Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces mitochondrial damage and apoptosis in human leukemia cells

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Abstract

Interactions between histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo2 ligand, were examined in human leukemia cells (e.g., U937, Jurkat, and HL-60). Simultaneous exposure of cells to 100-ng/ml TRAIL with either 1-mm sodium butyrate or 2-μM suberoylanilide hydroxamic acid resulted in a striking increase in leukemic cell mitochondrial damage, caspase activation, and apoptosis. Lethal effects were significantly diminished in U937 cells ectopically expressing dominant-negative caspase-8, dominant-negative Fas-associated death domain, CrmA (receptor pathway), or Bcl-2 or Bcl-X₇ (mitochondrial pathway). Analysis of mitochondrial events in U937 cells exposed to TRAIL/HDAC inhibitors revealed enhanced Bid activation and Bax translocation, loss of mitochondrial membrane potential, and cytoplasmic release of cytochrome c, Smac/DIABLO, and apoptosis-inducing factor. No changes were observed in expression of FLICE-like inhibitory protein, TRAIL receptors, or reactive oxygen species generation. TRAIL/HDAC inhibitor-induced apoptosis triggered caspase-dependent cleavage of p21ʷAFI/CIP¹; moreover, enforced expression of a nuclear localization signal deletant form of p21ʷAFI/CIP¹ significantly diminished lethality. Lastly, p27^KIP¹, pRB, X-linked inhibitor of apoptosis, and Bcl-2 displayed extensive proteolysis. These findings indicate that coadministration of TRAIL with HDAC inhibitors synergistically induces apoptosis in human myeloid leukemia cells and provide further evidence that simultaneous activation of the extrinsic and intrinsic pathways in such cells leads to a dramatic increase in mitochondrial injury and activation of the caspase cascade. (Mol Cancer Ther. 2003; 2(12):1273–1284)

Introduction

Recently, attention has focused on the development of histone deacetylase (HDAC) inhibitors as anticancer agents for the treatment of solid and hematological malignancies (1–4). The acetylation and deacetylation of histones of the core proteins of nucleosomes in chromatin play important roles in the regulation of gene expression (3, 5). Two classes of enzymes are involved in determining the acetylation state of histones: histone acetyl transferases and HDACs (6, 7). The observation that alterations in histone acetyl transferase or HDAC activity occur in numerous cancers (8–11) has prompted the search for pharmacological agents capable of inhibiting these enzymes. As a consequence, several HDAC inhibitors have been identified that inhibit tumor growth in vitro and in vivo at concentrations that exert relatively modest toxicity toward normal tissues (12, 13). The family of HDAC inhibitors includes short-chain fatty acids (e.g., sodium butyrate [SB]), phenylbutyrate, hydroxyamaminic acids (e.g., suberoylanilide hydroxamic acid [SAHA]), cyclin tetrapeptides (e.g., depsipeptide), and benzamides (e.g., MS-275; 3). Previous work from this and other laboratories have focused on the factors that determine whether HDAC inhibitors such as SB and SAHA induce apoptosis or differentiation in leukemia cells, with a particular emphasis on the cyclin-dependent kinase inhibitor (CDKI) p21ʷAFI/CIP¹ (14–17). For example, HDAC inhibitors including SB, SAHA, and MS-275 increase the expression of p21ʷAFI/CIP¹, which leads in turn to growth arrest in the G1 phase of the cell cycle and ultimately to differentiation (15, 18). Conversely, interference with p21ʷAFI/CIP¹ induction disrupts the maturation response and engages an alternative apoptotic program (15–17, 19).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF cytokine family and is homologous to other TNF-related proteins such as Fas ligand (Fas-L; 20). TRAIL, unlike Fas-L, appears to induce apoptosis in tumor cells preferentially versus normal cells (21). The TRAIL-induced apoptotic response is mediated by binding to TRAIL surface receptors. Four distinct TRAIL receptors have been identified: death receptors 4 (DR4) and 5 (DR5) and TRAIL-neutralizing decoy receptors (DcR) 1 and 2. DR4 and DR5 contain a functional death domain (21). The TRAIL-induced apoptotic response is mediated by binding to TRAIL surface receptors. Four distinct TRAIL receptors have been identified: death receptors 4 (DR4) and 5 (DR5) and TRAIL-neutralizing decoy receptors (DcR) 1 and 2.
region that anchors the receptor to the membrane but lack the intracellular tail required to initiate the death pathway (25). Activation of DR4 and DR5 recruits the adaptor protein Fas-associated death domain (FADD), which, along with procaspase-8, forms the death-inducing signaling complex (DISC; 26). The resulting cleavage of procaspase-8 to an active form results in activation of downstream effector caspases (e.g., procaspase-3) as well as Bid, which engages the intrinsic/mitochondrial apoptotic pathway (27). The two signaling TRAIL receptors, DR4 and DR5, induce apoptosis in a wide variety of cancer cells, whereas the DcRs, which are primarily expressed on normal cells, confer resistance against TRAIL (25).

Recent reports indicate that colon cancer and lung cancer cell lines are rendered more susceptible to TRAIL-induced apoptosis when arrested in G0/G1 phase compared with cells in late G1, S, or G2/M (28). Importantly, enhanced TRAIL-related induced apoptosis appears to be associated with an up-regulation of the CDKI p21\textsuperscript{WAF1/CIP1} (29). As HDAC inhibitors such as SB and SAHA are known to induce cell cycle arrest through the induction of p21\textsuperscript{WAF1/CIP1} (p21\textsuperscript{WAF1/CIP1-AS}), and their empty vector (EV) counterparts were obtained as reported previously (30), the notion that such agents might sensitize cells to TRAIL-induced lethality appears plausible. While interactions between TRAIL and HDAC inhibitors have recently been examined in a colon cancer cell model (31), no information is available concerning such interactions in malignant human hematological cells. In a preliminary communication, we reported that TRAIL potentiated HDAC inhibitor-mediated lethality in human leukemia cells (32). Here, we provide evidence that in human myeloid and lymphoid leukemia cells, simultaneous activation of the extrinsic/receptor-mediated cascade (i.e., by TRAIL) and the intrinsic/mitochondrial-mediated apoptotic pathway (i.e., by HDAC inhibitors) provides a highly potent cell death signal that involves induction of mitochondrial damage and activation of caspase cascades. These events are associated with Bid activation, Bcl-2 cleavage, and X-linked inhibitor of apoptosis (XIAP) down-regulation but not with up-regulation of DRs or diminished expression of FLICE-like inhibitory protein (FLIP). Furthermore, the expression/intracellular disposition of the CDKI p21\textsuperscript{WAF1/CIP1} appears to play a significant role in this process.

