The histone deacetylase inhibitor suberic bishydroxamate regulates the expression of multiple apoptotic mediators and induces mitochondria-dependent apoptosis of melanoma cells

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Abstract

Histone deacetylase (HDAC) inhibitors have attracted much interest because of their ability to arrest cell growth, induce cell differentiation, and in some cases, induce apoptosis of cancer cells. In the present study, we have examined a new HDAC inhibitor, suberic bishydroxamate (SBHA), for its effect on a panel of human melanoma cell lines. We report that it induces varying degrees of apoptosis in the melanoma lines but not in melanocytes and fibroblasts. Induction of apoptosis was caspase dependent and was associated with induction of changes in mitochondrial membrane permeability, which could be inhibited by overexpression of Bcl-2. The changes in mitochondria were independent of caspase activation and were associated with changes in conformation of Bax. SBHA down-regulated several key antiapoptotic proteins including X-linked inhibitor of apoptosis and the Bcl-2 family proteins, Bcl-X_L and Mcl-1. In contrast, it induced up-regulation of the Bcl-2 family proapoptotic proteins, Bim, Bax, and Bak. In addition, SBHA induced relocation of the protein Bim to mitochondria and its association with Bcl-2. *De novo* protein synthesis was required for initiation of apoptosis in that the protein synthesis inhibitor, cycloheximide, inhibited SBHA-induced conformational changes in Bax as well as changes in mitochondrial membrane permeability and activation of caspase-3. These results suggest that SBHA induces apoptosis by changing the balance between proapoptotic and antiapoptotic proteins in melanoma cells. The protein Bim may be a key initiator of apoptosis in cells treated with SBHA. [Mol Cancer Ther. 2004;3(4):425–435]

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Introduction

Histones are basic proteins that, by complexing with DNA, form nucleosomes leading to the compact structure of chromatin. Basic amino acids of the histones can be post-translationally modified with methyl, acetyl, or phosphate groups. The balance between histone acetylation and deacetylation regulated by histone acetyltransferase and histone deacetylases (HDACs) plays an important role in transcriptional regulation of genes (1–5). Acetylation of lysine residues of histones results in more open chromatin structure and activation of transcription, whereas hypoacetylation of histones is associated with a condensed chromatin structure resulting in the repression of gene transcription (4, 5). Inhibition of HDAC activity by HDAC inhibitors results in the accumulation of acetylated histones leading to altered transcription of several genes (6). This has been demonstrated to arrest cell growth (7–9) and to reverse neoplastic characteristics by inducing differentiation (10–13). In addition, HDAC inhibitors can induce apoptotic cell death in a variety of tumor cell lines (12, 14–16). HDAC inhibitors are therefore considered to be promising chemotherapeutic agents for treatment of malignant tumors. The potential significance of HDAC inhibitors as anticancer agents has been supported by studies in animal models and clinical trails showing antitumor activity with minor toxicity to normal tissues *in vivo* (17, 18).

Although several HDAC inhibitors have been shown to induce apoptosis of cultured tumor cells, the mechanism(s) underlying this appears to vary considerably. For example, explanations for the induction of apoptosis by the HDAC inhibitor sodium butyrate include alterations in Bcl-2 family protein expression (19–22), increased caspase activity (20), increased sensitivity to Fas/Fas ligand interaction (23, 24), and changes in the expression of genes such as c-*myc* and k-*ras* (25). Moreover, caspase-independent mechanisms have also been suggested as causative (21). It appeared that sodium butyrate-induced apoptosis was mediated through the mitochondrial pathway in that apoptosis was inhibitable by overexpression of Bcl-X_L (21). Studies with another HDAC inhibitor, apicidin, showed that this was associated with translocation of Bax to mitochondria and subsequent release of cytochrome *c* (26).

In the present study, we have examined the apoptosis-inducing potential of a new HDAC inhibitor, suberic bishydroxamate (SBHA), in human melanoma cell lines and dissected the SBHA-mediated apoptotic signaling pathway. We report that SBHA induces varying degrees of apoptosis in the majority of melanoma cell lines, but not in melanocytes and fibroblasts, via a mitochondrion-dependent pathway that is associated with relocation of Bax to

mitochondria. SBHA down-regulated the antiapoptotic proteins Bcl-X_L, Mcl-1, and X-linked inhibitor of apoptosis (XIAP) but up-regulated the proapoptotic proteins Bim, Bak, and Bax and, to a lesser extent, Bid, caspase-8, and caspase-3. The BH3-only protein Bim translocated to mitochondria and was associated with Bcl-2 in cells treated with SBHA. Initiation of apoptotic signaling requires *de novo* protein synthesis in that the protein synthesis inhibitor cycloheximide (CHX) inhibited SBHA-induced changes in Bax and mitochondria, caspase-3 activation, and apoptosis. These results are consistent with induction of apoptosis by changing the balance between proapoptotic and antiapoptotic proteins in melanoma cells. Activation of the BH3-only protein Bim may be the initiating factor of apoptosis in cells treated with SBHA.

