Novel Mechanisms of Apoptosis Induced by Histone Deacetylase Inhibitors[1]

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ABSTRACT

Histone deacetylase inhibitors (HDACIs) are a new class of chemotherapeutic drugs able to induce tumor cell apoptosis and/or cell cycle arrest; however, the molecular mechanisms underpinning their anticancer effects are poorly understood. Herein, we assessed the apoptotic pathways activated by three HDACIs, suberoylanilide hydroxamic acid, oxamflatin, and depsipeptide. We determined that all three drugs induced the accumulation of cells with a 4n DNA content and apoptosis mediated by the intrinsic apoptotic pathway. HDACI-induced mitochondrial membrane damage and apoptosis were inhibited by overexpression of Bcl-2, but not by the polycaspase inhibitor *N*-tert-butoxy-carbonyl-Val-Ala-Asp-fluoromethyl-ketone (zVAD-fmk). Moreover, induction of a G_1-S checkpoint through overexpression of $p16^{INK4A}$ or suppression of *de novo* protein synthesis also inhibited HDACI-induced cell death. Proteolytic cleavage of caspase-2, which is poorly inhibited by zVAD-fmk, was concomitant with HDACI-induced death; however, full processing of caspase-2 to the p19 active form was blocked by Bcl-2. Whereas all three drugs induce the activation of the proapoptotic Bcl-2 protein Bid upstream of mitochondrial membrane disruption, Bid cleavage in response to depsipeptide was significantly attenuated by zVAD-fmk. Suberoylanilide hydroxamic acid and oxamflatin could kill both P-glycoprotein (P-gp)+ MDR cells and their P-gp− counterparts, whereas depsipeptide was shown to be a substrate for P-gp and was less effective in killing P-gp+ cells. These data provide insight into the functional profile of three HDACIs and are important for the development of more rational approaches to chemotherapy, where information regarding the genetic profile of the tumor is matched with the functional profile of a given chemotherapeutic drug to promote favorable clinical responses.

INTRODUCTION

Structurally diverse chemotherapeutic drugs have disparate intracellular targets, and it is widely accepted that drug-induced cell death is primarily mediated through the activation of physiological apoptotic pathways (1). As a result, genetic alterations that disrupt apoptotic pathways frequently give rise to tumors that are resistant to chemotherapy. Consequently, there is a need to decipher the apoptotic signaling pathways activated by chemotherapeutic drugs to aid in the development of effective strategies that could circumvent or overcome lesions within apoptotic pathways that impede current anticancer therapies.

Chemotherapeutic drugs can induce cell death by activating either of two major apoptotic pathways (1). The death receptor pathway involves ligation of receptors (*i.e.* Fas, tumor necrosis factor receptor, and TRAILR) by their cognate ligands, resulting in binding of adaptor proteins (*i.e.* FADD and TRADD) and recruitment and activation of membrane proximal caspases (*i.e.* caspase-8 and caspase-10). These enzymes subsequently activate effector caspases (*i.e.* caspase-3), resulting in the cleavage of a number of nuclear and cytoplasmic substrates to induce the morphological changes characteristic of apoptosis. The second pathway, known as the intrinsic pathway, is primarily activated by stress stimuli such as chemotherapeutic drugs, ionizing radiation, and growth factor withdrawal (2). These stimuli induce the release of mitochondrial proteins such as cytochrome *c*, which, together with Apaf-1, induces caspase-9 activation, and HtrA2 and Smac/DIABLO, which neutralize the caspase-inhibitory actions of inhibitors of apoptosis proteins (IAP). Caspase-9 can then activate downstream effector caspases (*i.e.* caspase-3). The intrinsic pathway is primarily regulated by the Bcl-2 family of proteins, which includes the multidomain antiapoptotic (*i.e.* Bcl-2 and Bcl-X_L) and proapoptotic (*i.e.* Bak and Bax) members, and BH3 domain-only proapoptotic members (*i.e.* Bid, Bad, NOXA, PUMA, and Hrk). Interestingly, death receptors can also activate the intrinsic pathway through caspase-8-mediated activation of Bid. In this way, Bid serves as a molecular link between the two pathways and can amplify the death receptor-stimulated response.

HDACIs[3] are a novel class of chemotherapeutic agent initially identified by their ability to reverse the malignant phenotype of transformed cells. They have been shown to activate differentiation programs, inhibit the cell cycle, and induce apoptosis in a wide range of tumor-derived cell lines and to block angiogenesis and stimulate the immune system *in vivo* (3, 4). Whereas the mechanisms through which HDACIs exert these antitumor activities have not been fully delineated, induction of histone hyperacetylation and modulation of gene transcription through chromatin remodeling are thought to be primarily responsible, leading to the selective activation of genes associated with cell growth and survival. In addition, proteins other than histones, such as p53 and GATA-1, have been identified as targets for acetylation (5, 6), and it is possible that HDACIs may exert other effects independently of their direct effects on transcription through chromatin remodeling. Recently, there has been strong interest in HDACIs as anticancer agents due to their selective toxicity against tumor cells and synergistic activity with existing therapeutic agents, including retinoic acids (7), vitamin D analogues (8), and peroxisome proliferator-activated receptor ligands (9). A number of HDACIs are currently in Phase I and II clinical trials, and encouraging results have been reported for the treatment of both hematological malignancies and solid tumors (3, 4).

Previously, we reported that the HDACI SAHA mediated apoptosis via a novel mechanism involving activation of the intrinsic death pathway by inducing the caspase-independent cleavage and activation

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[3] The abbreviations used are: HDACI, histone deacetylase inhibitor; P-gp, P-glycoprotein; PI, propidium iodide; mAb, monoclonal antibody; PARP, poly(ADP-ribose) polymerase; MDR, multidrug resistance; CHX, cycloheximide; SAHA, suberoylanilide hydroxamic acid; dox, doxycycline; CBHA, *m*-carboxycinnamic acid bishydroxamide; zVAD-fmk, *N*-tert-butoxy-carbonyl-Val-Ala-Asp-fluoromethylketone; TRAILR, TNF related apoptosis inducing ligand receptor; FADD, Fas-associated death domain; TRADD, TNF receptor-associated death domain; PUMA, p53 upregulated modulator of apoptosis; ZFA-fmk, *N*-tert-butoxy-carbonyl-Phe-Ala-fluoromethylketone; TMRE, tetra-methyl rhodamine ethyl ester.

of Bid (10). The aim of this study was to determine whether the structurally diverse HDACIs oxamflatin and depsipeptide (officially called FR901228 or FK228) activate a similar apoptotic pathway to SAHA. Oxamflatin is a synthetic HDACI belonging to the same structural class (hydroxamic acid) as SAHA. Depsipeptide is a natural HDACI that was isolated from *Chromobacterium violaceum* and has a novel chemical structure (cyclic tetrapeptide). Both oxamflatin and depsipeptide exhibit potent cytotoxicity against various human tumor cell lines (11, 12) and *in vivo* efficacy against human tumor xenografts and/or murine tumors (12); however, little is known about the molecular events underpinning cell death by these drugs.

We have identified key molecular events necessary for apoptosis induced by these drugs and found that all three agents induced death marked by alteration of the cell cycle profiles with a loss of cells in G_1 and S phase and accumulation of cells with a 4n DNA content and the cleavage and activation of caspase-2 and the BH3-only Bcl-2 protein Bid. Inhibition of the death receptor pathway or addition of the polycaspase inhibitor zVAD-fmk, which can inhibit the activity of a number of key caspases (*i.e.* caspase-3, -7, -8, and -9) but is a poor inhibitor of caspase-2 (13), had little or no effect on tumor cell death by these drugs. However, consistent with these agents mediating cell death after mitochondrial disruption, overexpression of Bcl-2 inhibited HDACI-induced mitochondrial membrane disruption and the cytotoxic effects of these drugs. Interestingly, initial processing of caspase-2 to its p33 intermediate form still occurred in Bcl-2-overexpressing cells, indicating that HDACIs can induce the initial cleavage of caspase-2 upstream of Bcl-2; however, full cleavage to the active p19 form of caspase-2 required mitochondrial membrane perturbation. This is consistent with a model for caspase activation requiring the release of mitochondrial Smac/DIABLO and/or HtrA2 to sequester IAPs and allow full caspase activation.

Inhibition of cell cycle progression at the G_1-S boundary through overexpression of the cyclin-dependent kinase inhibitor p16^INK4A abrogated cell death mediated by HDACIs. Moreover, inhibition of *de novo* protein synthesis inhibited the cleavage of Bid and subsequent death induced by SAHA, oxamflatin, and depsipeptide, indicating that their mechanism of action is mediated through a direct (through histone hyperacetylation) or indirect effect on gene transcription. Whereas all three drugs had similar effects on cell proliferation and survival, depsipeptide-induced Bid cleavage was perturbed by zVAD-fmk, indicating differences in the mechanisms of action of depsipeptide compared with SAHA and oxamflatin. In addition, we have determined that depsipeptide is a substrate for the drug efflux protein P-gp and is less effective against P-gp-expressing, MDR cells. Taken together, these data provide a mechanistic profile of a novel class of chemotherapeutic drugs and indicate that although these structurally diverse compounds activate a number of common molecular pathways, subtle yet significant differences in their mechanisms of action exist.

MATERIALS AND METHODS

Drugs and Inhibitors. Oxamflatin was kindly provided by Dr. Anthony Dear (Monash University Department of Medicine, Box Hill Hospital, Australia), depsipeptide (FR901228/FK228) was obtained from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), and SAHA was kindly provided by Dr. Victoria Richon (Sloan Kettering Cancer Center, New York, NY). To induce p16^INK4A expression, cells were treated for 24 h with 100 ng/ml dox (Sigma, St. Louis, MO). To inhibit P-gp function, cells were pretreated for 1 h with 10 μM verapamil (Knoll Australia, Lane Cove, Australia). To inhibit the activation of caspases, cells were pretreated for 1 h with 20 μM peptidyl fluoromethylketones zVAD-fmk or zFA-fmk as a control (Enzyme Systems Products, Dublin, CA). Cells were pretreated with 5, 50, or 500 ng/ml cyclo-heximide (Sigma) for 1 h to inhibit protein synthesis.

