8, Bid, and PARP. Blots were subsequently stripped and reprobed with an antiactin antibody to ensure equivalent loading and transfer. Two additional studies yielded equivalent results. CF, cleavage fragment.

thality by TRAIL, empty-vector control cells as well as cells ectopically expressing DN caspase-8, CrmA, or DN FADD were exposed to these agents alone and in combination, after which apoptosis was assessed. As shown in Fig. 8A, ectopic expression of DN caspase-8 or CrmA abrogated, whereas DN FADD significantly reduced TRAIL-induced degradation of caspase-8, Bid, and PARP. Furthermore, whereas PD184352/UCN-01 induced apoptosis in ~60% of empty-vector control cells (3.1), the addition of TRAIL resulted in cell death in essentially 100% of cells ($P < 0.05$) (Fig. 8B). In marked contrast, coadministration of TRAIL failed to increase the lethality of the PD184352/UCN-01 regimen in cells ectopically expressing DN caspase-8, CrmA, or DN FADD ($P > 0.05$ in each case). These findings indicate that the activation of the extrinsic pathway by TRAIL plays a critical role in the ability of this molecule to enhance the antileukemic effects of the PD184352/UCN-01 regimen.

Discussion

Whereas conventional cytotoxic agents are believed to act primarily by activating the intrinsic, mitochondrial apoptotic pathway (Debatin et al., 2002), little is known about mechanisms of cell death triggered by more novel signal-transduction inhibitors, and even less is known concerning regimens in which such agents are combined. The results of recent studies indicate that exposure of human leukemia and myeloma cells to the Chk1 inhibitor UCN-01 leads to activation of MEK1/2 and extracellular signal-regulated kinase and that pharmacological inhibition of the latter processes results in a marked increase in caspase activation and apoptosis. We have also observed that treatment of leukemia cells with PD184352 and UCN-01 is accompanied by translocation of Bax to the mitochondria (Y. Dai and S. Grant, unpublished observations), an event that is believed to promote the release of cytochrome *c* and possibly other proapoptotic proteins into the cytosol (De Giorgi et al., 2002), thereby activating the apoptotic caspase cascade. The antiapoptotic proteins Bcl-2 and Bcl-xL act by blocking the release of cytochrome *c* by antagonizing the proapoptotic actions of the BH3-only domain members of the Bcl-2 family (i.e., Bid and Bad (Cheng et al., 2001). In view of these considerations, it is not surprising that ectopic expression of Bcl-2 or Bcl-xL afforded cells a high degree of protection from the lethal effects of the PD184352/UCN-01 regimen. Collectively, these data indicate that the highly synergistic induction of apoptosis by this drug combination is critically dependent on the mitochondrial pathway and that as a consequence, increased expression of antiapoptotic proteins such as Bcl-2 or Bcl-xL effectively confers high degrees of resistance to such regimens.

In previous studies, the activation of the extrinsic caspase-8–related apoptotic pathway has been shown to amplify the

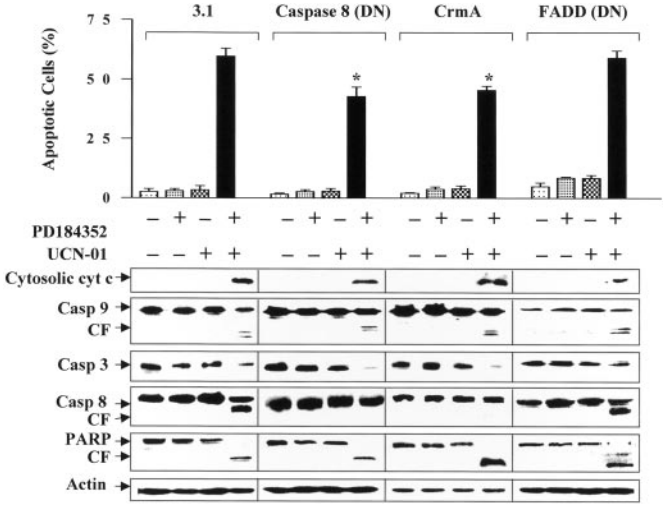

Fig. 7. U937 cells stably transfected with DN caspase-8, CrmA, DN FADD, or empty vector (pcDNA 3.1) were treated with 10 μM PD184352 ± 100 nM UCN-01 for 24 h, after which the percentage of apoptotic cells was determined as described under *Materials and Methods* (top). Results represent the means ± S.D. for three separate experiments performed in triplicate. ⋆, significantly lower than the value for cells with empty vector pcDNA3.1 ($P < 0.05$). In addition, cytosolic fractions were prepared as described above to monitor the release of mitochondrial cytochrome *c*; alternatively, cells were lysed and subjected to Western blot analysis to monitor cleavage/degradation of caspase-9, -8, and -3 and PARP (bottom). Blots were subsequently stripped and reprobed with an antiactin antibody to ensure equivalent loading and transfer. Two additional studies yielded equivalent results. CF, cleavage fragment.