Materials and Methods

Cell Lines

Human melanoma cell lines Me4405, Me1007, IgR3, Mel-FH, Mel-RMu, Mel-RM, Mel-CV, and MM200 have been described previously (27, 28). Melanocytes were kindly provided by Dr. P. Parson (Queensland Institute of Medical Research, Brisbane, Queensland, Australia) and cultured in medium supplied by Clonetics (Edward Kellar, Victoria, Australia). Human lung fibroblasts MRC-5 were obtained from Bio Whittaker (Walkersville, MD) and cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia).

Antibodies, Recombinant Proteins, and Other Reagents

SBHA was a kind gift from Dr. Parson. It was dissolved in distilled water and made up in a stock solution of 10 mg/ml. The cell-permeable pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F (z-VAD-fmk), caspase-9 inhibitor Z-Leu-Glu(OMe)-His-Asp(OMe)-CH_2F (z-LEHD-fmk), and caspase-3 inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (z-DEVD-fmk) were purchased from Calbiochem (La Jolla, CA). The rabbit polyclonal antibodies against caspase-3, caspase-8, and Bid, the mouse monoclonal antibodies (mAbs) against cytochrome *c*, poly(ADP-ribose)polymerase (PARP), $p21^{WAF1/CIP1}$, $p27^{Kip1}$, and cyclin A, and the rabbit mAb against the active form of caspase-3 were purchased from PharMingen (Bioclone, Marrickville, New South Wales, Australia). The rabbit polyclonal antibodies against inhibitor of caspase-activated DNase (ICAD) and Bak and the mouse mAbs against Bcl-2, Bcl-X_L, Mcl-1, and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse mAb against XIAP was purchased from Transduction Laboratories (Lexington, KY). The rabbit polyclonal antibody against Smac/DIABLO was from Calbiochem. The rabbit polyclonal antibody against cleaved caspase-9 was purchased from New England Biolabs (Beverly, MA). The rabbit polyclonal anti-Bax against amino acids 1–20 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit polyclonal antibody against Bim was from Imgenex (San Diego, CA). The mAb against cytochrome *c* oxidase subunit 4 (COX IV) was purchased from Molecular Probes (Eugene, OR). Isotype control antibodies used were the ID4.5 (mouse IgG2a) mAb against *Salmonella typhi* supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, South Australia, Australia), the 107.3 mouse IgG1 mAb purchased from PharMingen (San Diego, CA), and the rabbit immunoglobulin from Sigma Chemical Co. (Castle Hill, New South Wales, Australia).

Plasmid Vector and Transfection

Stable Mel-RM transfectants of Bcl-2 were established by electroporation of the PEF-puro vector carrying human Bcl-2 provided by Dr. David Vaux (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia) and described elsewhere (28).

Flow Cytometry

Immunostaining on intact and permeabilized cells was carried out as described previously (27, 28).

Apoptosis

Quantitation of apoptotic cells by the propidium iodide method was carried out as described elsewhere (27, 28). 4′,6-Diamidino-2-phenylindole (DAPI) staining was performed according to the manufacturer's instructions (Molecular Probes). Melanoma cells were seeded onto sterile coverslips in 24-well plates (Becton Dickinson, Lane Cove, New South Wales, Australia) overnight. Cells with or without treatment with SBHA for a further 24 h were washed with PBS, fixed with 3.7% paraformaldehyde, and stained with DAPI (300 nM) for 5 min. Coverslips were mounted in Gel-Mount (Biomedia, Foster City, CA) and examined using a Zeiss Axiophot microscope (Oberkochem, Germany).

Mitochondrial Membrane Potential

Tumor cells were cultured in 24-well plates and allowed to reach exponential growth for 24 h before treatment. MitoTracker Red CMXRos (Molecular Probes) was added at 100 nM during the last 30 min of treatment. The medium was removed into a 75-mm Falcon polystyrene tube (Becton Dickinson, Sunnyvale, CA) and the adherent cells were trypsinized and collected into the same tube. After washing with PBS, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) for MitoTracker uptake. Untreated cells were used as controls.

