The histone deacetylase inhibitor and chemotherapeutic agent suberoylanilide hydroxamic acid (SAHA) induces a cell-death pathway characterized by cleavage of Bid and production of reactive oxygen species

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Many chemotherapeutic agents induce mitochondrial-membrane disruption to initiate apoptosis. However, the upstream events leading to drug-induced mitochondrial perturbation have remained poorly defined. We have used a variety of physiological and pharmacological inhibitors of distinct apoptotic pathways to analyze the manner by which suberoylanilide hydroxamic acid (SAHA), a chemotherapeutic agent and histone deacetylase inhibitor, induces cell death. We demonstrate that SAHA initiates cell death by inducing mitochondria-mediated death pathways characterized by cytochrome *c* release and the production of reactive oxygen species, and does not require the activation of key caspases such as caspase-8 or -3. We provide evidence that mitochondrial disruption is achieved by means of the cleavage of the BH3-only proapoptotic Bcl-2 family member Bid. SAHA-induced Bid cleavage was not blocked by caspase inhibitors or the overexpression of Bcl-2 but did require the transcriptional regulatory activity of SAHA. These data provide evidence of a mechanism of cell death mediated by transcriptional events that result in the cleavage of Bid, disruption of the mitochondrial membrane, and production of reactive oxygen species to induce cell death.

Whereas the intracellular targets of disparate chemotherapeutic drugs seem to be many and varied, it now seems clear that a common mechanism of action of such agents is the exploitation of endogenous cell-death pathways to induce apoptosis and thereby eliminate tumor cells (1). Two functionally separable yet molecularly linked intracellular death pathways have been delineated (2, 3).

The first death pathway involves ligation of death receptors such as Fas and the tumor necrosis factor receptor to induce a cascade of protein–protein interactions mediated by proapoptotic cysteine proteases (caspases), culminating in the morphological changes that define apoptosis (4). The second pathway, usually stimulated by "stress stimuli" such as growth factor withdrawal, γ-irradiation, and certain chemotherapeutic drugs uses the mitochondria as a key component for the induction of cell death. Perturbation of the mitochondrial membrane results in the release of mitochondrial proteins, activation of caspases, and loss of the normal respiratory functions of the mitochondria (3). Members of the Bcl-2 family of proteins can both negatively and positively regulate apoptosis induced by this pathway, and the cleavage of Bid, a BH3-only Bcl-2 protein, by caspase-8 serves as a link between the death receptor and mitochondrial death pathways (5, 6). Bid also may be cleaved and activated by other cellular proteins such as the cytotoxic T cell granule protein granzyme B (GzB; refs. 5 and 7) and lysosomal proteases (8) to induce cell death, indicating that this might be an important proapoptotic event initiated by a number of different stimuli.

Whereas many downstream effects of mitochondrial membrane damage occur in a caspase-dependent manner, it is now clear that mitochondrial perturbations that occur in the absence of caspase activation may be sufficient in many cases to induce caspase-independent cell death (9). For example, cell death induced by perforin/GzB, staurosporine (STS), the proapoptotic Bcl-2 family member, Bax, and the hybrid polar compound hexamethylene bisacetamide (HMBA) induce caspase-independent cell death mediated by mitochondrial membrane disruption (10–13).

The effectiveness of chemotherapeutic drugs can be affected by alterations in apoptotic pathways, and knowledge of the molecular basis for this possibility is becoming an important factor in determining the most effective chemotherapeutic regime for some cancer treatments. For example, mutations in the p53 pathway (14), overexpression of antiapoptotic Bcl-2 family members (Bcl-2/Bcl-X_L; ref. 15), blockade of the Fas/FasL pathway (16), and mutations in caspases (17) and/or caspase-regulatory proteins such as Apaf-1 (18) can all induce chemoresistance. In addition, tumor cells may up-regulate the ATP-dependent efflux protein, P-glycoprotein (P-gp), in response to cytotoxic drugs, thereby conferring broad-spectrum multidrug resistance (19). Importantly, we have recently shown that P-gp also can prevent apoptosis by inhibiting the activation of caspases (13, 20, 21), thereby conferring additional resistance to chemotherapeutic drugs.

Histone deacetylase (HDAC) inhibitors have emerged recently as promising chemotherapeutic agents (22). One such

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Abbreviations: GzB, granzyme B; SAHA, suberoylanilide hydroxamic acid; ROS, reactive oxygen species; P-gp, P-glycoprotein; HDAC, histone deacetylase; PARP, poly(ADP-ribose) polymerase; tBid, truncated Bid; Act D, actinomycin D; CHX, cycloheximide; STS, staurosporine; Vin, vincristine; CEM-CCRF; acute T cell leukemia cell line; ZFA-fmk and ZVAD-fmk, peptidyl fluoromethyl ketones; NAC, *N*-acetylcysteine; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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agent, suberoylanilide hydroxamic acid (SAHA), has been shown to induce differentiation and/or apoptosis in a variety of cell types (23–26) and is currently in phase I clinical trials (24). Acetylation of histones is a key process in activating transcription (27), and SAHA has been reported to induce selectively the expression of specific genes such as the $p21^{WAF1/CIP1}$ cyclin-dependent kinase inhibitor to effect cell-cycle arrest (28). No link between SAHA-mediated transcription regulation and cell death has previously been identified.