**Materials and Methods**

**Cells**

U937, HL-60, and Jurkat cells were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured and maintained in logarithmic growth phase in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, l-glutamate, penicillin, streptomycin, and 10% fetal bovine serum (Life Technologies, Inc./Invitrogen, Carlsbad, CA). U937 cells stably overexpressing Bcl-2, Bcl-x\_\text{L}, CrmA (CrmA), dominant-negative FADD (FADD-DN), dominant-negative caspase-8 (C8-DN), antisense p21\textsuperscript{WAF1/CIP1} (p21\textsuperscript{WAF1/CIP1-AS}), and their empty vector (EV) counterparts were obtained as reported previously (33, 34) and maintained as described above in the presence of the corresponding selection antibiotics. Cells expressing cytoplasmic p21\textsuperscript{WAF1/CIP1} (amino acids 1–140; a deletion mutant lacking the nuclear localization signal [p21\textsuperscript{WAF1/CIP1-ΔNLS}]) were obtained as described by Asada et al. (35) All experiments were performed using cells in logarithmic phase growth suspended at 2.5 × 10\textsuperscript{6} cells/ml.

**Drugs and Chemicals**

TRAIL (20, 21, 36) was kindly provided by Amgen (Seattle, WA) and stored in aliquots at −80°C. SB was supplied as a powder (Calbiochem, La Jolla, CA) and dissolved in PBS before use. SAHA was purchased from Alexis (Carslsbad, CA), and stock solutions were prepared in DMSO at a concentration of 10\textsuperscript{-2} m and dissolved in PBS before use. The pan-caspase inhibitor BOC-D-fmk was purchased from Enzyme System Products (Livermore, CA) and dissolved in DMSO. Human recombinant TNF (Calbiochem) was dissolved in medium and kept at room temperature for 20 min before use.

**Morphological Assessment of Apoptosis**

Apoptotic cells were evaluated by both morphological assessment of Wright-Giemsa-stained cytospin preparations and annexin V/propidium iodide (PI) staining as described previously (17). Annexin V/PI analysis of cell death was carried out as per the manufacturer's instructions.

**Cell Cycle Analysis**

Analysis of cell cycle was made by flow cytometry as described previously (15) using a Becton Dickinson (Mansfield, MA) FACScan flow cytometer and Verity Winlist software (Verity Software, Topsham, ME).

**Assessment of Mitochondrial Membrane Potential**

At the indicated intervals, cells were harvested and 2 × 10\textsuperscript{5} cells were incubated with 40-nM DiOC\textsubscript{6} for 15 min at 37°C. Analysis was then carried out on Becton Dickinson FACScan flow cytometer. The percentage of cells exhibiting low levels of DiOC\textsubscript{6} was determined as described previously (37).

**Determination of Clonogenicity**

Pelleted cells were washed extensively and prepared for soft agar cloning as described previously (38). Cells were resuspended in cold PBS and seeded in 35-mm culture plates at a fixed density (400 cells/ml/well) in complete RPMI 1640 containing 20% FCS, 10% 5637 conditioned media, and 0.3% Bacto agar (DIFCO Laboratories, Detroit, MI). Cultures were maintained for 10–12 days in a 37°C, 5% CO\textsubscript{2} incubator after which colonies, defined as groups of ≥50 cells, were scored.

**Analysis of Cytosolic Cytochrome \(e\) and Cytosolic Proteins**

A previously described technique was employed (37) where the S-100 or cytosolic fraction was prepared as described with minor modifications. For each condition, 30 μg of the S-100 fraction were loaded on the gel and probed with the corresponding antibody.

**Western Blot Analysis**

Whole cell pellets were washed twice in PBS, resuspended in PBS, and lysed by the addition of 1 volume of
loading buffer (Invitrogen) as described previously (17). Thirty micrograms of total proteins per point were separated by 4–12% Bis-Tris NuPAGE precast gel system (Invitrogen) and electroblotted to nitrocellulose. After incubation with the corresponding primary and secondary antibodies, blots were developed by enhanced chemiluminescence (New England Nuclear, Boston, MA). Where indicated, blots were stripped and reprobed with antibodies directed against actin.

**Antibodies for Western Blot Analysis**

Primary antibodies for the following proteins were used at the designated dilutions: p21WAF1/CIP1 (1:1000; PharMingen-Transduction Laboratories, Lexington, KY); Bcl-XL (1:1000; Trevigen, Gaithersburg, MD); XIAP (1:1000; Cell Signaling Technology, Beverly, MA); poly (ADP-ribose) polymerase (1:1000; BioMol, Plymouth Meeting, PA); procaspase-3 (1:1000; PharMingen-Transduction Laboratories); cytochrome c; cyclins D1, A, and E; Mcl-1; pRb and underphosphorylated Rb (1:1000; PharMingen); Bcl-2 (1:2000; DAKO, Glostrup, Denmark); caspase-8 (1:2000 Alexia Corporations, San Diego, CA); Bid (1:1000; Cell Signaling Technology); actin (1:2000 Sigma Chemical Co., St. Louis, MO); and apoptosis-inducing factor (AIF), Bax, and Bak (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

**Measurement of Reactive Oxygen Species Production**

Cells were treated with 20-μM 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) for 30 min at 37°C, fluorescence measured by flow cytometry on a FACScan, and analyzed with CELLQuest software (17).