Cell Culture. The acute T-cell leukemia cell line CEM-CCRF and its doxorubicin-selected and P-gp$^+$ derivative CEM-P-gp and the colon carcinoma cell line LoVo and its Adriamycin-selected P-gp$^+$ derivative Lovo-adr have been described previously (14, 15). CEM cells expressing CrmA and p16^INK4A have been described previously (16, 17). CEM cells overexpressing human Bcl-2 were a gift from David Huang (Walter and Eliza Hall Institute, Melbourne, Victoria). All cells were grown in RPMI 1640 supplemented with 10% (v/v) FCS, 2 mm/liter glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY). CEM-p16^INK4A cells were additionally cultured in 250 ng/ml hygromycin B (Amersham, Castle Hill, Australia). The cell surface expression of P-gp was confirmed and monitored by fluorescence analysis using the anti-Pgp monoclonal antibody MRK 16 (Kamiya Biochemical Co., Thousand Oaks, CA).

Apoptosis Assays. Cells were cultured for 0.5–48 h with 0–10 μM oxamflatin, depsipeptide, or SAHA or 0.5–100 ng/ml anti-Fas antibody clone CH-11 (Upstate Biotechnology, Lake Placid, NY). Cell death was assessed by ^{51}Cr release assay as described previously (18). The spontaneous release of ^{51}Cr was determined by incubating cells with medium alone. The maximum release was determined by adding HCl to a final concentration of 1N. The percentage of specific lysis was calculated as follows: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Trypan blue exclusion assays were performed as described previously (19). In all assays, cells from at least four different fields of view were counted for each data point, and data were calculated as the mean ± SD and are representative of at least two separate experiments. The number of apoptotic or dead cells (blue cells) was expressed as a percentage of the total cell number. Translocation of the membrane phospholipid phosphatidylserine from the inner face of the plasma membrane to the outer leaflet was detected using Annexin-V-FLUOS according to the manufacturer's protocol (Roche, Mannheim, Germany). Briefly, cells treated for 18 h with various apoptotic stimuli were washed in PBS, stained with Annexin-V-FLUOS for 10 min at room temperature, and then analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences, North Ryde, Australia). Colony assays were performed on cells treated for 24 h with various apoptotic stimuli as described previously (19).

Cell Cycle Analysis. Cells (2×10^5) treated with various apoptotic stimuli were assessed for DNA content by PI staining followed by flow cytometry as described previously (20). Briefly, cells were washed with PBS, fixed by incubation in 50% ice-cold ethanol/PBS for 30 min on ice, washed again with PBS, and resuspended and incubated in PI solution (69 mM PI, 388 nM sodium citrate, 100 μg/ml RNase A) for 15 min at 37°C.

DEVDase Assay. Cells (2×10^6) treated with or without drug for 24 h were harvested, washed twice with PBS, and lysed in ice-cold NP40 lysis buffer without protease inhibitors. Lysate (45 μg) was incubated with 1.25 mM Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA) substrate (Bachem, Bubendorf, Switzerland) in BAADT buffer (pH 7.3) containing 0.1 M HEPES and 0.05 M CaCl$_2$ at 37°C, and absorbance at (405 nm) was read every 15 min. Relative fold activity was determined by dividing absorbance readings of samples containing drug by absorbance of control untreated samples.

Permeabilized Cell Assay for ΔΨm. Experiments to examine ΔΨm in the presence and absence of HDACIs were performed as described previously (21). CEM or CEM-Bcl-2 cells (2×10^5) incubated for 24 h at 37°C in the presence or absence of SAHA (2.5 μM), depsipeptide (0.5 μM), or oxamflatin (2.5 μM) were subsequently incubated in media containing TMRE (200 nM) for 20 min at 37°C. Cells were then analyzed by flow cytometry with TMRE fluorescence detected in FL2.

Anti-Bid mAb Production. A mAb reactive against human Bid was produced as described previously (22). Briefly, BALB/c mice received i.p. injection with 50 μg of recombinant glutathione *S*-transferase-Bid fusion protein in Freund's complete adjuvant and were then boosted twice at fortnightly intervals with 50 μg of glutathione *S*-transferase-Bid in Freund's incomplete adjuvant. Ten days after fusion, hybridoma culture supernatants were tested for the secretion of antibody reactive with Bid by ELISA.

Western Blot Analysis. Cells (6×10^5) were washed with PBS and lysed in ice-cold NP40 lysis buffer as described previously (19). Proteins (5–15 μg) were separated on 10% or 15% SDS-polyacrylamide gels electroblotted onto Immobilon-P nylon membranes (Millipore, Bedford, MA). Membranes were incubated with anti-acetylated histone H3 (Upstate Biotechnology), anti-acetylated histone H4 (Upstate Biotechnology), anti-caspase-2 (23), antihuman Bid mAb, antihuman PARP (Boehringer Mannheim), and antihuman α-tubulin

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mAb (Sigma) overnight at 4°C, followed by subsequent incubation with horseradish peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham).

To assess the release of cytochrome, cells (1×10^6) were harvested by centrifugation, washed once in PBS, and lysed in 100 μl of ice-cold plasma membrane permeabilization buffer (400 μg/ml digitonin in PBS containing 80 mM KCl and 250 mM sucrose) for 5 min (21). Aliquots were analyzed by trypan blue to ensure that greater than 95% of the cells had permeabilized plasma membranes. The cells were centrifuged (800 $\times$ g for 5 min at 4°C), and the supernatant was analyzed by Western blotting using an anti-cytochrome c mAb (PharMingen).

Extraction of Histones. Histones were isolated by acid extraction. Cells (5×10^6) treated with or without drug for varying times were harvested and washed with PBS. Cells were lysed in ice-cold lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, and 1.5 mM phenylmethyl-sulfonyl fluoride], and 5 M H$_2$SO$_4$ was added to 0.4 N. After incubation on ice for 1 h, the suspension was centrifuged, and the supernatant was harvested, mixed with acetone at a ratio of 9:1, and incubated at -20°C overnight. After centrifugation, the pellet was washed with 70% ethanol and air dried, and the acid-soluble histone fraction was dissolved in H$_2$O. A BCA protein assay was then used for quantitation (Pierce), and histones were analyzed by Western blot.

RESULTS

Oxamflatin and Depsipeptide Induce Death of CEM Cells. The HDACIs SAHA, sodium butyrate, and trichostatin A have all been shown to induce apoptosis of CEM cells (10, 24). We found that treatment of CEM cells for 24 h with oxamflatin or depsipeptide induced apoptosis of cells in a dose-dependent manner (data not shown). Doses of drug that induced approximately 50% cell death by 24 h were then used for a time course experiment (Fig. 1*A*). Death induced by oxamflatin (1 μM) and depsipeptide (0.5 μM) was compared with that induced by SAHA (2.5 μM). Significant death was observed after treatment with all three HDACIs by 12 h and steadily
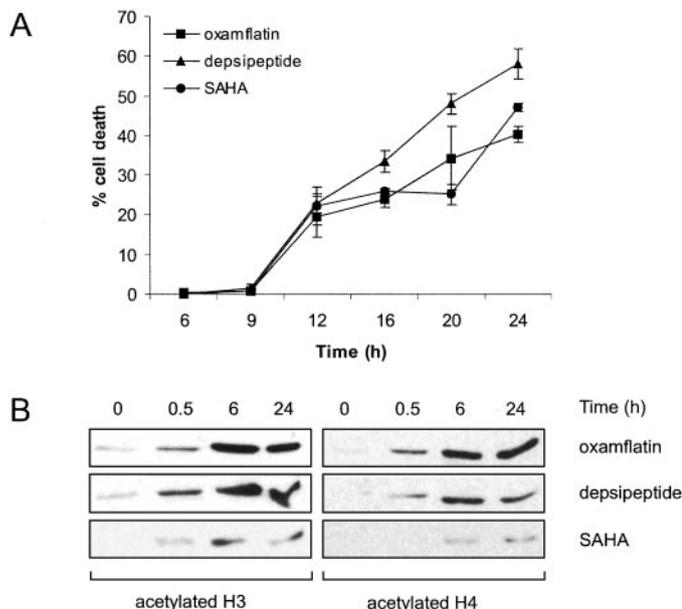