Western Blot Analysis

Methods used were as described previously (27, 28). The densities of the Western blot bands in some experiments were quantitated on a Macintosh computer using the public domain NIH Image Program, which is available on the Internet (http://rsb.info.nih.gov/nih-image).

Immunoprecipitation

Methods used were as described previously with minor modification (28). Briefly, 100 μl of lysates were precleared by incubation with 20 μl of a mixture of protein A-Sepharose and protein G-Sepharose packed beads (Santa Cruz Biotechnology) in a rotator at 4°C for 2 h and then with 20 μl of fresh packed beads in a rotator at 4°C overnight. Twenty micrograms of anti-Bcl-2 mAb or control immunoglobulin was then added to the lysate and rotated at 4°C for 2 h. The beads were then pelleted by centrifugation and washed five times with ice-cold lysate

buffer before elution of the proteins from the beads in lysate buffer at room temperature for 1 h. The resulted immunoprecipitates were then subjected to SDS-PAGE and Western blot analysis.

Preparation of Mitochondrial and Cytosolic Fractions

Methods used for subcellular fraction were similar to the methods described previously (28).

Real-Time PCR

Total RNA was isolated with SV Total RNA Isolation System (Promega, Sydney, New South Wales, Australia). Reverse transcription-PCR (RT-PCR) was carried out using Moloney murine leukemia virus transcriptase and Oligo d(T) and the resulting cDNA products were used as templates for real-time PCR assays. Real-time RT-PCR was performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Scoresby, Victoria, Australia). Twenty-five microliters of mixture were used for reaction, which contains 5 μl cDNA sample (0.5–1 μg/μl), 300 nM forward primers for Bcl-X_L (GGTCGCAT TGTGGCCTTT) or Bax (TGGAGCTGCAGAGGATTG), 300 nM reverse primers for Bcl-X_L (TCCTTGTCTACG CTTTCCACG) or Bax (GAAGTTGCCGTCAGAAAAC ATG), 200 nM probes for Bcl-X_L (VIC-ACAGTGCCCCGCC-GAAGGAGA-TAMRA) or Bax (VIC-AGAGGTCTTTTTCC-GAGTGG CAGCTG-TAMRA), and 9 mM $MgCl_2$. The probe was designed to cross the boundary of intron and exon of Mcl-1 cDNA. Analysis of cDNA for β-actin was included as a control. After incubation at 50°C for 2 min followed by 95°C for 10 min, the reaction was carried out for 40 cycles of the following: 95°C for 15 s and 60°C for 1 min. The threshold cycle value was normalized against β-actin cycle numbers. The relative abundance of mRNA expression of a control sample was arbitrarily designated as 1 and the values of the relative abundance of mRNA of other samples were calculated accordingly.