Whereas SAHA has been demonstrated to kill tumor-cell lines and can be inhibited by antiapoptotic Bcl-2 family members (23, 25), the molecular events upstream and downstream of mitochondrial membrane perturbation by SAHA have not been delineated. Herein, we have defined a mechanism of death triggered by SAHA. Our data implicate a role for the transcriptionally regulated cleavage of the Bcl-2 family member, Bid, and the production of reactive oxygen species (ROS) in SAHA-induced cytotoxicity. Furthermore, SAHA-induced cell death is not affected by functional caspase inhibitors and does not require activation of the death receptor pathway or the expression of functional p53. Therefore, we have defined a pathway for drug-induced apoptosis that may be targeted by new chemotherapeutic agents to induce tumor-cell death.

Materials and Methods

Cell Culture. The acute T cell leukemia cell line CEM-CCRF and its doxorubicin-selected and P-gp$^+$ derivative CEM-P-gp have been described (29). CEM cells overexpressing human Bcl-2 were a gift from David Huang (Walter and Eliza Hall Institute, Melbourne, Australia). CEM cells expressing CrmA have been described (30). Parental FDC-P1 cells and cells overexpressing Bid, Bcl-2, and Bid plus Bcl-2 were a kind gift from D. Huang and were cultured as described (7). All cells were grown in RPMI medium 1640 supplemented with 10% (vol/vol) FCS/2 mM glutamine/100 units/ml penicillin/100 μg/ml streptomycin (GIBCO), with the exception of the CrmA subclones, which were grown in the presence of 1 mg/ml G418 (GIBCO). Cells were cultured for 4–24 hr with 0–10 μM SAHA/100–500 ng/ml doxorubicin/100–500 ng/ml vincristine (Vin) or 10–100 ng/ml anti-Fas antibody clone CH-11 (Upstate Biotechnology, Lake Placid, NY). Doxorubicin and Vin were obtained from Phillip Kantharidis (Peter MacCallum Cancer Institute, East Melbourne, Australia). SAHA was kindly provided by Victoria Richon (Sloan–Kettering Cancer Center, NY). To inhibit the activation of caspases, cells were pretreated for 60 min with peptidyl fluoromethyl ketones (ZFA-fmk or ZVAD-fmk, Enzyme Systems Products, Livermore, CA) at a final concentration of 0–40 μM. To inhibit transcription or translation, cells were pretreated for 30 min with 10 ng/ml actinomycin D (Act D) or 500 ng/ml cycloheximide (CHX), respectively.

Cytotoxicity and Viability Assays. Cell death was assessed by ^{51}Cr-release assay as described (31). The spontaneous release of ^{51}Cr was determined by incubating the target cells with medium alone. The maximum release was determined by adding SDS to a final concentration of 5%. The percent specific lysis was calculated as follows: $100 \times$ [(experimental release $-$ spontaneous release)/(maximum release $-$ spontaneous release)]. Cells were cultured at 2×10^5 cells per ml in the presence or absence of cell-death stimuli for various times. Trypan blue exclusion assays were performed as described (24). In all assays, 150–300 cells were counted for each data point; data were calculated as the means $\pm$ SE of triplicate samples and are representative of at least three separate assays. The number of apoptotic or dead cells (blue cells) was expressed as a percentage of the total cell number.

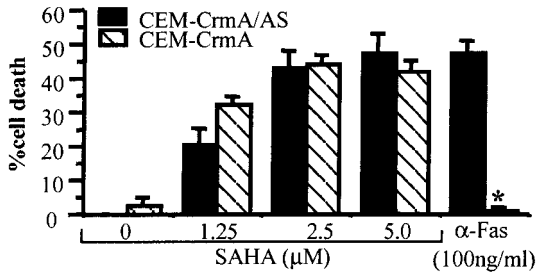

Fig. 1. SAHA-induced cell death was not mediated by death receptors. CEM cells expressing sense (CEM-CrmA) or antisense (CEM-CrmA/AS) CrmA cDNA were treated for 24 hr with 0–5 μM SAHA or 12 hr with 100 ng/ml anti-Fas antibody clone CH-11; cell death was assessed by trypan blue. The results shown are representative of three experiments with a number of different CEM-CrmA and CEM-CrmA/AS cell lines. For all cell-death assays, statistical differences ($P < 0.05$ as determined by the Mann–Whitney U test) between samples are denoted by *.