**Statistical Analysis**

The significance of differences between experimental conditions was determined using the Student’s t test for unpaired observations. To assess the interaction between agents, Median Dose Effect analysis (39) was used with a commercially available software program (CalcuSyn; Biosoft, Ferguson, MO). The combination index (CI) was calculated for a two-drug combination involving a fixed concentration ratio. Using these methods, CI values less than 1.0 indicate a synergistic interaction.

**Results**

**Exposure of U937 Cells to HDAC Inhibitors Results in p21WAF1/CIP1 Induction, pRb Dephosphorylation, and Delayed G1 Arrest**

As shown in Fig. 1A, no major changes in cell cycle distribution were observed following exposure of U937 cells to 1-mM SB or 2-μM SAHA during the first 24 h, although a robust induction of the CDK1 p21WAF1/CIP1 and a modest increase in underphosphorylated pRb was noted at this time point (Fig. 1B). However, after 48 h, treated cells exhibited a marked increase in the percentage of the cell population arrested in G0/G1 (e.g., 94.3% and 87.3% for SB and SAHA, respectively). A modest increase in the subdiploid (apoptotic) fraction was also observed, particularly in cells exposed to SAHA. These events were accompanied by marked up-regulation of p21WAF1/CIP1 and a clear increase in the underphosphorylated form of pRb (Fig. 1B).

Simultaneous but not Sequential Exposure of U937 Cells to HDAC Inhibitors and TRAIL Results in a Striking Increase in Apoptosis

In view of evidence that arrest of colon tumor cells in G1 sensitizes them to TRAIL (28) and that TRAIL-induced apoptosis is associated with induction of p21WAF1/CIP1 (29),
we sought to determine whether pretreatment of leukemic cells with HDAC inhibitors would increase their susceptibility to TRAIL lethality. To this end, U937 cells were preexposed to SB or SAHA as above followed by a 24- or 48-h exposure to TRAIL (50 or 100 ng/ml) after which apoptosis was assessed. Results of these studies revealed at best additive interactions. For example, when cells were pretreated 24 h with 1 mM SB or 2 μM SAHA followed by 24 h in combination with 100 ng/ml TRAIL, the extent of apoptosis was B: 40 ± 5.4%, S: 45 ± 4.3%, T: 7 ± 1.3%, TB: 55 ± 5.8%, and TS: 44 ± 3.8%, respectively. Similar interactions were noted for other sequential exposure intervals (i.e., 48 h; data not shown). Thus, pretreatment of human leukemia cells with HDAC inhibitors did not result in a major increase in TRAIL-related lethality.

However, very different results were obtained when cells were simultaneously exposed to HDAC inhibitors with TRAIL. In marked contrast to results obtained following sequential drug treatment, simultaneous 24-h exposure of cells to a subtoxic concentration of TRAIL (i.e., 100 ng/ml) in conjunction with 1 mM SB or 2 μM SAHA, which were only modestly toxic by themselves, resulted in a very dramatic increase in apoptosis (Fig. 2A). For example, values for drugs given alone were TRAIL 100 ng/ml: 7 ± 1.3%, SB (1 mM): 5.3 ± 1.3%, and SAHA (2 μM): 5.4 ± 1.4%, whereas values for the combination were TRAIL 100 B1: 60.1 ± 5.2% and TRAIL 100 S2: 54.7 ± 4.8%. Furthermore, combined treatment resulted in ~90% apoptosis after 48 h (Fig. 2B).

Moreover, the marked increase in apoptosis was abrogated by coadministration of the caspase inhibitor BOC-D-fmk (20 μM), demonstrating the caspase dependence of this interaction (Fig. 2B). In addition, Median Dose Effect analysis over a range of TRAIL (50–150 ng/ml) and HDAC inhibitor (SB: 0.5–1.5 mM and SAHA: 1–3 μM) concentrations yielded CI values considerably less than 1.0 (CI = 0.055), corresponding to a highly synergistic interaction (Fig. 2C).

Together, these observations suggest that simultaneous but not sequential exposure of U937 cells to HDAC inhibitors in conjunction with TRAIL results in a striking increase in cell death through a caspase-dependent process.

**TNF-α and Anti-Fas Antibody Markedly Potentiate SAHA and SB Lethality**

To determine whether other activators of the extrinsic pathway could similarly enhance HDAC inhibitor-associated lethality, U937 cells were simultaneously exposed to either SB or SAHA in combination with TNF-α or anti-Fas antibody. As shown in Fig. 2D, while each of the agents given alone was minimally toxic, coadministration of anti-Fas

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**Figure 2.** Simultaneous exposure of U937 cells to HDAC inhibitors and TRAIL as well as other extrinsic pathway activators results in increased apoptosis. **A**, U937 cells in logarithmic phase were suspended at 2.5 × 10⁶ cells/ml in the absence (control: C) or presence of 50- or 100-ng/ml TRAIL (T) in combination with 1- or 2-μM SB (B1 and B2, respectively) and 1- or 2-μM SAHA (S1 and S2, respectively) for 24 h after which apoptosis was monitored by annexin V/PI staining as described in “Materials and Methods.” Columns, means for three separate experiments performed in triplicate; bars, SD. **B**, U937 cells were incubated for 24 or 48 h with 1 mM SB (B), 2 μM SAHA (S), 100-ng/ml TRAIL (T), or the corresponding combinations ± the pan-caspase inhibitor BOC-D-fmk (24 h). The percentage of apoptotic cells was determined by annexin V/PI staining. Columns, means for three separate experiments performed in triplicate; bars, SD. **C**, U937 cells were exposed to various concentrations of HDAC inhibitors and TRAIL at a fixed ratio for 24 h after which the percentage of apoptotic cells was monitored as described in “Materials and Methods.” The CI was determined in relation to the fraction of cells affected using Median Dose Effect analysis to characterize drug interactions. CI values less than 1.0 correspond to synergistic interactions. Results are representative of three separate experiments. **D**, U937 cells were exposed to either 50-ng/ml anti-Fas antibody (F50) or 1-ng/ml TNF-α (T1) in combination with 1 mM SB (F50-B and T1-B, respectively) or 2 μM SAHA (F50-S and T1-S, respectively). Apoptosis was determined after 24-h treatment by annexin V/PI staining and analyzed by flow cytometry as described previously. Columns, means for three separate experiments performed in triplicate; bars, SD.