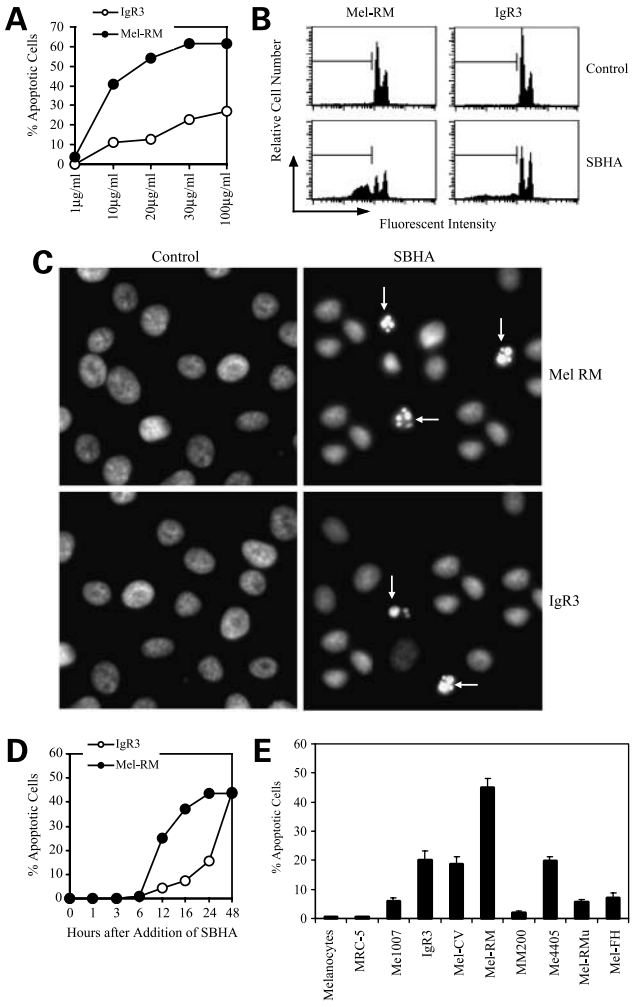

Figure 1. SBHA induces apoptosis of melanoma. **A,** titration of SBHA on induction of apoptosis. IgR3 and Mel-RM cells were treated with SBHA at indicated doses for 24 h before assay of apoptosis by the propidium iodide method using flow cytometry. Data are representative of three individual experiments. **B,** representative flow cytometry histograms of apoptosis assays by the propidium iodide method. IgR3 and Mel-RM cells were treated with SBHA at 30 μg/ml for 24 h. **C,** representative microphotographs of DAPI staining. IgR3 and Mel-RM cells were treated with SBHA at 30 μg/ml for 24 h. *Arrows,* apoptotic cells. **D,** kinetics of induction of apoptosis by SBHA. IgR3 and Mel-RM cells were exposed to SBHA at 30 μg/ml at the indicated time periods before assay of apoptosis by the propidium iodide method using flow cytometry. Data are representative of three individual experiments. **E,** SBHA induced apoptosis in a panel of melanoma cell lines but not in melanocytes and MRC-5 fibroblasts. Cells were treated with SBHA at 30 μg/ml for 24 h and apoptosis was measured by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE.

Results

SBHA Induces Apoptosis of Melanoma Cells

We examined the apoptosis-inducing potential of SBHA in melanoma cells by treating IgR3 and Mel-RM cells with a range of concentrations of the compound for 24 h. As shown in Fig. 1A, SBHA induced apoptosis of the melanoma cells even when used at concentrations as low as 10 μg/ml with the percentage of apoptotic cells peaking at 30 μg/ml for Mel-RM. Figure 1B shows the representative flow cytometry histograms of propidium iodide staining. Apoptosis was confirmed by visualization of DNA fragmentation by DAPI staining (Fig. 1C). Figure 1D shows that while apoptosis could be detected by 12 h after treatment in both IgR3 and Mel-RM cells, the kinetics differed between the two lines. The percentage of apoptotic cells in Mel-RM peaked at 24 h after treatment, whereas the kinetics of apoptosis of IgR3 cells appeared markedly delayed with a peak being observed at 48 h. As shown in Fig. 1E, there was a wide variation in sensitivity of melanoma cells to SBHA-induced apoptosis with negligible cell death in MM200 but nearly 50% apoptotic cells in Mel-RM cells. SBHA did not induce apoptosis in melanocytes and fibroblasts (Fig. 1E) even when the cells were treated for prolonged periods (data not shown).

Induction of Apoptosis of Melanoma by SBHA Is Caspase Dependent

Figure 2, A and B, shows that the pan-caspase inhibitor, z-VAD-fmk, completely inhibited SBHA-induced apoptosis,

whereas a caspase-3 specific inhibitor, z-DEVD-fmk, partially inhibited SBHA-induced apoptosis. The involvement of the caspase cascade in SBHA-induced apoptosis was confirmed by examining caspase-3 activation status in IgR3 and Mel-RM cells using a mAb that specifically recognizes the active form of caspase-3 as shown in Fig. 2, C and D. Caspase-3 was activated by 12 h with peak activation at 16 h after exposure to SBHA. Analysis of caspase-3 activation in Western blots showed that cleavage of the proenzyme form of caspase-3 appeared later than that detected by flow cytometry with degradation of procaspase-3 being detected at 48 h in IgR3 cells and 24 h in Mel-RM cells after exposure to SBHA (Fig. 2E). A slight increase in the procaspase-3 levels was detected in both cell lines at 3 h after SBHA treatment (Fig. 2E).

Figure 2E shows that SBHA induced cleavage of the long form of ICAD ($ICAD_L$) in Mel-RM cells by 16 h and it was barely detectable by 24 h. In contrast, reduction in $ICAD_L$ in IgR3 cells was only evident by 48 h after treatment. Figure 2E also shows that there was nearly complete disappearance of PARP expression at 16 and 24 h after SBHA treatment in Mel-RM cells and the p85 fragment of cleaved