Fig. 1. Oxamflatin and depsipeptide induce death of CEM cells. *A*, CEM cells were treated with oxamflatin (1 μM), depsipeptide (0.5 μM), or SAHA (2.5 μM) for 6, 9, 12, 16, 20, and 24 h; collected at each time point; and assessed for death by ^{51}Cr release. Data are presented as the mean $\pm$ SD of two separate experiments. *B*, histones were isolated by acid extraction from cells treated for 0.5, 6, and 24 h with oxamflatin (1 μM), depsipeptide (0.5 μM), or SAHA (2.5 μM). Five μg of protein were separated by 10% SDS-PAGE, and Western blot analyses were performed with anti-acetylated histone H3 or H4 antibodies. Equivalent protein loading was confirmed by Ponceau S staining (data not shown).
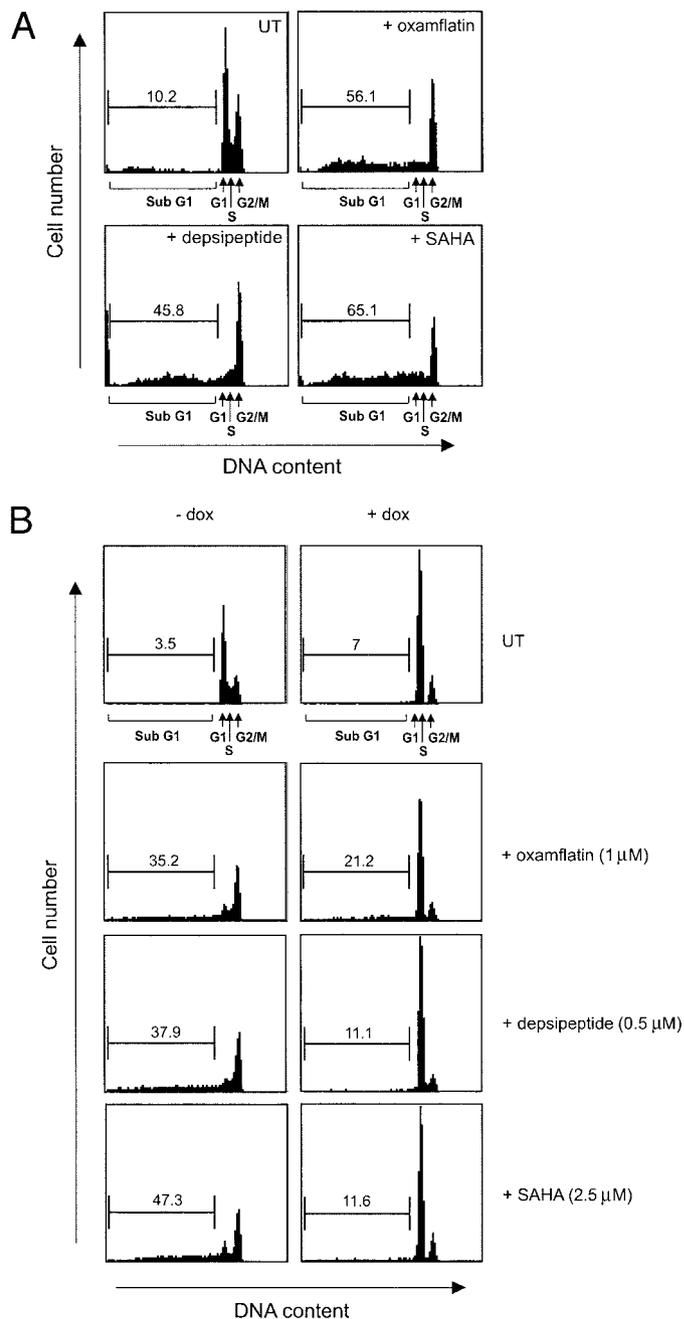

Fig. 2. Oxamflatin and depsipeptide induce G$_2$-M arrest in CEM cells, which is inhibited by overexpression of p16^{INK4A}. *A*, CEM cells were treated for 24 h with oxamflatin (1 μM), depsipeptide (0.5 μM), or SAHA (2.5 μM); fixed in 50% ethanol; and stained with PI. DNA content was analyzed by flow cytometry. The populations of cells in the G$_1$, S, and G$_2$-M phases of the cell cycle are indicated. The percentage of cells with a DNA content less than G$_1$ (sub-G$_1$) is indicated in each histogram. *B*, CEM cells overexpressing p16^{INK4A} in a dox-inducible manner were pretreated for 24 h with 100 ng/ml dox and then treated with 1 μM oxamflatin, 0.5 μM depsipeptide, or 2.5 μM SAHA for 24 h. PI-stained cells were analyzed as described above.

increased over the 24-h time period (Fig. 1*A*). Similar results were obtained using DNA fragmentation/PI staining, trypan blue exclusion, and Annexin V staining as markers of cell death (data not shown).

Oxamflatin, depsipeptide, and SAHA have all been shown to inhibit the enzymatic activity of histone deacetylases (11, 25, 26). Thus, we examined the effect of these drugs on the acetylation of histone H3 and H4 in CEM cells by Western blot using acetylated histone type-specific antibodies. As shown in Fig. 1*B*, treatment with oxamflatin, depsipeptide, or SAHA led to a marked increase in both

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Table 1 *HDACIs induce apoptosis and accumulation of CEM cells with a 4n DNA content*

Treatment	CEM cells (%)											
	24 h				36 h				48 h			
	Sub-G_1	G_1	S	G_2-M	Sub-G_1	G_1	S	G_2-M	Sub-G_1	G_1	S	G_2-M
Untreated	3.6	55	13.9	27.5	3	54.7	17.1	25.2	7	51.8	15.1	26.1
Oxamflatin	43	9	6	42	74	7.8	6.3	11.9	90.7	4.2	2.5	2.6
Depsipeptide	39.2	5.9	4.8	50.1	77.6	8.2	4.8	9.4	87.9	5.2	2.9	4.0
SAHA	29.6	10.9	10.2	49.3	65.8	9.7	7.9	16.6	90.8	4.2	2.6	2.4

acetylated histones H3 and H4 within 30 min. The accumulation of acetylated histones remained elevated to 24 h. Thus, similarly to SAHA, oxamflatin and depsipeptide induce apoptosis of CEM cells in a dose- and time-dependent manner, preceded by the accumulation of acetylated histones H3 and H4.

Apoptosis Induced by HDACIs Requires Cell Cycle Progression. Because HDACIs can affect both cell proliferation and cell survival, we assessed the effect of oxamflatin, depsipeptide, and SAHA on cell cycle progression. CEM cells were treated with the different HDACIs for 24 h and stained with PI. Flow cytometric analysis revealed that, in addition to inducing apoptosis as denoted by the increase in the percentage of the sub-G_1 population of cells, treatment with oxamflatin, depsipeptide, or SAHA caused an accumulation of cells with 4n DNA content, consistent with a G_2-M cell cycle arrest (Fig. 2*A*). A time course analysis revealed that the percentage of cells with 4n DNA content decreased over time, concomitant with an increase in the percentage of apoptotic cells (Table 1). These data indicate that the movement of cells into and through the G_2-M phases of the cell cycle may precede apoptosis induced by these HDACIs.

To determine whether cell cycle progression is necessary for HDACI-induced apoptosis, CEM cells that overexpress p16^{INK4A} in a dox-inducible manner were used. CEM-p16^{INK4A} cells were treated for 24 h with or without dox to induce p16^{INK4A} expression and G_1 arrest before the addition of oxamflatin, depsipeptide, or SAHA. As shown in Fig. 2*B*, p16^{INK4A}-expressing CEM cells were almost completely arrested in G_1 and were significantly less sensitive to death induced by the three HDACIs compared with cycling CEM cells. Treatment of CEM-p16^{INK4A} (−dox) cells with oxamflatin, depsipeptide, or SAHA led to an accumulation of cells with a 4n DNA content and induction of apoptosis after 24 h. In contrast, there was no increase in cells within G_2-M, and there was a marked reduction (>50%) in the sub-G_1 population of the HDACI-treated p16^{INK4A}-expressing cells. Remarkably, these cells remained largely protected from HDACI-induced death over 48 h (data not shown). Comparable results were also obtained by trypan blue exclusion assays, and similar effects were seen with an independent dox-inducible, p16^{INK4A}-expressing clone (data not shown). Thus, it appears that transition through the cell cycle is necessary for HDACI-mediated apoptosis and, taken together with Table 1, suggests that these drugs induce apoptosis during or immediately after movement of cells through G_2-M.

Oxamflatin, but not Depsipeptide, Induces Death of P-gp$^+$ and P-gp$^-$ Cells. MDR is a major obstacle for anticancer therapy, and one common mechanism of MDR is overexpression of the multidrug transporter P-gp. P-gp can protect cells from drug-induced apoptosis through its combined ability to efflux drugs and inhibit caspase activation (19). However, not all drugs are inhibited by P-gp because we have demonstrated that both hexamethylene bisacetamide and SAHA, which are not P-gp substrates and function in a caspase-independent manner, can equivalently kill P-gp$^+$ and P-gp$^-$ tumor cells (10, 20). To determine whether oxamflatin and depsipeptide were able to kill P-gp-expressing cells, we treated two paired P-gp$^+$

and P-gp$^-$ cell lines with various doses of oxamflatin or depsipeptide for 24 h and measured cell death. The expression of P-gp on these cell lines was assessed before and during the course of the assays by immunofluorescence analysis using the anti-P-gp mAb MRK 16. As shown in Fig. 3*A*, P-gp was highly expressed on CEM-P-gp and Lovo-adr cells, weakly expressed on Lovo cells, and not expressed at all on CEM cells. Treatment of the cells with oxamflatin or depsipeptide did not alter the expression levels of P-gp over the 24-h time period (data not shown). As seen in Fig. 3*B*, oxamflatin induced equivalent death of P-gp$^+$ and P-gp$^-$ cells in a dose-dependent manner. In contrast, death induced by depsipeptide was significantly inhibited in P-gp$^+$ cells. The protection by P-gp against depsipeptide-induced death but not oxamflatin-induced death was confirmed in sensitive long-term clonogenic assays (data not shown). Verapamil, a pharmacological inhibitor of P-gp, reversed the resistance of the P-gp$^+$ cells to depsipeptide (Fig. 3*C*), supporting the notion that depsipeptide is a substrate for P-gp (27).

In addition to effluxing chemotherapeutic drugs, P-gp can inhibit the activation of caspases (19, 28, 29). We therefore tested whether P-gp affected activation of caspases after treatment with different HDACIs. Caspase-3 was activated in CEM cells undergoing apoptosis induced by oxamflatin, depsipeptide, and SAHA, but not in CEM-P-gp cells, as determined by a caspase activity assay using a colorimetric caspase-3 substrate (Fig. 3*D*). Because depsipeptide is a substrate for P-gp, we expected a loss of caspase-3 activity in depsipeptide-treated CEM-P-gp cells. However, CEM-P-gp cells treated with SAHA or oxamflatin also show a strong decrease in caspase-3 activity, even though these cells are as sensitive to the drugs as the parental CEM cells. These data support our previous findings that P-gp can inhibit the activation of caspases by drugs that are not substrates for P-gp (29) and suggest that caspase-3 activation is not required for death induced by SAHA, depsipeptide, or oxamflatin.