Western Blot Analysis. Cells (2×10^5) were lysed in 50 μl of ice-cold Nonidet P-40 lysis buffer, as described (13). Protein determinations were performed by means of a Bradford reaction. Proteins (10–20 μg) were separated on SDS/10%, 12%, or 15% polyacrylamide gels electroblotted onto nylon membranes. Blots were probed with anti-human caspase-3 mAb (Transduction Laboratories, Lexington, KY), anti-human poly(ADP-ribose) polymerase (PARP; Roche Molecular Biochemicals), anti-human α-tubulin mAb (Sigma), anti-human Bid mAb (Junying Yuan, Harvard Medical School, Boston) and visualized by enhanced chemiluminescence (Amersham Pharmacia). Cell lysates from Jurkat cells treated with perforin and GzB were prepared as described (7).

Cytochrome *c* Release. Cytosolic extracts from cells (2×10^5) treated for 24 hr with 2.5 μM SAHA were prepared as described (13). Cytosolic proteins were separated on an SDS/15% polyacrylamide gel and transferred onto nylon membranes. Blots were probed with anti-human cytochrome *c* mAb (PharMingen).

ROS Measurements. Cells (2×10^5) were washed twice in 5 mM Hepes-buffered saline (pH 7.4) at 37°C. Cells were then resuspended in 37°C 5 mM Hepes-buffered saline alone or with 10 ng/ml H_2DCFDA dye (C-400, Molecular Probes) and incubated at 37°C for 15 min. Cells then were washed with ice-cold Hepes/saline and placed on ice. Fluorescence was measured with a FACScalibur fluorescence-activated cell sorter (Becton Dickinson) at an excitation wavelength of 480 nm and an emission wavelength of 525 nm. To examine the effects of antioxidants *N*-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) and the electron chain uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP), cells either were coincubated with 2.5 μM SAHA and 0–100 μM NAC, 0–5 μM PDTC, or 0–10 μM CCCP or were treated for 20 hr with 2.5 μM SAHA and then incubated for 30 min with 20 mM NAC.

Results

SAHA-Induced Cell Death Is Not Mediated by Death Receptors or Key Caspases. Some chemotoxins, including the SAHA-related compound m-carboxy-cinnamic acid bis-hydroxamide, have been reported to induce apoptosis via the death receptor pathway (32, 33). To determine whether SAHA-induced cell death via this pathway, we used CEM cells transfected with the cowpox virus protein CrmA, which inactivates membrane-proximal initiator caspases-8 and -10 to inhibit the death receptor pathway (34). CEM-CrmA cells treated with SAHA displayed equivalent cell death when compared with control cells expressing antisense

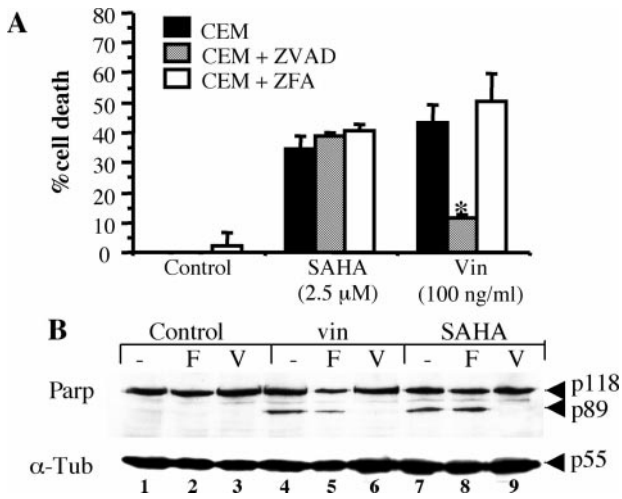

Fig. 2. SAHA-induced cell death is caspase-independent. (*A*) CEM cells were pretreated with 40 μM ZVAD-fmk or ZFA-fmk for 1 hr and then cultured for 24 hr with 2.5 μM SAHA or 100 ng/ml Vin. Cell death was assessed by trypan blue exclusion. (*B*) Whole-cell lysates treated with 2.5 μM SAHA in the presence or absence of ZVAD-fmk (V) or ZFA-fmk (F) as above were assessed for PARP cleavage and α-tubulin expression by Western blotting.

CrmA mRNA (CEM-CrmA/AS; Fig. 1). In contrast, CrmA effectively blocked Fas-mediated cell death in both short-term death assays (Fig. 1) and long-term clonogenic assays (data not shown). Therefore, these data indicate that SAHA does not use the death receptor pathway to induce cell death.