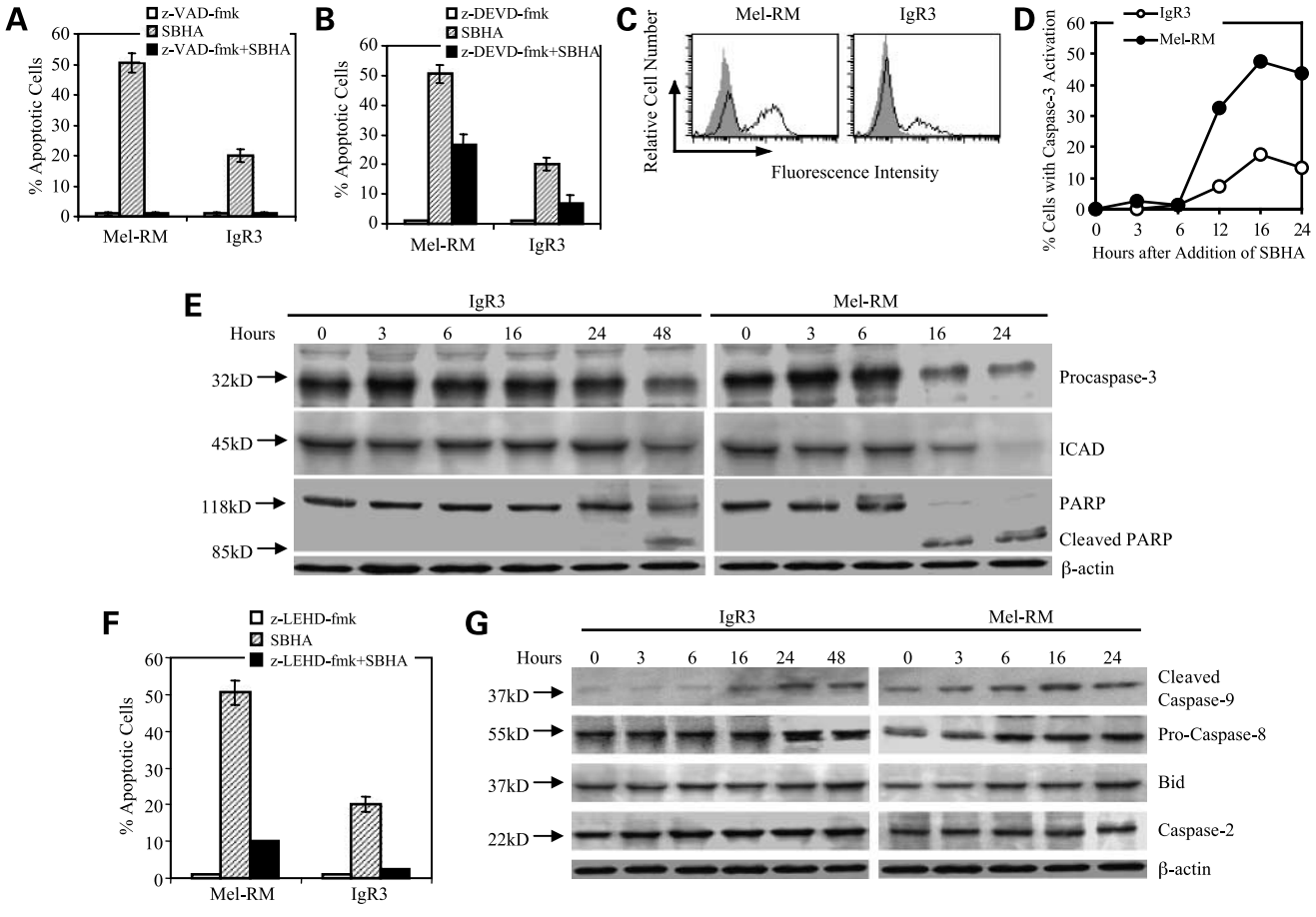

Figure 2. Induction of apoptosis by SBHA is caspase dependent. **A** and **B,** inhibition of SBHA-induced apoptosis by a pan-caspase inhibitor, z-VAD-fmk (**A**), or a caspase-3 inhibitor, z-DEVD-fmk (**B**). Mel-RM and IgR3 cells were treated with either z-VAD-fmk (20 μM) or z-DEVD-fmk (30 μM) 1 h before adding SBHA (30 μg/ml) for another 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. **C,** representative flow cytometry histograms of assays of caspase-3 activation induced by SBHA. Mel-RM and IgR3 cells were treated with SBHA (30 μg/ml) for 16 h before activated form of caspase-3 was measured in permeabilized cells using flow cytometry. *Filled histograms,* isotype controls. **D,** kinetics of SBHA-induced caspase-3 activation. Mel-RM and IgR3 cells were treated with SBHA (30 μg/ml) at the indicated time periods. Caspase-3 activation was measured as described in **C**. Data are representative of three individual experiments. **E,** SBHA induced cleavage of procaspase-3, ICAD, and PARP. Mel-RM and IgR3 cells were treated with SBHA (30 μg/ml) at the indicated time periods. Whole cell lysates were subjected to Western blot analysis. Western blot analysis of β-actin levels was included to show that equivalent amounts of protein were loaded in each lane. **F,** inhibition of SBHA-induced apoptosis by a caspase-9 inhibitor, z-LEHD-fmk. Mel-RM and IgR3 cells were treated with z-LEHD-fmk (30 μM) 1 h before adding SBHA (30 μg/ml) for another 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. **G,** SBHA induced caspase-9 activation but not cleavage of caspase-8, Bid, and caspase-2. Mel-RM and IgR3 cells were treated with SBHA (30 μg/ml) at the indicated time periods. Whole cell lysates were subjected to Western blot analysis using an antibody that specifically recognizes the cleaved form of capsase-9 and antibodies against procaspase-8, Bid, and procaspase-2, respectively. Western blot analysis of β-actin levels was included to show that equivalent amounts of protein were loaded in each lane.

PARP was clearly detected. PARP cleavage was however only detected in IgR3 cells at 24 and 48 h.