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antibody and particularly TNF-α resulted in marked increase in the extent of cell death. Together, these observations suggest that combining agents that activate the receptor-mediated pathway with HDAC inhibitors provides a very potent apoptotic stimulus in human leukemia cells.

**Coadministration of TRAIL with HDAC Inhibitors Potently Induces Apoptosis in Multiple Malignant Human Hematopoietic Cell Lines**

To determine whether the previous findings were restricted to U937 cells, parallel studies were performed using several other malignant human hematopoietic cell types (e.g., promyelocytic leukemia cells [HL-60], Jurkat T-human lymphoblastic leukemia cells, and human multiple myeloma cells [U266]). Cells were exposed for 24 h to 1-mM SB or 2-μM SAHA ± 100-ng/ml TRAIL after which Western blot analysis of cell lysates was performed. In each case, 30 μg of protein were separated on SDS-PAGE gels after which blots were probed with the corresponding antibodies. To ensure equivalent loading and transfer, the blots were stripped and reprobed with an antibody to actin. Results of a representative study are shown; two additional experiments yielded similar results.

**Figure 3.** TRAIL/HDAC inhibitor-mediated cell death is associated with caspase activation and Bid cleavage but not with perturbations in expression of FLIP or TRAIL receptors. A and B. U937 cells were exposed for 24 h to 1-mM SB (B) or 2-μM SAHA (S) ± 100-ng/ml TRAIL (T) after which Western blot analysis of cell lysates was performed. In each case, 30 μg of protein were separated on SDS-PAGE gels after which blots were probed with the corresponding antibodies. To ensure equivalent loading and transfer, the blots were stripped and reprobed with an antibody to actin. Results of a representative study are shown; two additional experiments yielded similar results.

**TRAIL/HDAC Inhibitor-Mediated Cell Death Involves Activation of Caspase-3 and -8 and Bid Cleavage but Is Not Associated with Perturbations in FLIP or TRAIL Receptors**

Western blot analysis of lysates obtained from cells coexposed to TRAIL and HDAC inhibitors revealed that enhanced cell death was accompanied by increased degradation of the caspase-3 substrate poly(ADP-ribose) polymerase, enhanced cleavage/activation of procaspase-8 and -3, and decreases in levels of the caspase-8 substrate Bid (Fig. 3A). Such findings are consistent with the finding that TRAIL/HDAC inhibitor-induced apoptosis was caspase dependent (Fig. 2B).

Recent studies have shown that sensitization of human colon cancer cells to TRAIL-mediated apoptosis by SB involved diminished expression of FLIP (31), a protein that interferes with DISC activation (40). Attempts were therefore undertaken to determine whether a similar mechanism might be involved in TRAIL/HDAC inhibitor-mediated lethality in human leukemia cells. As shown in Fig. 4A, reductions in FLIP expression in cells exposed to HDAC inhibitors ± TRAIL were not observed. Similarly, it has previously been reported that increased sensitivity to TRAIL-induced cell death may stem from increased expression of TRAIL receptors (41–43). As shown in Fig. 4B, exposure to SB or SAHA ± TRAIL failed to modify expression of DR4, DR5, or DcR2, while DcR1 was essentially undetectable (data not shown) as reported previously (44). Together, these results argue against the possibilities that alterations in the expression of FLIP or TRAIL receptors are involved in potentiation of cell death by the TRAIL/HDAC inhibitor regimens in human leukemia cells.

**Potentiation of Apoptosis in U937 Cells Exposed to TRAIL/HDAC Inhibitors Is Associated with Enhanced Loss of Δψm and Release of Cytochrome c but not with Increased Reactive Oxygen Species Generation**

Early mitochondrial dysfunction has been shown to play a key role in apoptotic events (44). Previous studies have demonstrated that both TRAIL and HDAC inhibitors target the mitochondria in drug-induced cell death (14, 15, 27, 46). To investigate the effects of the TRAIL/HDAC inhibitor regimen on mitochondrial function, cytoplasmic cytochrome c release and uptake of the lipophilic fluorochrome DiOC₆ reflecting maintenance of Δψₘ were monitored. Exposure of cells to the HDAC inhibitors SB and SAHA alone had no effect on Δψₘ or release of cytochrome c into the cytosolic S-100 fraction, whereas TRAIL alone modestly increased the percentage of cells showing a loss of Δψₘ and induced cytoplasmic cytochrome c release (Fig. 4, A and B, respectively). However, coexposure of U937 cells to TRAIL/HDAC inhibitors resulted in a marked increase in loss of Δψₘ and the release of cytochrome c. Interestingly, the loss of Δψₘ in TRAIL/HDAC inhibitor-treated cells was largely blocked by the caspase inhibitor BOC-D-fmk (Fig. 4A), whereas the latter had no effect on the release of cytochrome c to the cytosol (Fig. 4B). This indicates that...
loss of $\Delta \psi_m$ and cytochrome c release represent a secondary and a primary event, respectively, in TRAIL/HDAC inhibitor-mediated mitochondrial injury in human leukemia cells.