Although SAHA does not use the death receptor pathway, there is evidence that caspases can be activated after SAHA treatment (23). Therefore, we determined whether caspase activity was necessary for SAHA-mediated cell death by using the polycaspase inhibitor ZVAD-fmk and the cathepsin B inhibitor ZFA-fmk as a control. The effectiveness of this caspase inhibitor in our experimental systems was demonstrated by enzymatic assays using colorimetric caspase-3 substrate assays (data not shown) and Western blotting for PARP cleavage (see Fig. 2*B*). It was demonstrated recently that caspase-3 and -7 are the main effector caspases responsible for cleavage of the caspase substrate PARP downstream of caspase-9 and -8 activation (35). CEM cells treated with SAHA in the presence of ZVAD-fmk displayed equivalent cell death when compared with cells treated with SAHA alone or SAHA plus ZFA-fmk (Fig. 2*A*). As reported (20, 21), Vin-induced death in P-gp$^-$ CEM cells was inhibited by ZVAD-fmk (Fig. 2*A*). Whereas ZVAD-fmk did not affect SAHA-induced cell death, SAHA did mediate caspase activation, as demonstrated by the cleavage of PARP. PARP cleavage was completely inhibited by ZVAD-fmk but not by the cathepsin B inhibitor ZFA-fmk (Fig. 2*B*). Consistent with the data shown in Fig. 2*A*, Vin-mediated cleavage of PARP also was inhibited by ZVAD-fmk (Fig. 2*B*). Therefore, although caspases indeed can be activated during SAHA-induced cell death (23, Fig. 2*B*), in the presence of a functional polycaspase inhibitor that effectively blocked apoptosis induced by Vin, SAHA-induced cell death was unperturbed.

The action of many cytotoxic drugs can be inhibited by functional P-gp affecting drug efflux and caspase activation (13, 19–21). However, we have demonstrated recently that agents such as hexamethylene bisacetamide that function in a caspase-independent manner equally can kill P-gp$^+$ and P-gp$^-$ tumor cells (13). Therefore, we tested whether SAHA could induce death in P-gp-expressing cell lines. P-gp$^-$ and drug-induced P-gp$^+$ CCRF-CEM cells that lack functional p53 (30) were treated for 24 hr with SAHA or Vin as a control. Consistent with

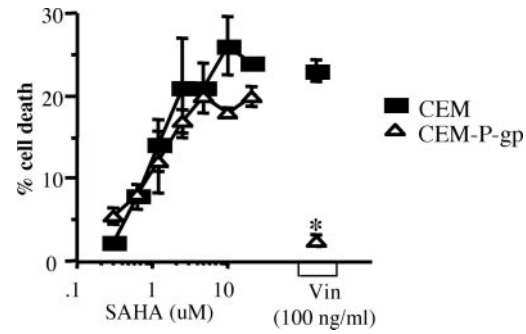

Fig. 3. SAHA induces equivalent cell death in P-gp$^+$ and P-gp$^-$ cells. CEM-P-gp (P-gp$^+$) and CEM (P-gp$^-$) cells were cultured for 24 hr with 0–10 μM SAHA or 100 ng/ml Vin. Cell death was assessed by ^{51}Cr release.

the results shown in Fig. 2, SAHA induced equivalent death in both P-gp$^+$ and P-gp$^-$ CEM cells in a dose-dependent manner, whereas only P-gp$^-$ cells were sensitive to Vin (Fig. 3). Thus, SAHA-induced cell death was not regulated by functional P-gp. Furthermore, SAHA did not affect P-gp expression or efflux function, and SAHA-mediated HDAC activity was equivalent in P-gp$^+$ and P-gp$^-$ cells (data not shown).

Bcl-2 Inhibits SAHA-Mediated Cytochrome *c* Release and Cell Death. Given that the caspase-dependent death receptor pathway was not necessary for SAHA-mediated apoptosis, we assessed the importance of mitochondrial membrane disruption in SAHA-induced death. A hallmark of the mitochondrial death pathway is the ability of Bcl-2 to block effectively this form of cell death (3), and therefore we investigated whether Bcl-2 could inhibit SAHA-induced death of CEM cells. Consistent with previous reports that Bcl-2 can inhibit death by SAHA of a myeloid cell line (23), Bcl-2 inhibited SAHA-mediated cell death at concentrations as high as 5 μM in both short-term assays (Fig. 4*A*) and long-term clonogenic assays (data not shown). By contrast, overexpression of Bcl-2 did not significantly affect death induced by Fas ligation (Fig. 4*A*).