Inhibition of caspase-9 activity by treatment of cells with a caspase-9 specific inhibitor, z-LEHD-fmk, 1 h before the addition of SBHA almost completely blocked SBHA-induced apoptosis in melanoma cells (Fig. 2F). Caspase-9 activation by SBHA was confirmed by using an antibody that specifically recognizes the p38 fragment of the cleaved caspase-9. As shown in Fig. 2G, treatment of IgR3 and Mel-RM cells for up to 6 h resulted in weak expression of the cleaved caspase-9, but at 16 and 24 h, there was a marked increase in the expression levels. Procaspase-8 was increased in Mel-RM by 6 h after exposure to SBHA and remained relatively stable thereafter (Fig. 2G). Only small increases in procaspase-8 were seen in the IgR3 cell line. The p43 and p18 cleaved forms of caspase-8 were not detected (data not shown). Figure 2G also shows that the expression of Bid was slightly upregulated in Mel-RM cells by 6 h but was less evident in IgR3 cells after treatment with SBHA. The truncated form of Bid was not detected after treatment with SBHA (data not shown). The levels of expression of procaspase-2 remained unchanged in melanoma cells before and after treatment with SBHA (Fig. 2G).

SBHA Induces Changes in Mitochondrial Membrane Permeability

The activation of caspase-9 suggested that SBHA may be acting to induce changes in mitochondrial membrane permeability leading to release of mitochondrial apoptogenic proteins. Figure 3A shows that treatment of melanoma cells with SBHA induced varying degrees of changes in the mitochondrial membrane potential ($\Delta\Psi_m$) in melanoma cells, which were detectable at 12 h and peaked at 16 h after exposure to SBHA. As shown in Fig. 3B, inhibition of caspase activity had no effect on changes in $\Delta\Psi_m$ induced by SBHA. Figure 3C shows that after exposure to SBHA for 16 h, both cytochrome *c* and Smac/DIABLO were observed in the cytosolic fractions with a corresponding decrease in the mitochondrial fractions. Release of cytochrome *c* and Smac/DIABLO were caspase independent as shown in Fig. 3D.

Overexpression of Bcl-2 Inhibits SBHA-Induced Apoptosis of Melanoma

To confirm the role of mitochondria in SBHA-induced apoptosis, we transfected cDNA encoding Bcl-2 into Mel-RM cells (Fig. 4A). Figure 4B shows that the levels of SBHA-induced apoptosis in Bcl-2 transfected cells were markedly decreased compared with those in vector alone transfected cells. As shown in Fig. 4C, SBHA-induced changes in the $\Delta\Psi_m$ were reversed in Bcl-2-transfected cells but not in cells transfected with the vector alone. Similarly, SBHA-induced caspase-3 activation was also inhibited by Bcl-2 overexpression (Fig. 4D). Consistent with this, cleavage of ICAD and PARP by SBHA treatment was almost completely blocked in Bcl-2 overexpressing cells (Fig. 4E).