In view of recent evidence that in CEM-CCRF T-lymphoblastic leukemia cells, SAHA-induced cell death stems, at least in part, from increased levels of reactive oxygen species (ROS; 46), studies were performed to determine whether the TRAIL/HDAC inhibitor regimen resulted in increased ROS generation. However, exposure of U937 cells to 1-mM SB or 2-$\mu$m SAHA $\pm$ 100-ng/ml TRAIL for 24 h failed to modify the percentage of cells exhibiting an increase in ROS levels (i.e., <5% in each case; data not shown). Such findings indicate that synergistic interactions between HDAC inhibitors and TRAIL do not stem from increased ROS generation.

**Combined Treatment with TRAIL/HDAC Inhibitors Induces Multiple Perturbations in Levels of Proapoptotic and Antiapoptotic Proteins**

Because cell death decisions are tightly regulated by the balance between proapoptotic (e.g., Bak and Bax) and antiapoptotic (e.g., Bcl-2, Bcl-XL, Mcl-1, XIAP, etc.) Bcl-2 family members (47, 48), attempts were made to determine what effect combined exposure to TRAIL and HDAC inhibitors might have on such proteins. As shown in Fig. 5A, HDAC inhibitors or TRAIL given individually for 24 h had little or no effect on expression of Bcl-2 or XIAP. However, in cells exposed to TRAIL in combination with HDAC inhibitors, a rapidly migrating Bcl-2 species, presumably corresponding to a proapoptotic Bcl-2 cleavage product (49), was clearly discernible. Coadministration of the caspase inhibitor BOC-D-fmk blocked this phenomenon, supporting the notion that this fragment corresponds to a Bcl-2 degradation product. Recent reports have highlighted the critical role inhibitor of apoptosis (IAP) family members exert on TRAIL-mediated apoptosis (50). It is noteworthy that combined exposure of leukemic cells to TRAIL in conjunction with HDAC inhibitors resulted in the complete down-regulation of XIAP, an event that was only partially restored by coexposure to the caspase inhibitor BOC-D-fmk (Fig. 5A).

Figure 4. Enhanced apoptosis in TRAIL/HDAC inhibitor-treated cells is associated with increased loss of $\Delta \psi_m$ and the caspase-independent cytosolic release of cytochrome c. U937 cells were exposed for 24 h to 1-mM SB (B) or 2-$\mu$m SAHA (S) $\pm$ 100-ng/ml TRAIL (T) in the absence (TB and TS) or presence (TB* and TS*) of the pan-caspase inhibitor BOC-D-fmk (25 $\mu$m). A, the loss of $\Delta \psi_m$ was monitored by determining the increase in the percentage of cells expressing low $\Delta \psi_m$, reflected by diminished uptake of DiOC$_{20}$ relative to untreated controls. Columns, means for three separate experiments performed in triplicate; bars, SD. *, $P < 0.05$, significantly greater than control. **, $P < 0.01$, significantly greater than values obtained for agents given individually. ***, $P < 0.02$, significantly less than values for TB or TS. B, after treatment with SB (B) or SAHA (S) $\pm$ TRAIL (T) as above, U937 cells were pelleted, lysed, and protein extracted from the cytosolic S-100 cell fraction followed by SDS-PAGE separation. In each case, 30 $\mu$g of protein were analyzed by Western blot and probed with the corresponding cytochrome c antibody. Subsequently, blots were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. Results of a representative study are shown; two additional experiments yielded similar results.

Exposure of cells to HDAC inhibitors alone or in combination with TRAIL for 24 h induced a moderate decrease on total cellular levels of Bcl-XL while had little effect on Bax, Bak, or Mcl-1 (Fig. 5B). However, analysis of the cytosolic S-100 cell fractions showed a marked increase in cytosolic release of the proapoptotic mitochondrial proteins AIF and Smac/DIABLO and a marked depletion of cytosolic Bax in cells exposed to the combination of HDAC inhibitors and TRAIL (Fig. 5C). Together, these and the preceding findings indicate that enhanced apoptosis in leukemic cells exposed to HDAC inhibitors in conjunction with TRAIL is associated with Bcl-2 and Bid cleavage, XIAP down-regulation, moderate decrease on Bcl-XL, cytosolic release of cytochrome c, AIF, and Smac/DIABLO, and cytosolic depletion of Bax.

**Synergistic Induction of Apoptosis in Leukemic Cells Exposed to TRAIL/HDAC Inhibitor-Induced Apoptosis Involves Activation of Both the Extrinsic (Receptor-Mediated) and the Intrinsic (Mitochondrial-Mediated) Pathways**

TRAIL and other members of the TNF ligand family induce apoptosis/cell death by binding to their cognate DRs on the cell surface (27, 51). On the other hand, previous studies from this and other laboratories have demonstrated that SB- and SAHA-associated lethality primarily involves activation of the mitochondrial pathway (16, 17, 46). To investigate the role of the receptor-mediated pathway in TRAIL/HDAC inhibitor interactions, transfectant U937 cell lines ectopically expressing CrmA, C8-DN, or FADD-DN were employed. As shown in Fig. 6A, ectopic expression of CrmA, C8-DN, or FADD-DN significantly reduced apoptosis following TRAIL/HDAC inhibitor exposure (24 h) compared with EV controls. In parallel studies, Western blot analysis revealed that a corresponding reduction in Bid cleavage and Bax translocation was observed in each of the transfectant cell lines (data not shown). These findings indicate that activation of the extrinsic pathway plays a critical role in synergistic antileukemic interactions between TRAIL and HDAC inhibitors.