To demonstrate that SAHA induced mitochondrial mem-

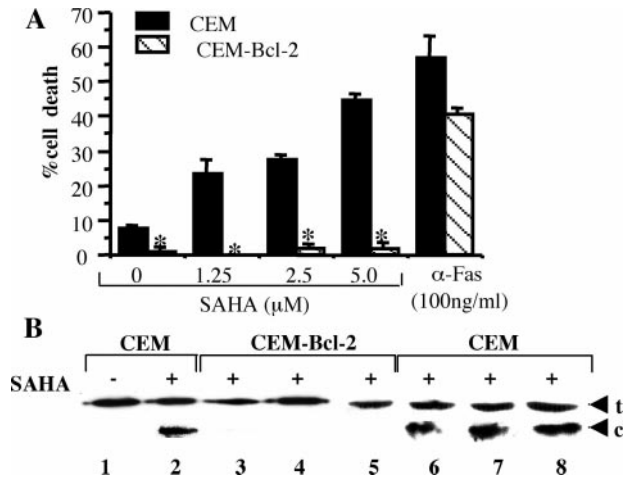

Fig. 4. Bcl-2 inhibits SAHA-induced cell death. (*A*) CEM and CEM-Bcl-2 cells were treated with 0–5 μM SAHA for 24 hr or 100 ng/ml anti-Fas antibody for 12 hr; cell death was assessed by trypan blue exclusion. (*B*) Western blots of cytosolic extracts from CEM and CEM-Bcl-2 cells treated with or without 2.5 μM SAHA were probed with mAbs to cytochrome *c* (c) and α-tubulin (t). In some wells, CEM cells were pretreated with 40 μM ZVAD-fmk (lanes 4 and 7) or ZFA-fmk (lanes 5 and 8) for 1 hr and then cultured for 24 hr with 2.5 μM SAHA.

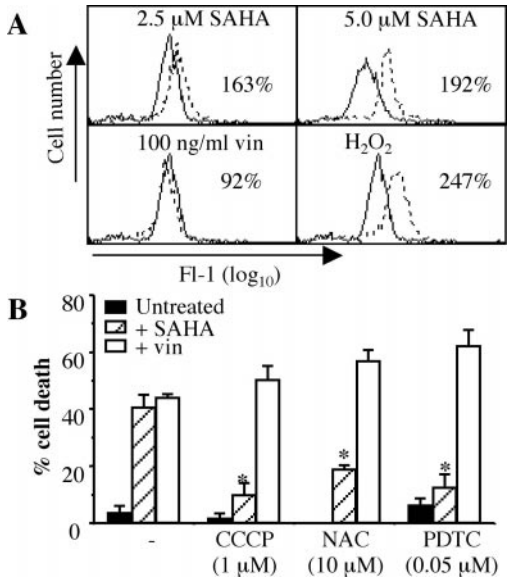

Fig. 5. Production of ROS is central to SAHA-mediated cell death. (*A*) CEM cells were cultured for 24 hr in the presence or absence of 2.5 and 5.0 μM SAHA or 100 ng/ml Vin, labeled with oxidative-sensitive dye C-400, and analyzed by flow cytometry for increases in Fl-1 fluorescence. As a control, cells were labeled with C-400 in the presence of 10 μM H_2O_2. Values were calculated as the percent mean fluorescence compared with control (untreated cells). (*B*) Cells were pretreated with CCCP, NAC, or pyrrolidine dithiocarbamate (PDTC) for 1 hr and then cultured for 24 hr with 2.5 μM SAHA; cell death was assessed by trypan blue exclusion.

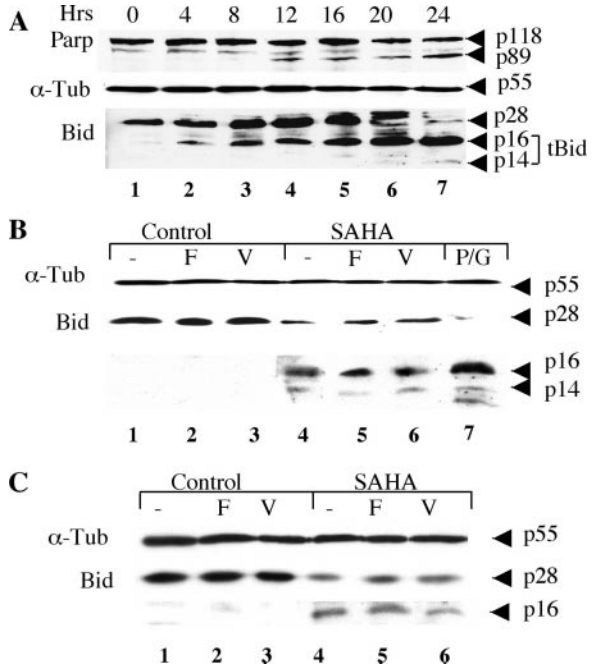

Fig. 6. SAHA induces caspase-independent Bid cleavage in the presence of Bcl-2. (*A*) Whole-cell lysates from CEM cells cultured for 0–24 hr with 2.5 μM SAHA were assessed for Bid and PARP cleavage by Western blotting. (*B*) Whole-cell lysates from CEM cells cultured for 24 hr with 2.5 μM SAHA were assessed for Bid cleavage and α-tubulin by Western blot analysis. In some lanes, cells were pretreated for 1 hr with 40 μM ZVAD-fmk (V) or ZFA-fmk (F) and then cultured for 24 hr with 2.5 μM SAHA. As a control for Bid cleavage, whole-cell lysates from Jurkat cells treated with a sublytic dose of perforin in combination with 60 nM GzB (P/G) were run on the same gel. The middle panel is a 30-sec exposure showing the decrease in p28 full-length Bid protein. The bottom panel is a 5-min exposure showing the tBid products p16 and p14. (*C*) Whole-cell lysates from CEM-Bcl-2 cells treated as above were assessed for Bid cleavage and α-tubulin by Western blotting.