Oxamflatin and Depsipeptide Do Not Require an Intact Death Receptor Pathway or zVAD-fmk-inhibitable Caspases to Induce Cell Death. A number of chemotherapeutic drugs, including the HDACIs apicidin and CBHA, have been reported to induce cell death via activation of the death receptor pathway (30, 31), whereas others, such as SAHA and butyrate, show no requirement for this pathway (10, 24). To determine whether oxamflatin or depsipeptide utilize this pathway to induce cell death, we used CEM cells expressing the cowpox virus protein CrmA, which inhibits caspase-8 and -10, effectively short circuiting the death receptor pathway. As shown in Fig. 4*A*, expression of CrmA had no effect on apoptosis induced by either oxamflatin or depsipeptide, indicating that they do not require a functional death receptor pathway to induce apoptosis. This result was confirmed in long-term clonogenic assays (Fig. 4*B*). As expected, cell death mediated by anti-Fas antibody was significantly inhibited in the CrmA-expressing cells in both short- and long-term cell death assays, whereas CrmA had no effect on SAHA-mediated death (Fig. 4*A*; Ref. 10). Thus, similarly to SAHA, oxamflatin and depsipeptide do not require activation of the death receptor pathway for their cytotoxic action.

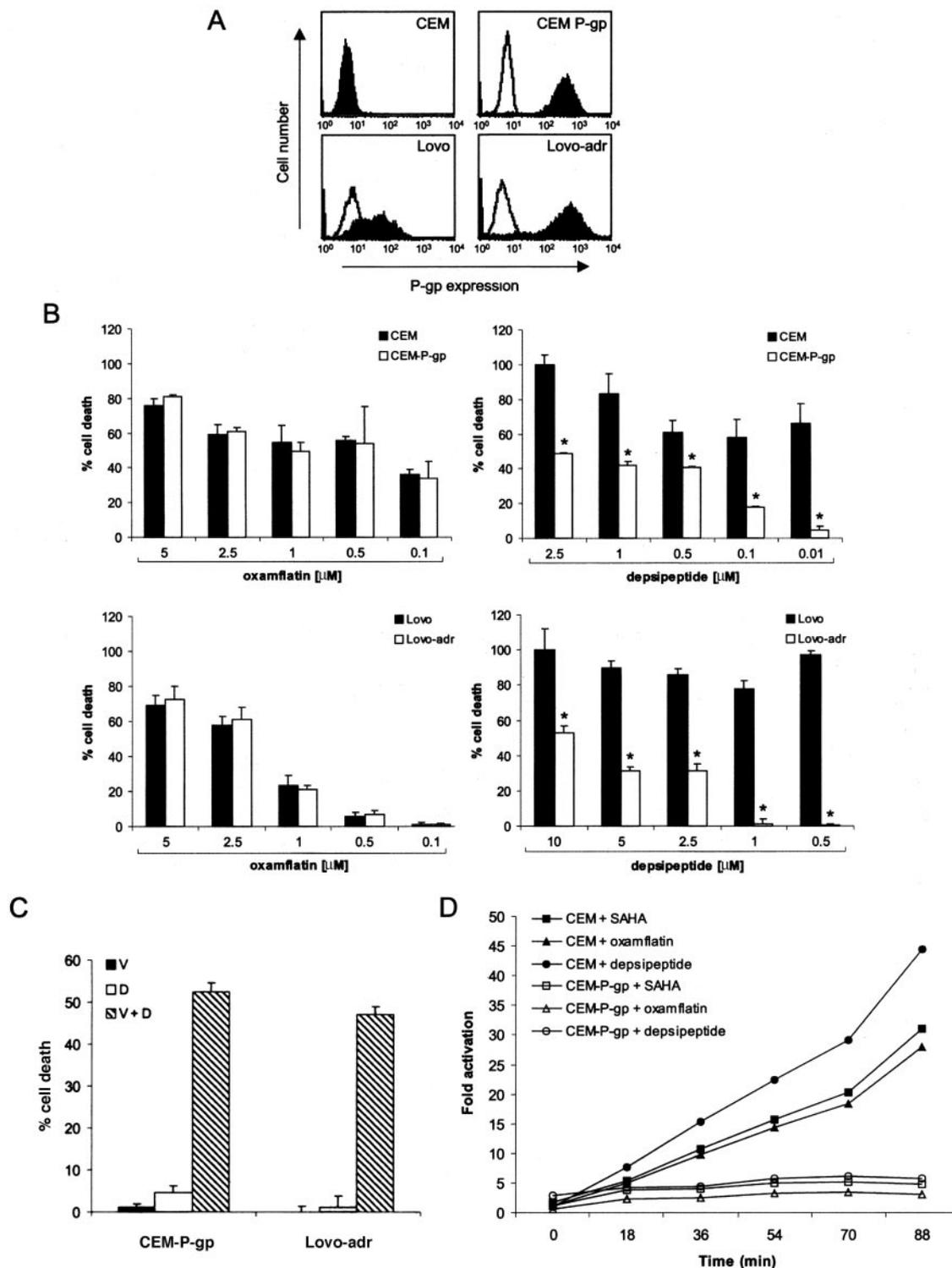We next determined whether oxamflatin and depsipeptide were

Fig. 3. Oxamflatin, but not depsipeptide, induces equivalent death of P-gp[+] and P-gp[−] tumor cell lines. *A*, CEM and Lovo cell lines and their P-gp-expressing partner lines CEM-P-gp and Lovo-adr were examined for expression of P-gp by flow cytometry using MRK 16 mAb (*filled histogram*) or an isotype control antibody (*empty histogram*). *B*, P-gp[+] and P-gp^{−} cells were treated with various concentrations of oxamflatin or depsipeptide for 24 h, and cell death was assessed by ^{51}Cr release. Data shown are representative of at least three separate experiments. In these and other assays, statistical differences between samples ($P < 0.05$) were determined using the Mann-Whitney U test and are denoted by an *asterisk*. *C*, CEM-P-gp and Lovo-adr cells were treated with 1 (*CEM-P-gp*) or 0.5 μM (*Lovo-adr*) depsipeptide (*D*) for 24 h in the presence or absence of verapamil (*V*), and cell death was measured by ^{51}Cr release. *D*, cytosolic lysates from CEM and CEM-P-gp cells treated with 2.5 μM SAHA, 0.5 μM depsipeptide, or 1.0 μM oxamflatin for 24 h were prepared in the absence of protease inhibitors. Lysates were incubated with the caspase-3 substrate Ac-DEVD-pNA for the given period of time, and substrate cleavage measured by change in absorbance. Relative fold activity was determined by dividing absorbance readings of samples containing drug by absorbance of control untreated samples.

able to induce death in the presence of the poly-caspase inhibitor zVAD-fmk. We have previously shown that although caspases are activated after treatment of CEM cells with SAHA, zVAD-fmk does not inhibit SAHA-induced cell death (10). In addition, the results shown in Fig. 3 indicate that caspase-3 activity is not necessary for SAHA- or oxamflatin-induced death. CEM cells were pretreated with zVAD-fmk or the cathepsin B inhibitor zFA-fmk for 1 h before the addition of oxamflatin, depsipeptide, SAHA, or anti-Fas antibody as

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Fig. 4. Oxamflatin- and depsipeptide-induced cell death is not mediated by death receptors. *A*, CEM cells expressing CrmA sense (*CEM-CrmA*) or antisense (*CEM-CrmA/AS*) cDNA were treated with the indicated concentrations of oxamflatin or depsipeptide, 2.5 µM SAHA, or 100 ng/ml anti-Fas antibody for 24 h, and cell death was examined by ^{51}Cr release. Data shown are representative of at least three separate experiments. *B*, after incubation with 0.5 to 0.01 µM oxamflatin, 10 to 1 nM depsipeptide, or 100 ng/ml anti-Fas antibody for 24 h, cells were plated out in soft agar. Colonies were counted after 10 days in culture.
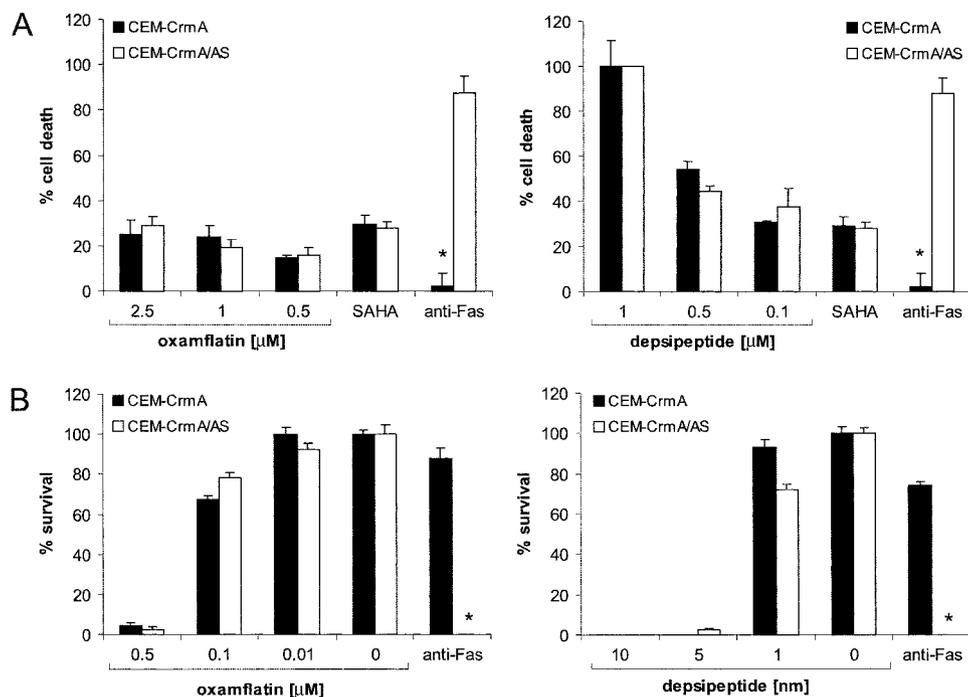
a control, and cell death was then assessed by Annexin V staining (Fig. 5*A*). Inhibition of caspase activity by zVAD-fmk had no effect on the ability of oxamflatin, depsipeptide, or SAHA to induce apoptosis, and the effectiveness of zVAD-fmk in inhibiting caspase activation in our assay systems was confirmed because Fas-mediated apoptosis was significantly inhibited in the presence of this inhibitor. Similar results were also obtained by trypan blue exclusion (data not shown) and clonogenic assays (Fig. 5*B*). Taken together with the results seen in Figs. 3 and 4 and previous published data (10, 29), these data indicate that although oxamflatin, depsipeptide, or SAHA do induce caspase activation, the activity of caspases that are blocked by zVAD-fmk are not necessary for the cytotoxic activities of these drugs.