To characterize the role of the intrinsic/mitochondrial-mediated pathway in these events, U937 cells ectopically expressing Bcl-2 or Bcl-XL were employed. These proteins
regulate the release of proapoptotic mitochondrial molecules to the cytosol, thereby blocking activation of the caspase cascade (52). As shown in Fig. 6B, cells ectopically expressing Bcl-2 or Bcl-XL were significantly more resistant to TRAIL/HDAC inhibitor-induced cell death than their EV counterparts, although Bcl-XL was more effective than Bcl-2 in this regard. Western blot analysis revealed that ectopic expression of Bcl-2 or Bcl-XL attenuated TRAIL/HDAC inhibitor-mediated cytosolic cytochrome c release and Bid cleavage (data not shown). Taken together with the preceding results, these findings indicate that synergistic induction of apoptosis in leukemic cells by combined treatment with TRAIL and HDAC inhibitors involves both intrinsic and extrinsic apoptotic cascades.

Further studies were performed with these genetically modified cells (i.e., C8-DN, Bcl-2, or Bcl-XL overexpressing cells) tending to evaluate the role of the both apoptotic pathways in the TRAIL/HDAC inhibitor-induced cell death. Consistent with the marked reduction in the apoptotic response depicted in Fig. 6A, 24-h exposure to the drug combination reduced minimally the capacity of C8-DN cells to form colonies (Fig. 6C). By contrast, 24-h treatment of both Bcl-XL and Bcl-2 cells essentially abrogated the clonogenic survival to the levels observed with the corresponding U937/EV control cells (Fig. 6C). These data suggest that despite the blockade observed in the levels of apoptosis after 24-h exposure to TRAIL/HDAC inhibitors, either Bcl-2 or Bcl-XL is unable to block the final proapoptotic response to the drug combination.

Figure 5. Cotreatment of leukemia cells with SB or SAHA with TRAIL results in Bcl-2 cleavage, XIAP degradation, release of AIF and Smac/DIABLO, and depletion of cytosolic Bax. U937 cells were incubated for 24 h with 1-mM SB (B) or 2-mM SAHA (S) ± 100-ng/ml TRAIL (T) in the absence (TB and TS) or presence (T* and T**) of the pan-caspase inhibitor BOC-D-fmk (25 µM) after which they were pelleted, lysed, and protein extracted from either whole cell lysates (Bcl-2, XIAP, Bax, Bak, Bcl-XL, and Mcl-1) or cytosolic S-100 fraction (Smac, AIF, Bax, and Bak) as described in “Materials and Methods.” In each case, lanes were loaded with 30 µg of protein and separated by SDS-PAGE. Blots were then probed with the corresponding antibodies after which they were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. Results of a representative study are shown; two additional experiments yielded similar results. CF, cleavage fragment.

Figure 6. Apoptosis induced by TRAIL and HDAC inhibitors involves activation of both the extrinsic and the intrinsic pathways. Stable U937 transfectant cell lines were exposed to 1-mM SB (B) or 2-mM SAHA (S) ± 100-ng/ml TRAIL (T) after which cell death/apoptosis was evaluated by annexin V/PI staining and analyzed by flow cytometry as described in “Materials and Methods.” A, the role of the extrinsic/receptor-mediated pathway in cell death was evaluated using U937 cells stably transfected with either an EV (U937/EV) or plasmids encoding CrmA, C8DN, or FADD DN. B, to determine the role of the intrinsic/mitochondrial-related apoptotic pathway, U937 cells ectopically expressing either Bcl-2 or Bcl-XL were treated as above and cell death was determined by annexin V/PI staining. C, transfected EV, C8DN, Bcl-XL, or Bcl-2 cells were exposed to 1-mM SB/100-ng/ml TRAIL (TB) for 24 h, washed free of drug, and plated in soft agar as described in “Materials and Methods.” At the end of 10–12 days of incubation, colonies consisting of groups of ≥50 cells were scored under light microscopy using an inverted microscope. *P < 0.05, significantly less than values obtained for EV control cells. **P < 0.02, significantly less than values obtained for EV control cells.
Synergistic Antileukemic Interactions Between HDAC Inhibitors and TRAIL Are Associated with p21WAF1/CIP1 Cleavage and Inhibited by Ectopic Expression of a p21WAF1/CIP1-ΔNLS

Recent studies have highlighted the role of the CDKI p21WAF1/CIP1 in regulating TRAIL- and HDAC inhibitor-induced apoptosis (29, 53, 54). In particular, the cleavage of p21WAF1/CIP1 has been implicated in apoptosis induced by cytotoxic agents in combination with TRAIL (40). As shown in Fig. 7A, 1-mM SB and 2-μM SAHA (24 h each) robustly induced expression of p21WAF1/CIP1 whereas TRAIL alone (100 ng/ml) had little effect. However, when HDAC inhibitors were coadministered with TRAIL, a p21WAF1/CIP1 cleavage product was clearly discernible. The appearance of this fragment was abrogated by coadministration of BOC-D-fmk. Similarly, the CDKI p27KIP1 also underwent caspase-dependent cleavage following cotreatment with HDAC inhibitors and TRAIL (Fig. 7A).

To investigate further the role of p21WAF1/CIP1 in TRAIL/HDAC inhibitor-induced apoptosis, effects of these regimens were examined in U937 cells ectopically expressing either p21WAF1/CIP1-AS (34) or p21WAF1/CIP1-ΔNLS. As shown in Fig. 7B, p21WAF1/CIP1-AS cells were significantly more sensitive than EV controls to a 24-h exposure to 1-mM SB or 2-μM SAHA, consistent with our previous findings (15, 17). p21WAF1/CIP1-AS cells also displayed increased sensitivity to TRAIL-induced lethality (100 ng/ml; 24 h). However, ectopic expression of p21WAF1/CIP1-AS did not modify the sensitivity of cells to either TRAIL or HDAC inhibitor regimen (P > 0.05 in each case). Diametrically opposed results were obtained in cells ectopically expressing the p21WAF1/CIP1-ΔNLS mutant. For example, ectopic expression of this mutant protein failed to modify the sensitivity of cells to HDAC inhibitors or TRAIL given individually. However, p21WAF1/CIP1-ΔNLS cells were significantly less sensitive to the lethal effects of combined exposure to TRAIL and HDAC inhibitors than their EV counterparts (P < 0.002 in each case). Two different clones, ΔNLS-1 and ΔNLS-43, were used to validate the cells and to exclude clonal variation. These findings suggest that enhanced antileukemic activity of the TRAIL/HDAC inhibitor regimen does not require p21WAF1/CIP1 induction and that enforced expression of the p21WAF1/CIP1-ΔNLS mutant, which localizes to the cytoplasm (35), can protect cells from the lethal effects of the TRAIL/HDAC inhibitor combination.