brane disruption, we examined cytosolic extracts from CEM cells treated for 24 hr with SAHA for release of mitochondrial cytochrome *c*. Cytochrome *c* was present in cytosolic extracts from CEM cells cultured with SAHA in both the presence and absence of ZVAD-fmk and control ZFA-fmk (Fig. 4*B*). In contrast, overexpression of Bcl-2 completely inhibited cytochrome *c* release (Fig. 4*B*). Therefore, these data indicate that SAHA can induce the mitochondrial death pathway in the absence of the activation of key caspases to kill the target cell.

Production of ROS Is Central to SAHA-Induced Cell Death. Thus far, our results established that mitochondrial membrane perturbation played a pivotal role in SAHA-mediated apoptosis, and that polycaspase inhibitors did not affect the cytotoxic effect of SAHA. Therefore, we sought to identify the events induced by the mitochondrial death pathway necessary for the cytotoxic action of SAHA. The production of ROS has been implicated in mitochondrial membrane depolarization and cell death (36–38). Therefore, we examined the effect of SAHA treatment on the production of ROS and the importance of ROS in SAHA-induced cell death. SAHA treatment of CEM cells increased production of ROS in a dose-dependent manner (Fig. 5*A*) as early as 6 hr after treatment with SAHA (data not shown). Interestingly, treatment of cells with Vin did not increase ROS production (Fig. 5*A*), suggesting that ROS production does not merely correlate with cell death. Furthermore, SAHA-induced ROS production was inhibited by the antioxidants pyrrolidine dithiocarbamate and NAC, and by the electron chain uncoupler CCCP, which prevents the production of mitochondrial ROS, but not by ZVAD-fmk or ZFA-fmk (data not shown). Importantly, and in contrast to caspase inhibitors, antioxidants and CCCP inhibited SAHA-induced cell death but had no effect on Vin-mediated cell death (Fig. 5*B*). Consistent with the results seen in Figs. 4 and 5, overexpression of Bcl-2 inhibited SAHA-induced ROS production (data not shown). Taken together, these data suggest that the generation of ROS does not merely correlate with cell death, but is an important effector of death induced by SAHA.

SAHA Induces Cleavage of Bid That Is Not Blocked by Bcl-2 and Not Dependent on Key Caspases. To explore the mechanism of mitochondrial membrane disruption by SAHA, we examined proteins known to be upstream of the mitochondrial death pathway. The BH3-only Bcl-2 family member Bid can be processed to its active pore-forming products (≈14–16 kDa) by active caspase-8, GzB, and lysosomal proteases (6–8, 39), resulting in the insertion of truncated Bid (tBid) into the mitochondrial membrane and induction of cell death. SAHA induced Bid cleavage in a time-dependent manner, with the active p16 product apparent by 4 hr after SAHA treatment (Fig. 6*A*, Bid), whereas PARP cleavage was not detected until 12 hr after incubation with SAHA. SAHA treatment resulted in the loss of full-length Bid (p28) and the production of characteristic tBid cleavage products (p16 and p14) similar in size to those produced by GzB that directly process Bid at the Asp residue at position 75 (D75; ref. 7; Fig. 6*B*). Furthermore, Bid was equivalently processed in CEM and CEM-Bcl-2 cells, indicating that Bid cleavage is upstream of mitochondria membrane damage (Fig. 6 *B* and *C*). Importantly, the processing of Bid was not inhibited by ZVAD-fmk in either CEM or CEM-Bcl-2 cells (Fig. 6 *B* and *C*); however, the effectiveness of ZVAD-fmk at this concentration and time point was demonstrated by Western blotting for PARP cleavage and caspase-3 activity assays using specific colorimetric peptides