HDACIs Induce Mitochondrial Membrane Depolarization and Cytochrome c Release That Is Blocked by Overexpression of Bcl-2. Because the death receptor pathway was not required for oxamflatin- or depsipeptide-mediated cell death, we next evaluated the importance of mitochondrial membrane disruption for HDACI-induced death using CEM cells overexpressing Bcl-2, which blocks the intrinsic apoptotic pathway. CEM-Bcl-2 and parental CEM cells were treated with SAHA, oxamflatin, or depsipeptide for 24 h, and mitochondrial membrane disruption was assessed by analysis of ΔΨm and release of cytochrome *c* from the mitochondria into the cytosol. The overexpression of Bcl-2 in CEM-Bcl-2 cells was confirmed by intracellular fluorescence-activated cell sorting (data not shown). As shown in Fig. 6*A*, treatment of cells with oxamflatin, depsipeptide, or SAHA induced a loss of TMRE staining indicative of mitochondrial membrane disruption and a loss of mitochondrial transmembrane potential. The effect of these HDACIs on ΔΨm was completely inhibited by overexpression of Bcl-2. The activation of the intrinsic apoptotic pathway by oxamflatin, depsipeptide, and SAHA was confirmed by Western blotting to assess the release of cytochrome *c* from the mitochondria into the cytosol. As expected, all three HDACIs induced the release of mitochondrial cytochrome *c* that was effectively blocked by overexpression of Bcl-2 (Fig. 6*B*).

The induction of cell death in the presence of zVAD-fmk is not unprecedented, and this is usually considered to be a consequence of

the effect of mitochondrial dysfunction that is not inhibited by zVAD-fmk (1). Consistent with this, we demonstrated that HDACI-induced cytochrome *c* release could occur in the presence of zVAD-fmk (Fig. 6*C*). By contrast, the release of cytochrome *c* mediated by cross-linking of Fas was inhibited by zVAD-fmk (Fig. 6*C*). Taken together, these data indicate that oxamflatin, depsipeptide, and SAHA can perturb the mitochondrial membrane that can be blocked by overexpression of Bcl-2 but not by the inhibition of caspase activation using zVAD-fmk.

Overexpression of Bcl-2 Inhibits Oxamflatin- and Depsipeptide-induced Cell Death but not Cell Cycle Arrest. To determine the role of mitochondrial membrane perturbation in death induced by HDACIs, CEM-Bcl-2 and parental CEM cells were treated with SAHA, oxamflatin, or depsipeptide for 24 h, and cell death was measured by ^{51}Cr release. Overexpression of Bcl-2 significantly inhibited death mediated by oxamflatin and depsipeptide (Fig. 7*A*) over various doses of drug, indicating that mitochondrial membrane perturbation is required for death induced by these drugs. As seen previously (10), overexpression of Bcl-2 also inhibited SAHA-induced cell death. Interestingly, although Bcl-2 increased survival of drug-treated cells in short-term assays, the clonogenic potential of the CEM-Bcl-2 cells was diminished (Fig. 7*B*). PI staining of the HDACI-treated CEM-Bcl-2 cells revealed that although the cells were less sensitive to DNA damage induced by HDACIs the effects on the cell cycle, marked by accumulation of cells with a 4n DNA content, were not affected (Fig. 7*C*), suggesting that the cytostatic effects of these drugs cannot be blocked by Bcl-2. Because clonogenic assays are a measure of both cell survival and proliferation, it is likely that the alteration in cell cycle progression is responsible for the loss of clonogenicity of the CEM-Bcl-2 cells.

HDACIs Induce the Processing of Caspase-2. The studies outlined above indicated that the release of cytochrome *c* from the mitochondria and loss of cell viability after treatment with SAHA, oxamflatin, or depsipeptide did not require the activation of caspase-3 or other caspases that can be effectively inhibited by zVAD-fmk. It has been shown that caspase-2 is poorly inhibited by zVAD-fmk and that caspase-2 can function as an initiating caspase to activate the
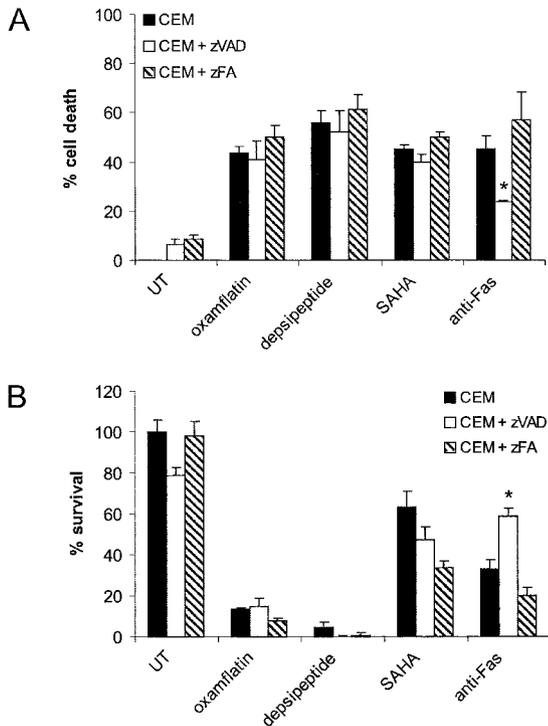
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Fig. 5. zVAD-fmk does not inhibit HDACI-induced cell death. *A*, CEM cells were untreated (*UT*) or treated with 2.5 μM oxamflatin, 1 μM depsipeptide, 2.5 μM SAHA, or 100 ng/ml anti-Fas antibody for 18 h. In some wells, cells were pretreated for 1 h with 20 μM zVAD-fmk (*zVAD*) or zFA-fmk (*zFA*). Cells were stained with Annexin V and analyzed by flow cytometry. The percentage of Annexin V-positive cells is presented, and data are representative of three separate experiments. *B*, after incubation with 0.5 μM oxamflatin, 5 nM depsipeptide, 0.5 μM SAHA, or 0.5 ng/ml anti-Fas antibody in the presence or absence of 20 μM zVAD-fmk of zFA-fmk for 24 h, cells were plated out in soft agar. Colonies were counted after 10 days in culture.

intrinsic apoptotic pathway and induce cell death (32). Furthermore, it has been hypothesized that caspase-2 may additionally target proteins downstream of the mitochondria to more directly induce apoptosis (33). We therefore determined whether HDACIs induced the cleavage and activation of caspase-2. Treatment of CEM cells with SAHA (Fig. 8*A*), oxamflatin (Fig. 8*B*), or depesipeptide (Fig. 8*C*) resulted in the processing of pro-caspase-2 (p48) to produce the intermediate (p33) and active (p19) proteins. Interestingly, the production of the p33 form of caspase-2 still occurred after treatment of CEM-Bcl-2 cells with SAHA, oxamflatin, or depesipeptide; however, further cleavage to produce p19 caspase-2 was inhibited by Bcl-2. These data demonstrate that the initiation of caspase-2 activation after treatment with SAHA, oxamflatin, or depsipeptide can occur upstream of the mitochondria, indicating that caspase-2 processing may be an important step for the induction of cell death after treatment with these drugs.

Cleavage and Activation of Bid in Response to HDACIs. We have previously shown that SAHA induced Bid cleavage in CEM cells in the presence of zVAD-fmk and that Bid can be cleaved upstream of mitochondrial membrane perturbation (10). To determine whether oxamflatin and depsipeptide also induce Bid cleavage, CEM cells were treated for 24 h with these drugs alone and in the presence of zVAD-fmk or zFA-fmk, and whole cell lysates were analyzed by Western blot. As shown in Fig. 9*A*, all three HDACIs induced the cleavage of Bid to its characteristic M_r 16,000 activated tBid form. The molecular weight of tBid produced in response to the HDACIs was indistinguishable from that produced after addition of anti-Fas antibody. Cleavage of Bid by SAHA and oxamflatin was not affected by zVAD-fmk (Fig. 9*A*); however, depsipeptide-induced Bid cleavage was significantly attenuated after the addition of zVAD-fmk, to an

extent similar to that seen following the cross-linking of Fas in the presence of zVAD-fmk. This suggests that depsipeptide may be more reliant on caspases to cleave Bid than are oxamflatin and SAHA. As expected, Fas-induced Bid cleavage was inhibited by zVAD-fmk. The effectiveness of zVAD-fmk in inhibiting caspase activity at this time point and concentration was demonstrated by Western blotting for cleavage of the prototypic caspase substrate PARP. PARP was cleaved after treatment of cells with SAHA, oxamflatin, depsipeptide, and Fas, both alone and in the presence of zFA-fmk; however, cleavage was significantly inhibited after treatment with all HDACIs or with anti-Fas antibodies when zVAD-fmk was present (Fig. 9*A*).