Analysis of other cell cycle-related proteins revealed that U937 cells exposed to 100-ng/ml TRAIL ± 1-mM SB or 2-μM SAHA for 24 h exhibited a marked increase in caspase-dependent pRb cleavage products and the virtual disappearance of cyclin D1, an effect that was not antagonized by the caspase inhibitor BOC-D-fmk (data not shown). No changes were observed in levels of expression of cyclins E and A. Thus, coexposure of U937 cells to TRAIL in combination with HDAC inhibitors resulted in multiple perturbations in cell cycle regulatory events that might contribute to enhanced lethality, including cleavage of p21WAF1/CIP1 and p27KIP1, degradation of pRb, and down-regulation of cyclin D1.

Discussion

The present findings indicate that simultaneous administration of TRAIL with HDAC inhibitors potently induces mitochondrial damage, caspase activation, and apoptosis in human leukemia cells. The present studies were prompted by recent reports that TRAIL-induced cell death/apoptosis was enhanced in gastrointestinal cancer cells by induction of p21WAF1/CIP1 (29) or in cells growth arrested in G1 (28). In view of evidence that HDAC inhibitors induce G1 arrest through induction of p21WAF1/CIP1 (14, 15, 17, 18), the possibility that such treatment would sensitize leukemic cells to TRAIL-induced lethality appeared plausible. However, whereas sequential exposure of cells to HDAC inhibitors followed by TRAIL failed to increase apoptosis substantially, simultaneous exposure to these agents resulted in a striking increase in cell death. These findings suggest that induction of p21WAF1/CIP1 or G1 arrest per se do not potentiate TRAIL-mediated lethality, at least in human leukemia cells.
Interactions between TRAIL and other cytotoxic agents have previously been related to perturbations in FLIP, a protein that structurally resembles caspase-8 but lacks proteolytic activity (55, 56), or modulation of expression of TRAIL receptors (26, 27). For example, diminished expression of FLIP has been implicated in the sensitization of colon cancer cells to TRAIL by SB (31) and in sensitization of SHEP neuroblastoma cells to CD95 by cycloheximide and actinomycin D (57). Alternatively, up-regulation of the DR4 and DR5 in cells exposed to TRAIL and cytotoxic agents has been reported (44, 58, 59). However, the failure to detect diminished FLIP expression or up-regulation of DR4 or DR5 in cells exposed to HDAC inhibitors/TRAIL argues against the involvement of such mechanisms in synergistic interactions between these agents in human leukemia cells.

On binding to its receptors, TRAIL transmits a caspase-activating signal through its cytoplasmic death domain (27, 60). This pathway involves the formation of the DISC, a complex formed by the adaptor protein FADD and procaspase-8 (61, 62). Depending on the extent of DISC formation, caspase-8 either directly activates the caspase cascade or triggers cleavage of the BH3 domain-only proapoptotic protein Bid (60). Bid interacts with the proapoptotic Bcl-2 relatives Bax and Bak, thereby inducing their translocation to the mitochondria and resulting in the release of factors such as cytochrome c that promote activation of the caspase cascade (26, 60, 63). The findings that coadministration of TRAIL and HDAC inhibitors resulted in caspase-8 cleavage, Bid degradation, and Bax translocation, along with the observations that toxicity was significantly attenuated in cells ectopically expressing CrmA, FADD-DN, or C8-DN, strongly implicates the extrinsic pathway in synergistic interactions between these agents. In this regard, while some HDAC inhibitors (e.g., apicidin) appear primarily to activate the extrinsic/receptor pathway (64), several recent studies have shown that HDAC inhibitors (e.g., SB and SAHA) activate the apoptotic cascade through the intrinsic/mitochondrial pathway (16, 17, 46). The finding that ectopic expression of Bcl-2 or Bcl-XL, which act principally by blocking mitochondrial damage (65), similarly attenuated TRAIL/HDAC inhibitor-related lethality indicates that the intrinsic/mitochondrial pathway also plays a critical role in enhanced antileukemic activity of this regimen. Taken together, these observations are compatible with the notion that simultaneous activation of the intrinsic pathway (i.e., by HDAC inhibitors) and extrinsic cascade (i.e., by TRAIL) results in amplification of the apoptotic process, a phenomenon that has been described in conventional cytotoxic agents (66).

The observation that overexpression of the antiapoptotic proteins Bcl-2 and Bcl-XL in the first 24 h blocked TRAIL/ HDAC inhibitor-induced cell death was ineffective in the long term. Aberrant expression of either Bcl-2 or Bcl-XL is in general associated with a poor response to chemotherapy and decreased overall survival (67–70). Although recent reports suggest that TRAIL can bypass apoptosis resistance caused by Bcl-2 in CEM cells and human multiple myeloma cells (71–73), Bcl-2 conferred protection against TRAIL in neuroblastoma, glioblastoma, or breast carcinoma cell lines (74). Moreover, at the doses of either TRAIL or HDAC inhibitors used in the present work, both parental and overexpressing Bcl-2 or Bcl-XL cells were not affected by the individual drugs. Only when combined that these compounds induce high levels of apoptosis. These data suggest that the combination of TRAIL with HDAC inhibitors may represent an important alternative to kill Bcl-2-positive cells that are usually resistant to other class of chemotherapeutic agents. Moreover, TRAIL/HDAC inhibitor-mediated cell death was accompanied with a marked cleavage of Bcl-2. Caspase-mediated cleavage of Bcl-2, other than reducing the levels of the protein, also generates a 23-kDa proapoptotic fragment that promotes cytochrome c release (75) and could plausibly contribute to enhanced lethality of the TRAIL/HDAC inhibitor regimen.