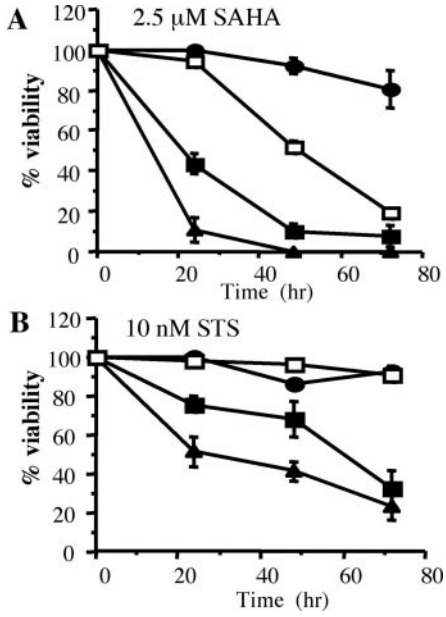

Fig. 7. Cooverexpression of Bid reverses Bcl-2's block of SAHA-mediated cell death. FDC-P1 cells (■) and FDC-P1 cells CO-overexpressing Bid (▲), Bcl-2 (●), or Bid and Bcl-2 (□) were cultured for 0–72 hr in the presence of 2.5 μM SAHA (*A*) or 10 nM STS (*B*). Cell death was assessed by trypan blue exclusion. These results are representative of six separate experiments.

(data not shown). We have also demonstrated that SAHA can efficiently induce Bid cleavage in CrmA-overexpressing cells, further indicating that Bid cleavage is not caused by caspase-8 activation (data not shown). These data indicate that SAHA induces the rapid cleavage of Bid in the presence of functional caspase inhibitors. Furthermore, although Bcl-2 does not prevent Bid cleavage, it does prevent cell death. These results demonstrate that SAHA-mediated cell death occurs through Bid-mediated disruption of the mitochondria.

Cooverexpression of Bid Reverses the Bcl-2 Block of SAHA-Mediated Cell Death. If Bid is a crucial upstream component of SAHA-mediated cell death, we hypothesized that co-overexpression of Bid may be able to reverse the block in SAHA-mediated cell death mediated by overexpression of Bcl-2 alone. We used a system used previously to demonstrate the importance of Bid cleavage in GzB-mediated cell death (7). Uncloned FDC-P1 cells that overexpressed Bid and/or Bcl-2 were treated with 2.5 μM SAHA or 10 nM STS, which activates the mitochondrial death pathway independently of Bid processing as a control (7). Expression of Bcl-2 and/or Bid was confirmed by intracellular fluorescence-activated cell sorting and Western blotting, and Bcl-2 expression was consistently greater relative to Bid expression (data not shown). Cells overexpressing Bcl-2 were resistant to SAHA- and STS-induced cell death, whereas cells overexpressing Bid were more sensitive to SAHA and STS than were control parental cells, which is consistent with the notion that Bid is a proapoptotic molecule and sensitizes cells to death stimuli (Fig. 7*A*). However, coexpression of Bid and Bcl-2 restored sensitivity of the cells to SAHA-induced cell death (Fig. 7*A*). Importantly, STS-induced cell death was inhibited by Bcl-2; however, in contrast to SAHA, death by STS was not affected by coexpression of Bid (Fig. 7*B*). These results show that Bid is a key protein in SAHA-mediated cell death and demonstrate the important events upstream of mitochondrial membrane damage necessary for SAHA killing.

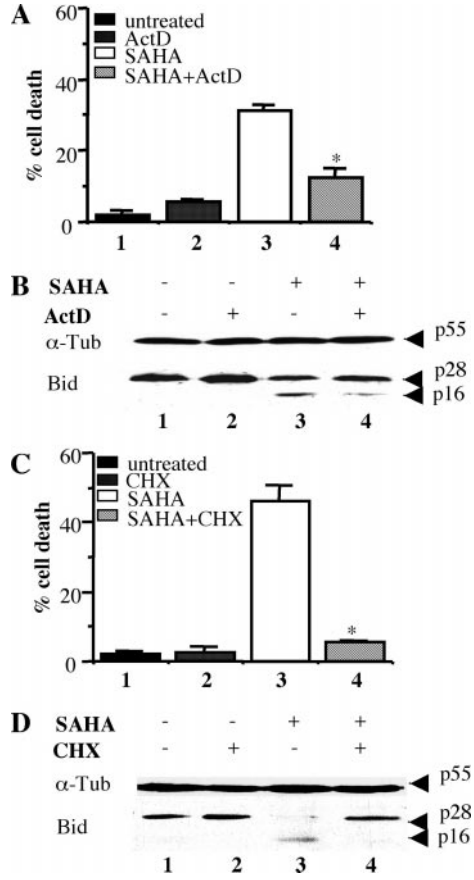

Fig. 8. New gene transcription is necessary for SAHA-mediated cell death. CEM cells were pretreated for 30 min with 10 ng/ml Act D (*A*) or 500 ng/ml CHX (*C*) and then cultured for 24 hr in the presence of SAHA. Cell death was assessed by trypan blue exclusion. (*B* and *D*) CEM cells were treated as above and whole-cell lysates were assessed for Bid cleavage and α-tubulin protein loading by Western blotting.