SBHA Induces Changes in the Conformation of Bax and Its Relocation from the Cytosol to Mitochondria

Bax translocation from the cytosol to mitochondria is believed to play a key role in induction of apoptosis by a variety of apoptotic stimuli (29). Bax translocation involves a conformational change that exposes the NH_2 terminus and the hydrophobic COOH terminus that targets mitochondria (30, 31). We studied the conformation status of Bax in melanoma cells with or without exposure to SBHA by using an antibody directed against the NH_2-terminal region of Bax in flow cytometry (32, 33). As shown in Fig. 5A, after exposure to SBHA for 16 h, there was a marked increase in Bax that had undergone conformational changes. The degree of increase in mean fluorescence intensity varied considerably among the cell lines with a

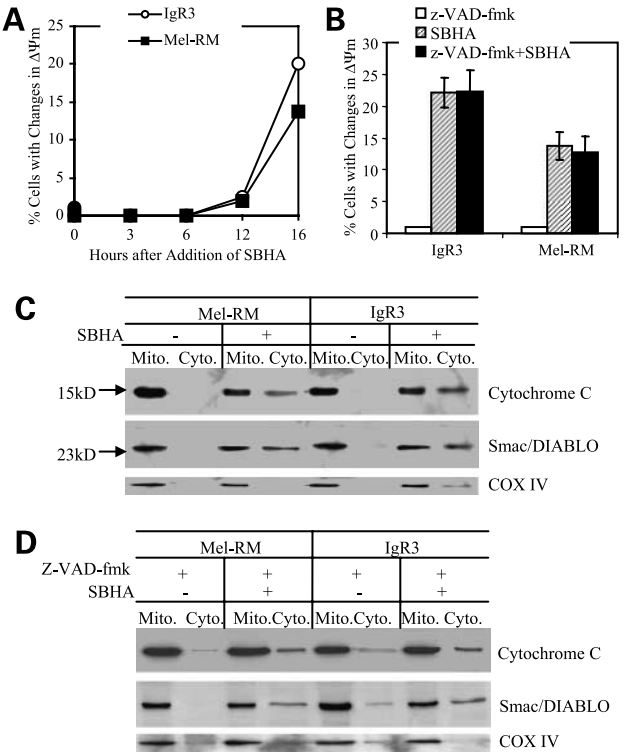

Figure 3. Induction of changes in mitochondrial membrane permeability by SBHA. **A,** kinetics of induction of changes in the $\Delta\Psi_m$ by SBHA. Mel-RM and IgR3 cells were treated with SBHA (30 μg/ml) at the indicated time periods. The $\Delta\Psi_m$ was measured by uptake of the MitoTracker Red CMXRos using flow cytometry. Data are representative of three individual experiments. **B,** SBHA-induced changes in $\Delta\Psi_m$ are independent of caspases. Mel-RM and IgR3 cells were treated with z-VAD-fmk (20 μM) 1 h before adding SBHA (30 μg/ml) for another 16 h. The $\Delta\Psi_m$ was measured as described in **A**. *Columns,* mean of three individual experiments; *bars,* SE. **C,** SBHA induced release of cytochrome *c* and Smac/DIABLO from mitochondria to the cytosol. Mel-RM and IgR3 cells were treated with SBHA (30 μg/ml) for 16 h before harvest. Mitochondrial and cytosolic fractions were subjected to Western blot analysis. Western blot analysis of COX IV levels was included to show relative purity of the mitochondrial fractions. Data are representative of two individual experiments. **D,** SBHA-induced release of cytochrome *c* and Smac/DIABLO is independent of caspases. Mel-RM and IgR3 cells with or without pretreatment with z-VAD-fmk (20 μM) for 1 h were treated with SBHA at 30 μg for 16 h. Mitochondrial and cytosolic fractions were subjected to Western blot analysis. Western blot analysis of COX IV levels was included to show relative purity of the mitochondrial fractions. Data are representative of two individual experiments.

marked increase in Me1007, IgR3, Mel-CV, Mel-RM, and MM200 but only minor changes in Me4405, Mel-RMu, and Mel-FH. This was clearly correlated with the levels of apoptosis induced by SBHA (Fig. 5, B and C).

Figure 5D shows that conformational changes in Bax induced by SBHA could not be inhibited by pretreatment with z-VAD-fmk. Similarly, studies on Bcl-2-transfected Mel-RM cells indicated that overexpression of Bcl-2 had only a minor effect on induction of conformational changes in Bax by SBHA (Fig. 5E). These results suggest that the conformational changes in Bax were upstream of changes in mitochondrial membrane permeability and activation of caspases.

We confirmed the role of Bax in SBHA-induced apoptosis of melanoma by examining the expression of Bax in different subcellular fractions of IgR3 and Mel-RM cells with or without exposure of cells to SBHA. As shown in Fig. 5F, before treatment, the Bax protein was predominantly in the cytosol with only a negligible amount being detected in the mitochondrial fractions. In contrast, 12 h after treatment with SBHA, a considerable amount of Bax was observed in the mitochondrial fractions with a corresponding decrease in the expression in the cytosol.