To determine whether HDACI-induced Bid cleavage occurred upstream of Bcl-2, lysates were prepared from CEM and CEM-Bcl-2 cells treated for 24 h with oxamflatin, depsipeptide, or SAHA and analyzed for Bid cleavage by Western blot (Fig. 9*B*). Treatment with increasing doses of all three HDACIs induced cleavage of Bid in a dose-dependent manner in both the CEM and CEM-Bcl-2 cells, as shown by loss of the M_r 22,000 pro-form and an increase in the M_r 16,000 tBid cleavage product. Thus, it appears that Bid is cleaved upstream of mitochondria membrane disruption by these drugs. As expected, there was less processing of Bid in the CEM-Bcl-2, compared with CEM cells, because overexpression of Bcl-2 would sup-
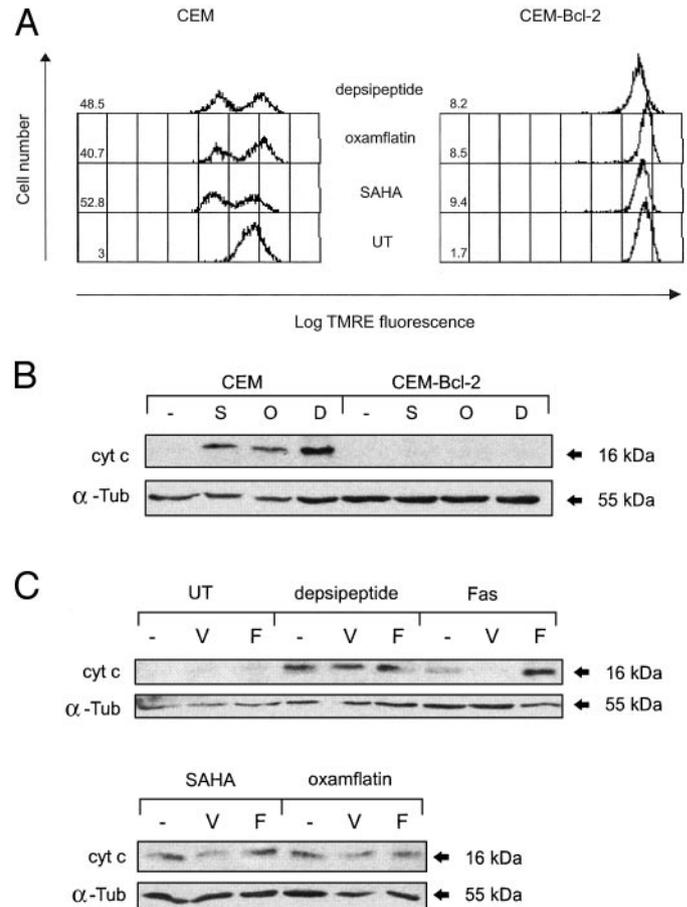

Fig. 6. HDACIs induce mitochondrial membrane perturbation that is inhibited by Bcl-2 but not by zVAD-fmk. *A*, CEM and CEM-Bcl-2 cells were untreated (*UT*) or incubated with 2.5 μM oxamflatin, 1 μM depsipeptide or 2.5 μM SAHA for 24 h. Cells were stained with TMRE (20 nM) and analyzed by flow cytometry. The percentage of cells with reduced $\Delta\Psi$m is indicated. *B*, Western blots of cytosolic extracts from CEM and CEM-Bcl-2 cells incubated with 2.5 μM oxamflatin (*O*), 1 μM depsipeptide (*D*), or 2.5 μM SAHA (*S*) for 24 h were probed with mAbs to cytochrome *c* (*cyt c*) and α-tubulin. *C*, Western blots of cytosolic extracts from CEM cells incubated with 2.5 μM oxamflatin, 1 μM depsipeptide, 2.5 μM SAHA, or 10 ng/ml anti-Fas mAb in the absence (−) or presence of zVAD-fmk (*V*) or zFA-fmk (*F*) for 24 h were probed with mAbs to cytochrome *c* (*cyt c*) and α-tubulin.
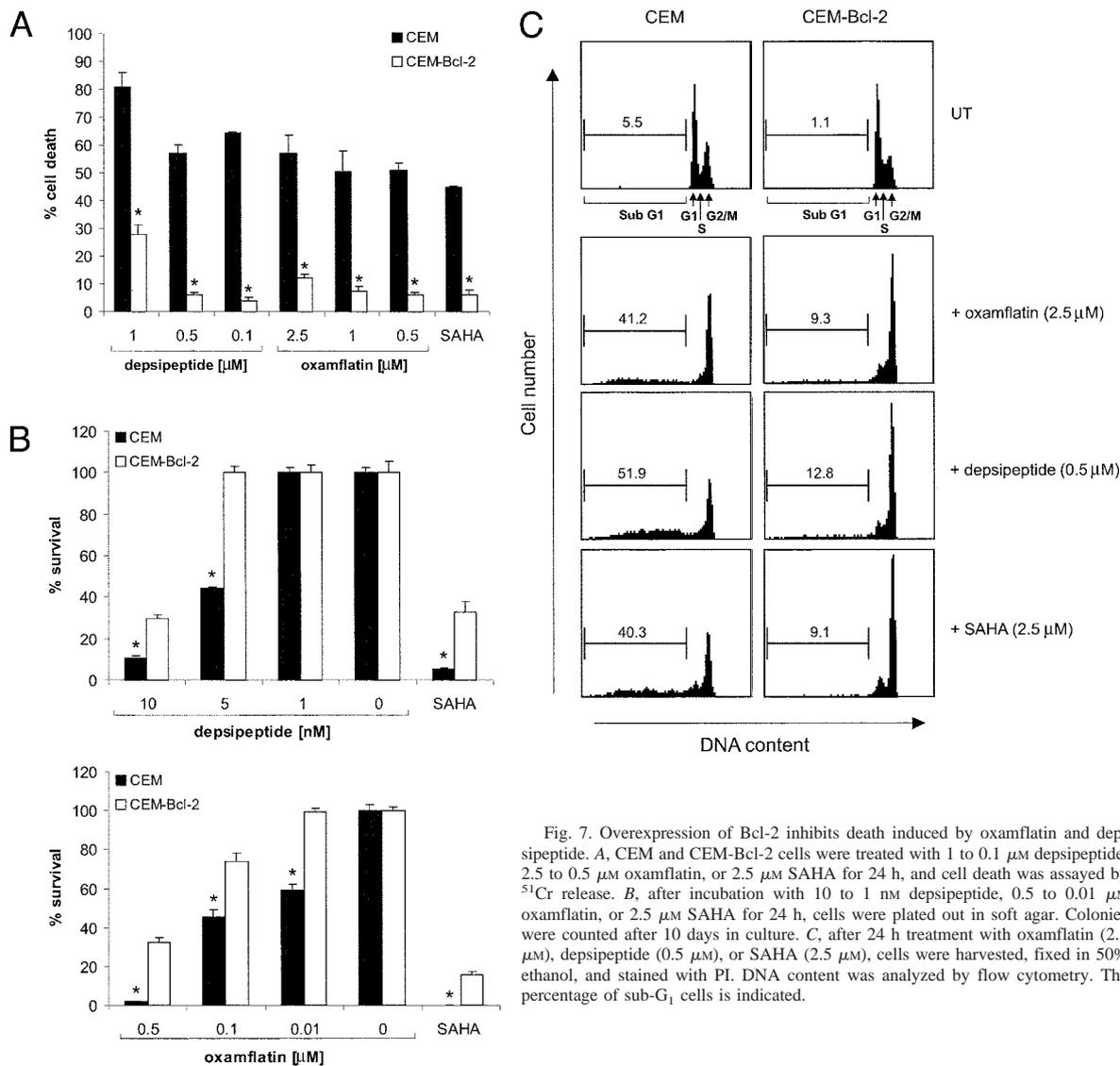
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Fig. 7. Overexpression of Bcl-2 inhibits death induced by oxamflatin and depsipeptide. *A*, CEM and CEM-Bcl-2 cells were treated with 1 to 0.1 μM depsipeptide, 2.5 to 0.5 μM oxamflatin, or 2.5 μM SAHA for 24 h, and cell death was assayed by ^{51}Cr release. *B*, after incubation with 10 to 1 nM depsipeptide, 0.5 to 0.01 μM oxamflatin, or 2.5 μM SAHA for 24 h, cells were plated out in soft agar. Colonies were counted after 10 days in culture. *C*, after 24 h treatment with oxamflatin (2.5 μM), depsipeptide (0.5 μM), or SAHA (2.5 μM), cells were harvested, fixed in 50% ethanol, and stained with PI. DNA content was analyzed by flow cytometry. The percentage of sub-G_1 cells is indicated.

press the release of caspase-activating molecules such as cytochrome *c*, HtrA2, and Smac/DIABLO from the mitochondria, thereby inhibiting Bid cleavage after secondary caspase activation.