New Gene Transcription Is Necessary for SAHA-Mediated Cell Death. The ability of SAHA to function as an HDAC inhibitor results in the derepression of specific cellular genes (22). To examine whether new gene expression was necessary for SAHA-induced cell death and cleavage of Bid, CEM cells were pretreated with Act D (Fig. 8*A*) or CHX (Fig. 8*C*) and then cultured for 24 hr with SAHA. Cell viability was not affected by this concentration of Act D or CHX alone; however, SAHA-induced cell death was significantly inhibited by both agents. Inhibition of SAHA-induced death by CHX depended on the dose of CHX used in the experiment (data not shown). Furthermore, cleavage of Bid was inhibited by Act D (Fig. 8*B*) and CHX (Fig. 8*D*), with the degree of Bid cleavage correlating with the percentage of cells undergoing apoptosis. Taken together, these data establish a dependence on new gene transcription and translation for the induction of Bid cleavage and cell death by SAHA.

Discussion

SAHA is a chemotherapeutic drug capable of inhibiting HDAC activity; by this means, it can regulate gene expression and induce cell-cycle arrest and cell death (23–26). To date, the molecular events necessary for SAHA-induced cell death have not been dissected, and a possible link between the gene-regulatory activity of SAHA and its ability to mediate cell death has not been assessed. By using CEM cells that lack functional p53, we demonstrate that SAHA induces cell death regardless of functional P-gp expression, and that SAHA does not proceed

through the death receptor pathway. However, SAHA-induced cell death was mediated by the activation of a mitochondrial death pathway characterized by caspase-independent cleavage of Bid, cytochrome *c* release, and ROS production. Inhibition of ROS and overexpression of Bcl-2 significantly affected SAHA-mediated cell death, indicating that disruption of mitochondrial function and production of ROS are crucial for cell death induced by SAHA. As expected, disruption of the mitochondrial membrane in SAHA-treated cells did result in caspase activation; however, addition of the polycaspase inhibitor ZVAD-fmk, which effectively inhibited caspase-3 activity and the cleavage of PARP, had no effect on SAHA cytotoxicity. These data indicate that whereas SAHA can induce activation of these apoptotic enzymes, key caspases such as caspase-8 and -3 are not necessary for SAHA-induced cell death.

We identified cleavage of the BH3-only proapoptotic Bcl-2 family member Bid as a key event upstream of SAHA-induced mitochondrial membrane disruption. Bid cleavage was not blocked by caspase inhibitors or overexpression of Bcl-2, but depended on the transcriptional regulatory function of SAHA. These findings indicate that drug-induced Bid cleavage can occur upstream of mitochondrial perturbation and demonstrate that Bid cleavage is an important process in SAHA-mediated cell death.

It has been reported that caspase-8 and -3 process Bid at position D59, GzB at position D75, and lysosomal proteases at position R65 (6–8, 39). Our data demonstrating the production of characteristic tBid products by SAHA in the presence of a functional polycaspase inhibitor and the absence of GzB indicate that a protease may induce SAHA-mediated Bid cleavage. The production of SAHA-induced p16 and p14 tBid products indicates that the Bid cleavage sites induced by SAHA are within, or close to, the region targeted by caspases, GzB, and lysosomal proteases. Whereas it is possible that the protease responsible for SAHA-induced Bid cleavage may be a caspase that is not efficiently inhibited by ZVAD-fmk and does not directly cleave PARP, it is unlikely to be the key caspase-8 or -3, as neither CrmA nor ZVAD-fmk, which clearly inhibited caspase activity in our cell systems, could inhibit Bid cleavage. That SAHA could induce Bid cleavage in Bcl-2-overexpressing cells provides further evidence that caspases are not required for this proteolytic event.

It is possible that SAHA-induced Bid cleavage is induced by the release of lysosomal proteases directly or indirectly regulated by SAHA at the level of gene transcription. Lysosomes are largely composed of cathepsins; however, the SAHA-induced cleavage of Bid is unlikely to be mediated by cathepsin B, as the cathepsin B inhibitor ZFA-fmk had no effect on Bid cleavage. Given that SAHA inhibited HDAC activity in CEM cells (data not shown), and that cell death and Bid cleavage were affected by Act D and CHX (Fig. 8), we propose that SAHA may directly affect the transcription of an unknown protease or protease regulator that can cleave Bid. It will be of great interest to identify the specific protease(s) necessary for SAHA-mediated Bid cleavage.

In conclusion, these studies demonstrate a mechanism of drug-induced apoptosis mediated by Bid cleavage and subsequent production of ROS. Activation of this pathway occurs in P-gp-expressing multidrug-resistant tumor cells containing functional mutations in p53 without the activation of key caspases. Therefore, we propose a model for the induction of tumor-cell death by HDAC inhibitors. It remains to be determined whether other chemotherapeutic drugs also may induce cell death by activating this pathway.

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