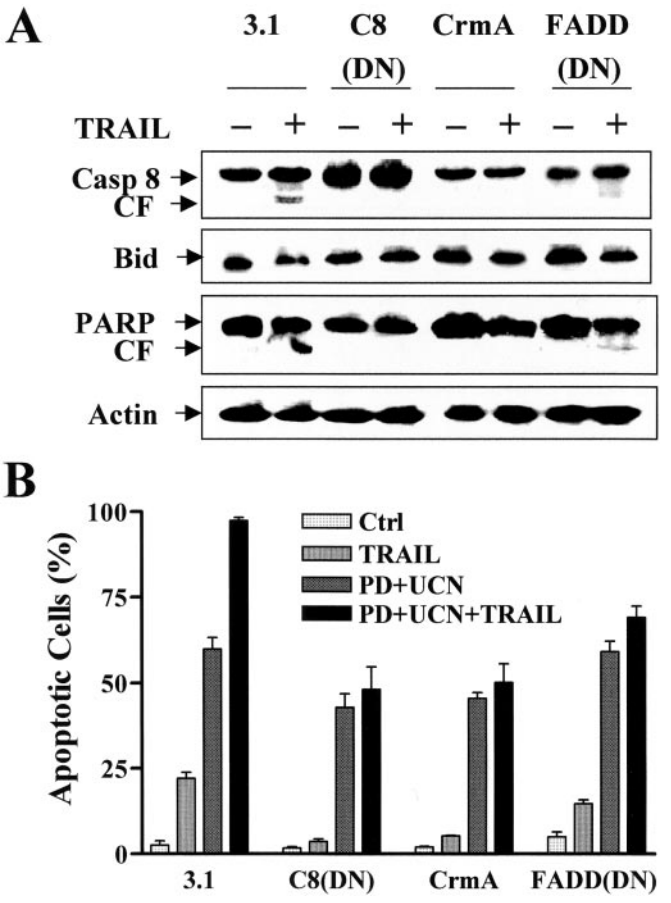

Fig. 8. A, U937 cells stably transfected with DN caspase-8, CrmA, DN FADD, or empty vector (pcDNA 3.1) were treated with 100 ng/ml TRAIL for 24 h, after which cells were lysed and subjected to Western blot analysis to monitor expression of caspase-8, Bid, and PARP. Blots were subsequently stripped and reprobed with an antiactin antibody for normalization. Two additional studies yielded equivalent results. B, U937 cells with stable transfection of DN caspase-8, CrmA, DN FADD, or empty vector (pcDNA 3.1) were treated with 10 μM PD184352 + 100 nM UCN-01 for 24 h in the presence or absence of 100 ng/ml TRAIL, after which the percentage of apoptotic cells was determined as described under *Materials and Methods*. Results represent the means ± S.D. for three separate experiments performed in triplicate.

lethal effects of certain cytotoxic drugs (e.g., VP-16) in leukemic cells (Engels et al., 2000). For example, activation of the extrinsic pathway can lead to cleavage and translocation of the pro-apoptotic BH3-only domain Bcl-2 family member Bid to the mitochondrion, in which it facilitates cytochrome *c* release (Luo et al., 1998). In addition, in epithelial carcinoma cells, the extrinsic caspase-8 signaling pathway has been shown to contribute to MEK1/2 inhibitor/UCN-01–induced cell-killing via the amplification of mitochondrial pathway and cytochrome *c* translocation (McKinstry et al., 2002). However, it seems that this phenomenon plays only a minor role in PD184352/UCN-01–induced lethality in leukemic cells. For example, ectopic expression of Bcl-2, Bcl-xL, or a loop-deleted form of Bcl-2 in U937 cells not only blocked PD184352/UCN-01–induced release of proapoptotic mitochondrial proteins in leukemic cells (i.e., cytochrome *c* and Smac/DIABLO) as well as activation of procaspase-9 and -3, but also attenuated procaspase-8 and Bid cleavage. This is consistent with the notion that attenuation of mitochondrial dysfunction by Bcl-2 family members prevented engagement of the extrinsic pathway by mitochondrial-activated caspases. In addition, disruption of the extrinsic pathway by DN caspase-8 or CrmA, which specifically prevent caspase-8 activation (Cartee et al., 2002), minimally reduced PD184352/UCN-01 lethality, whereas DN FADD, which interferes with the adaptor function of the death-inducing signaling complex apparatus (Sprick et al., 2002), was essentially ineffective. Taken together, such findings suggest that the activation of the extrinsic apoptotic pathway by this regimen in human leukemia cells represents a secondary phenomenon and plays only a minor role in amplifying the lethal consequences of PD184352/UCN-01–induced mitochondrial dysfunction.