***De Novo* Protein Synthesis Is Required for Oxamflatin- and Depsipeptide-induced Apoptosis.** HDACIs induce histone acetylation, resulting in chromatin remodeling and new gene transcription. We have shown previously that *de novo* protein synthesis is necessary for SAHA-induced cell death, and we therefore determined whether alteration of gene expression is necessary for the antitumor activities of oxamflatin and depsipeptide. Pretreatment of cells with 5, 50, or 500 ng/ml CHX had no effect on cell cycle progression of CEM cells over the 24-h time course of this experiment (data not shown). However, treatment of cells with CHX for 1 h before the addition of oxamflatin, depsipeptide, or SAHA significantly inhibited the ability of the drugs to affect the cell cycle (data not shown) and induce apoptosis (Fig. 10*A*), suggesting that oxamflatin and depsipeptide require new protein synthesis to exert their antitumor effects. The effectiveness of CHX in inhibiting protein synthesis was demonstrated by Western blotting for proteins with short half-lives (*i.e.* p27; data not shown).

We have demonstrated that CHX inhibits SAHA-induced Bid cleavage (10). Importantly, treatment with CHX also inhibited the cleavage and activation of Bid in response to depsipeptide or oxam-

flatin (Fig. 10*B*). These data are consistent with our previous studies demonstrating the importance of Bid cleavage in SAHA-induced apoptosis (10). Although depsipeptide-induced Bid cleavage was partially inhibited by zVAD-fmk, whereas the poly-caspase inhibitor did not affect cleavage of Bid by SAHA and oxamflatin, all three drugs required new protein synthesis to mediate Bid cleavage and cell death.

DISCUSSION

HDACIs are a novel and promising class of chemotherapeutic agent that can induce apoptosis and differention, inhibit cell cycle progression, and possess antiangiogenic and immune stimulatory properties (3, 4). Despite numerous studies demonstrating these activities in a range of tumor cell lines and animal models, little progress has been made in understanding the molecular mechanisms underlying their action. A number of HDACIs are currently being tested in early-phase clinical trials against a variety of cancers, and promising results are being reported, supporting the development of these compounds for clinical use. We are particularly interested in understanding the molecular mechanisms of HDACI-induced apoptosis. Previous studies by our laboratory with the hydroxamic acid-based HDACI SAHA led to the identification of a novel apoptotic pathway activated in response to this drug and the finding that SAHA could kill P-gp-expressing
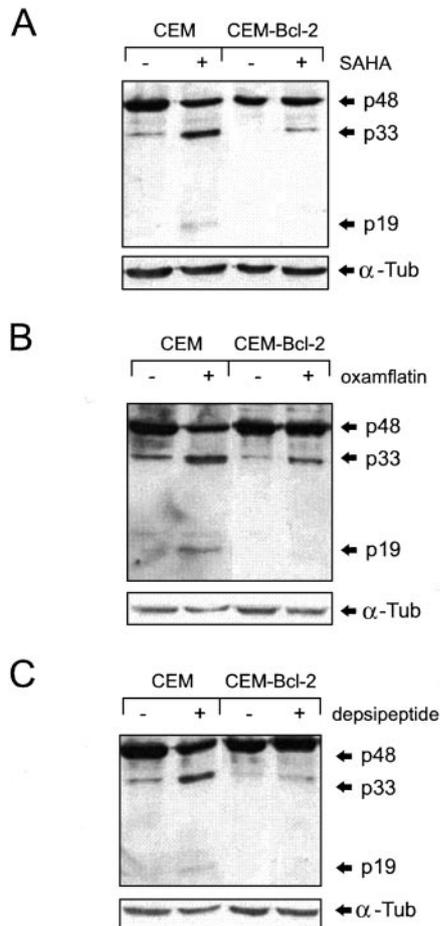
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Fig. 8. HDACI-induced cleavage of caspase-2. Western blots of whole cell lysates from CEM and CEM-Bcl-2 cells treated with 1 μM depsipeptide, 2.5 μM oxamflatin, or 2.5 μM SAHA for 24 h were probed with anti-caspase-2 and anti-α-tubulin mAb. The presence of pro-caspase-2 (p48), processed intermediate caspase-2 (p33), and active caspase-2 (p19) is indicated.

determinant responsible for this arrest (37, 38). The molecular events leading to the effect of HDACIs on cells during or after DNA replication have not yet been defined. It is possible that histone modification during DNA replication and cell division may be important for HDACI function. Histone acetylation in heterochromatin is tightly regulated during S phase, and disruption of this process triggers cell cycle arrest within G_2-M (39). The HDACI-associated accumulation of cells with a 4n DNA content may be related to hyperacetylation of the centromere, allowing release of heterochromatin proteins resulting in abnormal chromosomal segregation (40). In cancer cells, a proposed HDACI-associated G_2 checkpoint may be lost, leading to aberrant mitoses and apoptosis (34). Hence, it is formally possible that dysregulated histone acetylation during replicative S phase and/or mitosis may play an important role in the induction of HDACI-mediated cell death.

Progression through the G_1-S checkpoint was necessary for the induction of apoptosis by SAHA, oxamflatin, and depsipeptide because CEM cells overexpressing p16[INK4A] and arrested in G_1 were resistant to HDACI-induced death. A previous study using these p16[INK4A]-expressing CEM cells similarly reported that butyrate-induced cell death was abrogated by forced G_1 arrest (24). In contrast to these studies, Burgess et al. (41) demonstrated that melanoma cells arrested at G_1 using a similar inducible p16[INK4A] system remained sensitive to the HDACI azelaic bishydroxamic acid. However, we
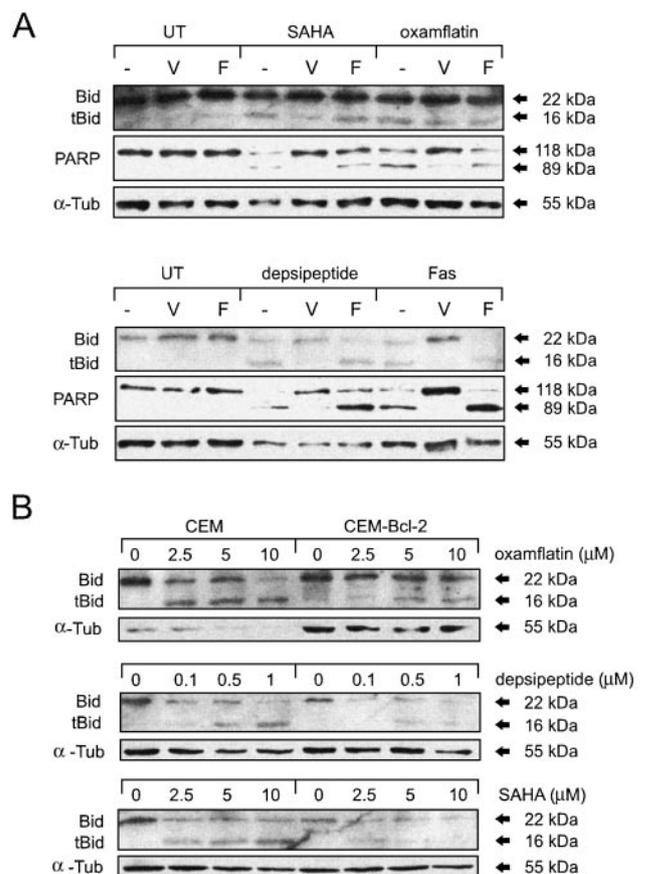

Fig. 9. Caspase-dependent and independent cleavage of Bid following treatment with HDACIs. A, total cell lysates (15 μg) prepared from CEM cells treated with 2.5 μM SAHA, 2.5 μM oxamflatin, 1 μM depsipeptide, or 10 ng/ml anti-Fas antibody for 18 h in the absence or presence of 20 μM zVAD-fmk (V) or zFA-fmk (F) were separated by 15% SDS-PAGE and analyzed by Western blotting using antibodies for Bid, PARP, and α-tubulin. Arrows indicate the positions of full-length Bid (M_r 22,000) and its p16 tBid cleavage product, as well as that of full-length PARP (M_r 118,000) and its M_r 89,000 cleavage product. B, CEM and CEM-Bcl-2 cells were untreated or treated with 2.5–10 μM oxamflatin, 0.1–1 μM depsipeptide or 2.5–10 μM SAHA for 24 h. Lysates were prepared and analyzed by Western blot as described above.

MDR cells (10, 29). However the question still remained as to whether other HDACIs activated a similar apoptotic pathway to induce cell death, and whether they were similarly effective against P-gp-expressing cells. In the present study, we used another hydroxamic acid-based HDACI, oxamflatin, and the structurally unrelated HDACI, depsipeptide, to answer these questions.

We found that all three HDACIs were comparable in their ability to alter the cell cycle, resulting in an accumulation of cells with a 4n DNA content, and all induced apoptosis via activation of the intrinsic apoptotic pathway, which was accompanied by the cleavage and activation of caspase-2 and Bid. However, differences emerged with relation to the protease(s) responsible for Bid cleavage induced by these drugs because zVAD-fmk inhibited Bid cleavage induced by depsipeptide, but not that induced by oxamflatin or SAHA. Furthermore, oxamflatin and SAHA, but not depsipeptide, were able to kill P-gp-expressing MDR cells.