Other potential contributors to TRAIL/HDAC inhibitor-mediated lethality include proteolytic cleavage and down-regulation of key apoptotic regulatory molecules (i.e., XIAP and p21WAF1/CIP1). Members of the IAP family of proteins, including XIAP, c-IAP, and c-IAP2, bind to and directly inhibit caspase-3, -7, and -9 (76–78). In this context, XIAP is an important modulator of the TRAIL activity in prostate cancer cells (50) as well as in U937 leukemia cells (70). The possibility that XIAP down-regulation and Bcl-2 cleavage cooperate to promote TRAIL/HDAC inhibitor-related lethality also cannot be excluded.

The role that the CDKI p21WAF1/CIP1 plays in the apoptotic response of cells to HDAC inhibitors ± TRAIL is complex and may be pleiotropic. For example, the results of several studies have suggested that cleavage of p21WAF1/CIP1 may be implicated in induction of apoptosis by various agents, including HDAC inhibitors and TRAIL (41, 79–82). Consequently, it is tempting to speculate that the cleavage of p21WAF1/CIP1 in TRAIL/HDAC inhibitor-treated cells contributed to enhanced lethality. On the other hand, previous studies have shown that p21WAF1/CIP1 protects cells from the lethal actions of certain HDAC inhibitors (e.g., apicidin; 83), and the observations that p21WAF1/CIP1-AS-expressing cells displayed enhanced sensitivity to SAHA- and SB-mediated apoptosis are consistent with results of our earlier reports (15, 17). Similarly, Xu and El-Deiry recently reported that p21WAF1/CIP1 blocked DR4 TRAIL receptor-induced apoptosis in epithelial tumor cells (84). Our finding that p21WAF1/CIP1-AS-expressing cells were more sensitive to TRAIL-induced apoptosis is compatible with these observations. However, the failure of p21WAF1/CIP1-AS-expressing cells to display enhanced susceptibility to the TRAIL/HDAC inhibitor regimen suggests that factors other than or in addition to p21WAF1/CIP1 dysregulation contribute to synergistic interactions between these agents. The mechanism underlying the putative cytoprotective actions of p21WAF1/CIP1 has not been fully elucidated but may involve the capacity of this CDKI to form a complex with caspase-3 and inhibit its activity (85). It is noteworthy that, consistent with recent reports involving vitamin D3-induced apoptosis (35), U937 cells constitutively expressing p21WAF1/CIP1ΔNLS
exhibited significant resistance to TRAIL/HDAC inhibitor-induced cell death. Such a finding suggests that while interference with p21WAF1/CIP1 induction may not further increase the sensitivity of leukemic cells to the combination of TRAIL/HDAC inhibitors, cytoplasmic actions of p21WAF1/CIP1 may nevertheless serve to protect cells from the lethal effects of this drug combination.

Coadministration of TRAIL with HDAC inhibitors also resulted in the down-regulation of cell cycle-related proteins (cyclin D1, p27Kip1, and pRb), phenomena that have previously been associated with increased susceptibility to apoptosis (15, 81, 86–88). These proteins also play key roles in the arrest of cells in G1, an event that is required for leukemic cell maturation (89). In this regard, synergistic induction of leukemic cell apoptosis by combination of the CDKI flavopiridol with HDAC inhibitors (e.g., SAHA and SB) or phorbol myristate acetate has also been associated with down-regulation of these proteins (16, 17, 19), raising the possibility that disruption of leukemic cell differentiation may cause cells to engage an alternative apoptotic program. A similar mechanism may be involved in cells exposed simultaneously to TRAIL and HDAC inhibitors. Finally, it may not be coincidental that activation of the TNF-related extrinsic pathway has recently been implicated in synergistic antileukemic interactions between flavopiridol and protein kinase C activators such as phorbol myristate acetate and bryostatin 1 (33, 90).

In summary, the present findings indicate that coadministration of TRAIL with HDAC inhibitors constitutes a potent apoptotic stimulus in human myeloid and lymphoid leukemia cells and provide further evidence that simultaneous activation of the extrinsic and intrinsic pathways in such cells leads to a dramatic increase in mitochondrial injury and activation of caspase cascades. These events are associated with multiple perturbations in cell cycle and apoptotic regulatory proteins, including degradation and/or cleavage of Bid, Bcl-2, XIAP, p21WAF1/CIP1, p27Kip1, cyclin D1, and pRb that might be responsible for or contribute to the marked potentiation of lethality. Previous studies have demonstrated that TRAIL enhances the antitumor activity of multiple conventional cytotoxic drugs, which primarily act through the intrinsic/mitochondrial pathway, including etoposide, cytosine arabinoside, cisplatin, doxorubicin, and paclitaxel (44, 91). The present results suggest that similar interactions occur in human leukemia cells exposed to TRAIL in combination with novel HDAC inhibitors that have recently entered the clinical arena (92). Given the current interest in the clinical development of TRAIL and related agents as well as the theoretical possibility that TRAIL-containing regimens, like TRAIL itself, may spare normal target tissues (26), the concept of combining TRAIL with HDAC inhibitors warrants attention as a new antileukemic strategy. Accordingly, further efforts to investigate this approach are currently under way.

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Antileukemic Synergism between TRAIL and HDAC Inhibitors


Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces mitochondrial damage and apoptosis in human leukemia cells

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