Despite evidence that the extrinsic pathway contributed minimally to PD184352/UCN-01–induced leukemic cell death, activation of this pathway, such as by TRAIL or by TNF-α, substantially increased lethality in cells ectopically expressing Bcl-2, ΔBcl-2, or Bcl-xL. Although in some systems such proteins are unable to protect cells from activation of the extrinsic pathway (i.e., by Fas ligand) (Huang et al., 1999), this has not been a universal phenomenon, and in other cell types, Bcl-2 attenuates apoptosis induced by agents that trigger the extrinsic cascade (Sun et al., 2002). In the present study, coadministration of TRAIL or TNF-α restored, at least in part, the ability of PD184352/UCN-01 to induce mitochondrial dysfunction (i.e., cytochrome *c* and Smac/DIABLO release), caspase activation, and apoptosis in cells ectopically expressing Bcl-2, ΔBcl-2, or Bcl-xL. The question arises regarding how activation of the extrinsic pathway might play a relatively minor role in PD184352/UCN-01–induced lethality but remain capable of overcoming, at least in part, resistance to this regimen conferred by ectopic expression of antiapoptotic family members. One possible explanation for these observations is that activation of the extrinsic pathway by PD184352/UCN-01, manifested by caspase-8 and Bid cleavage, reflects a secondary phenomenon stemming from mitochondrial dysfunction and activation of the procaspase-9 and -3 cascade. Consistent with this notion, attenuation of cytochrome *c* release and activation of procaspase-9 and -3 were accompanied by diminished cleavage/activation of procaspase-8 and Bid. On the other hand, activation of the extrinsic pathway through administration of TRAIL or TNF-α potentiated PD184352/UCN-01–mediated activation of procaspase-8 and Bid in otherwise resistant cells ectopically expressing antiapoptotic Bcl-2 family members. Because Bid is known to promote mitochondrial pathway and release of cytochrome *c* (Werner et al., 2002), it is tempting to speculate that engagement of the extrinsic pathway (i.e., by TRAIL or TNF-α) interferes with the ability of Bcl-2 and related proteins to block mitochondrial dysfunction and thus restore, at least in part, PD184352/UCN-01–induced lethality. In support of this concept, interference with the extrinsic pathway (e.g., in cells ectopically expressing DN caspase-8, CrmA, or DN FADD) effectively abrogated the ability of TRAIL to potentiate PD184352/UCN-01–mediated apoptosis.

In summary, the present studies indicate that a novel regimen combining Chk1 with MEK1/2 inhibition kills leukemic cells largely through the induction of mitochondrial dysfunction and activation of the intrinsic apoptotic cascade rather than through activation of the extrinsic pathway. As a consequence, this regimen is highly susceptible to the protective effects of Bcl-2 and related antiapoptotic Bcl-2 family members. However, coadministration of TRAIL, which directly activates the extrinsic pathway, substantially increases the ability of the PD184352/UCN-01 regimen to engage the mitochondrial pathway and to trigger apoptosis in otherwise resistant cells ectopically expressing Bcl-2 or Bcl-xL. These findings may have particular significance for disorders such as leukemia, in which increased expression of Bcl-2 has been associated with a poor response to chemotherapy (Schmitt et al., 2000). They also provide additional support for the notion that simultaneous activation of the intrinsic and extrinsic apoptotic pathways represents a potent apoptotic stimulus in leukemia cells. In view of evidence that TRAIL is selectively toxic to neoplastic cells (Pan et al., 1997; Kim et al., 2000), the strategy of using such an agent to enhance the lethality of conventional cytotoxic drugs has attracted considerable attention (Naka et al., 2002). The present findings suggest that this approach may also be appropriate for novel regimens that simultaneously interrupt survival signaling and cell-cycle regulatory pathways, and in so doing, promote mitochondrial dysfunction. Accordingly, further efforts to develop this antileukemic strategy are currently in progress.

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