Treatment of cells with oxamflatin, depsipeptide, or SAHA induced two cellular responses: (a) accumulation of cells with a 4n DNA content at what has been termed a G_2 cell cycle checkpoint (34) and (b) induction of apoptosis. With time, those cells that initially accumulate with a 4n DNA content also undergo apoptosis. A number of studies have demonstrated that the cell cycle inhibitor p21[WAF1/CIP1] is commonly induced in response to HDACIs (35, 36) and plays an important role in the G_1 cell cycle arrest of many cancer cells, although it is clear from studies with p21^{−/−} cells that it is not the sole
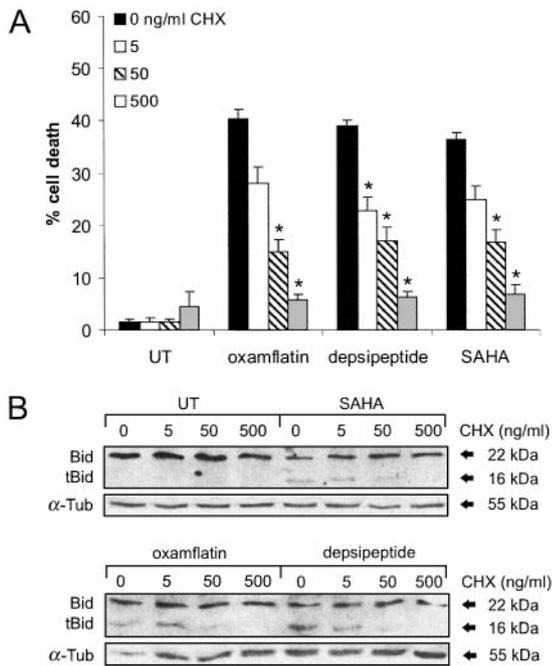
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Fig. 10. Inhibition of protein synthesis abrogates HDACI-induced apoptosis and Bid cleavage. CEM cells were pretreated for 1 h in the presence or absence of 5, 50, or 500 ng/ml CHX and then treated with 2.5 μM oxamflatin, 0.5 μM depsipeptide, or 2.5 μM SAHA. *A*, after 24 h, cells were assayed for death by trypan blue exclusion. Data shown are representative of three separate experiments. *B*, whole cell lysates were assessed for Bid cleavage and α-tubulin protein expression as a loading control by Western blotting.

have shown that when p16[INK4A]-expressing CEM cells were treated with azelaic bishydroxamic acid, death was inhibited to a similar extent to that seen with the HDACIs investigated in this study,[4] suggesting that these differences may relate to cell type. Interestingly, other chemotherapeutic drugs, including vincristine, staurosporine, and etoposide, were also ineffective in killing these p16[INK4A]-expressing CEM cells, whereas anti-Fas antibody could kill the cells to the same extent as cycling cells (data not shown), demonstrating that these cells are not resistant to all death stimuli and that diverse chemotherapeutic drugs rely on the ability of cells to cycle to exert their effects.

Apoptosis mediated by oxamflatin, depsipeptide, and SAHA did not require activation of the death receptor pathway. Likewise, another HDACI, sodium butyrate, has also been shown to induce apoptosis independently of this pathway in CEM cells (24). Interestingly, butyrate did not alter the expression of either the CD95 receptor or ligand (CD95L) in these cells but enhanced sensitivity to CD95-mediated apoptosis (42). These results are in contrast to studies with the HDACIs CBHA and apicidin, which have been reported to induce the expression of CD95/CD95L in neuroblastoma and promyelocytic leukemia cells, respectively, thereby activating the death receptor pathway (30, 31). Moreover, apicidin-induced apoptosis was abrogated when CD95 signaling was inhibited (30). Thus, the molecular pathway(s) initiated in response to HDACIs may be related to intrinsic characteristics of the particular cell type. Alternatively, it is possible that structurally similar HDACIs may preferentially target different HDACs and therefore initiate diverse molecular pathways to induce cell death.

With its broad substrate specificity, P-gp can efflux a wide variety of compounds, thereby conferring resistance to structurally diverse and functionally unrelated chemotherapeutic agents (43). In addition

to lowering intracellular drug concentration, P-gp can protect cells against caspase-dependent forms of apoptosis by inhibiting caspase activation after chemotherapeutic drug treatment (29). Consistent with this, we showed here that HDACI-induced caspase-3 activation was inhibited in P-gp-expressing tumor cells. The poly-caspase inhibitor zVAD-fmk did not attenuate killing induced by oxamflatin, depsipeptide, or SAHA, demonstrating the ability of these drugs to kill in the absence of the activation of certain caspases. Therefore, all three HDACIs had the potential to kill P-gp-expressing MDR cells. However, we found that whereas oxamflatin and SAHA induced equivalent death of P-gp-expressing and non-P-gp-expressing cells, depsipeptide was a substrate for P-gp and was therefore less effective against these cells. Our data and those of others (12, 27) indicate that the anticancer activities of depsipeptide may be somewhat diminished against P-gp-expressing MDR tumors. A recent report demonstrating that depsipeptide is a prodrug and is activated by glutathione-mediated reduction after cellular uptake indicates that this agent may be effective in killing tumors that have become resistant to agents such as cisplatin that are inhibited by high glutathione levels (44). Whether depsipeptide is a substrate for the drug efflux pump MRP1, which extrudes glutathione-conjugated xenotoxins from tumor cells, remains to be determined.

The poly-caspase inhibitor zVAD-fmk can inhibit the activity of most caspases; however, it is a poor inhibitor of caspase-2 (13). In addition, caspase-2 has recently been demonstrated to be an important initiator caspase, capable of activating the intrinsic apoptotic pathway in response to DNA damage (32), and recombinant caspase-2 can cleave and activate Bid *in vitro* (45). It is therefore possible that caspase-2 can mediate the cleavage of Bid induced by SAHA or oxamflatin and/or may be responsible for the induction of cell death induced by all three HDACIs in the presence of zVAD-fmk. SAHA, oxamflatin, and depsipeptide activated caspase-2 consistent with a putative role of this caspase in HDACI-mediated cell death. However, full processing and activation of caspase-2 were inhibited in CEM-Bcl-2 cells, whereas the cleavage of Bid still occurs in these cells, although to a lesser degree. Thus, active caspase-2, produced after treatment of cells with HDACIs, may cleave Bid; however, it appears that fully processed caspase-2 is not necessary for Bid activation. Whether the p33 intermediate form of caspase-2 is capable of cleaving Bid needs to determined following a thorough biochemical analysis. In addition, the molecular processes necessary for the activation and cleavage of caspase-2 in response to HDACI treatment also remain to be elucidated.

A number of studies have demonstrated that overexpression of Bcl-2 protects cells against diverse apoptotic stimuli that induce death via the intrinsic apoptotic pathway (1). Bcl-2 overexpression inhibited the reduction of $\Delta\Psi$m, release of mitochondrial cytochrome *c*, and subsequent apoptosis induced by oxamflatin, depsipeptide, and SAHA, suggesting that mitochondrial membrane disruption is important for HDACI-induced apoptosis of CEM cells. The proapoptotic BH3-only Bcl-2 family member Bid can activate the intrinsic apoptotic pathway and is involved in apoptosis mediated by SAHA (10). We found that Bid was also cleaved after treatment of cells with oxamflatin or depsipeptide, even in cells overexpressing Bcl-2, placing HDACI-induced Bid cleavage upstream of mitochondrial membrane perturbation. Interestingly, however, zVAD-fmk inhibited Bid cleavage in response to depsipeptide, but not oxamflatin or SAHA, suggesting that caspases may be primarily responsible for mediating Bid cleavage by depsipeptide, whereas an as yet unidentified protease may be activated and/or induced by oxamflatin and SAHA to cleave Bid. Identification of the protease responsible for the processing of Bid by each of these drugs should aid in dissecting the upstream signaling pathways leading to mitochondrial membrane disruption

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[4] M. J. Peart, A. A. Ruefli, and R. W. Johnstone, unpublished observations.

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triggered by these drugs. Interestingly, the accumulation of cells with a 4n DNA content by HDACIs was not affected by Bcl-2 overexpression, suggesting that HDACI-induced cell cycle alterations occur independently or upstream of mitochondrial membrane disruption. In addition to its role as an antiapoptotic protein, Bcl-2 has a separate function as a cell cycle inhibitor (46). Overexpression of Bcl-2 has been reported to delay transition from the M to G_1 phase (47) and prolong the G_0-G_1 phase (48). Movement through the cell cycle is required for HDACI-induced apoptosis; thus, in addition to its ability to inhibit the release of proapoptotic proteins from the mitochondria, the antiproliferative property of Bcl-2 could contribute to its protective effect against HDACI-induced apoptosis.

Our study demonstrated that oxamflatin, depsipeptide, and SAHA required *de novo* protein synthesis to induce their antitumor activities, supporting the notion that HDACIs mediate their effects through regulation of new gene expression. Similar results have been reported with other HDACIs, including butyrate, trichostatin A, CBHA, apicidin, and depudecin (30, 31, 49, 50). The induction of new gene expression by HDACIs is presumed to be mediated primarily through the inhibition of histone deacetylases, and whereas histones may be their primary targets, non-histone proteins (*i.e.* p53) can be acetylated and are therefore potential targets for HDACIs (3, 4). Thus it is unclear whether the cytostatic and cytotoxic effects of HDACIs are mediated through histone acetylation, thereby directly affecting gene expression, or by acetylation of other proteins that also require new gene expression to mediate their effects. Interestingly, we found depsipeptide to be a significantly more potent agent against P-gp⁻ cells compared with oxamflatin and SAHA. In addition, Bid cleavage after depsipeptide treatment was inhibited by zVAD-fmk, whereas oxamflatin- and SAHA-mediated Bid cleavage was unaffected by the poly-caspase inhibitor. These data highlight functional differences between these structurally dissimilar compounds that are thought to have the same intracellular targets.

These and our other recent studies (10, 29) identify novel mechanisms of apoptosis induced by HDACIs. Our findings demonstrate that oxamflatin, depsipeptide, and SAHA require activation of a number of common molecular pathways to induce both growth arrest and apoptosis. However, these HDACIs differed in their ability to kill P-gp-expressing cells, which is related to structural characteristics of the drugs, and in the molecular events triggered upstream of Bid cleavage and mitochondrial membrane disruption. This illustrates the value of profiling the mechanism of action of individual agents, information that will be important for the improvement of existing therapies. Ongoing studies in our laboratory are using microarray analysis to examine the gene expression changes induced in response to these drugs to identify key pathways and components linking histone deacetylase inhibition to cell cycle arrest and apoptosis induction to further elucidate the underlying mechanisms of action of HDACIs.

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