SBHA Down-Regulates Members of the Antiapoptotic Bcl-2 Family and XIAP but Up-Regulates Proapoptotic Bcl-2 Family Members

We evaluated expression of the antiapoptotic proteins Bcl-X_L, Bcl-2, and Mcl-1 together with the proapoptotic proteins Bax, Bak, and Bim in IgR3 and Mel-RM cells after treatment with SBHA at 30 μg/ml for varying time periods. As shown in Fig. 6A, Bcl-X_L expression was markedly down-regulated as early as 3 h. When the density of the bands was quantitated, the levels of Bcl-X_L expression appeared to be down-regulated by 98% in IgR3 cells and by 92% in Mel-RM cells by 6 h after incubation with SBHA. The expression of Mcl-1 was up-regulated at 3 and 6 h but was down-regulated thereafter. In contrast, no changes in the overall protein levels of Bcl-2 were observed even at 24 h after treatment. The expression levels of the multidomain proapoptotic Bcl-2 family members Bax and Bak were up-regulated with a peak being observed at 24 h. By 6 h after the addition of SBHA, the levels of Bax expression were increased by 54% for IgR3 cells and by 43% for Mel-RM cells and the levels of Bak expression were increased by 106% for IgR3 cells and by 168% in Mel-RM cells. Bim, a BH3-only protein of the Bcl-2 family, was markedly

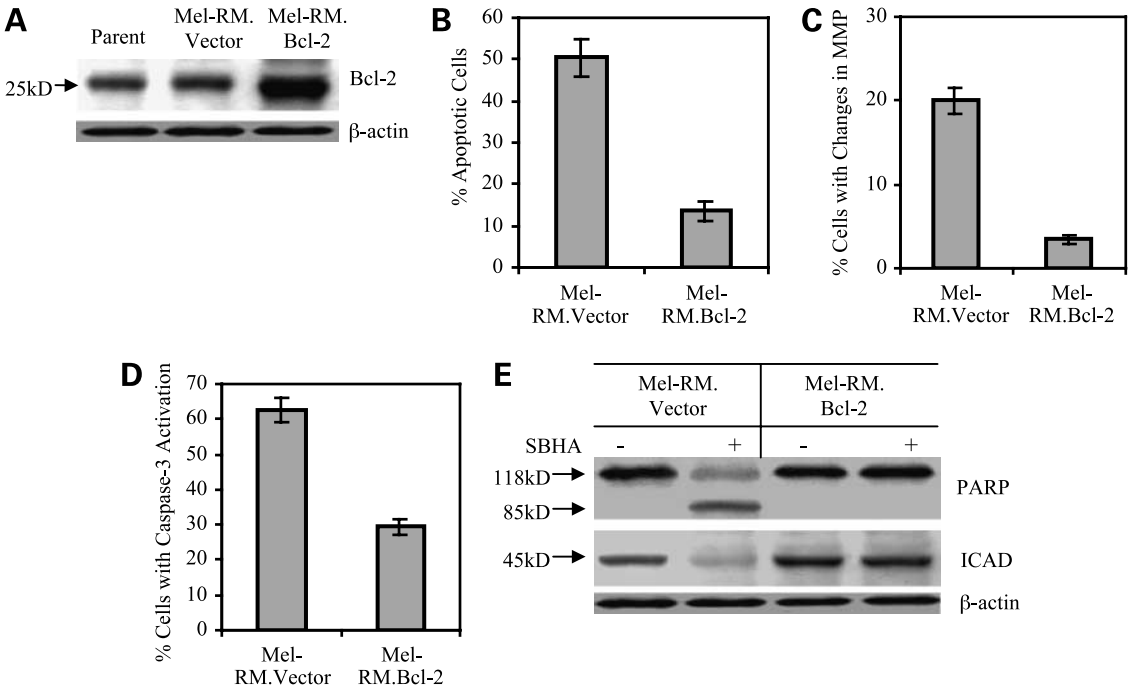

Figure 4. Overexpression of Bcl-2 suppresses SBHA-induced apoptotic events. **A,** Bcl-2 was overexpressed in Mel-RM cells transfected with the cDNA encoding Bcl-2 but not in the cells transfected with the vector alone. Whole cell lysates were subjected to Western blot analysis. Western blot analysis of β-actin levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. **B,** overexpression of Bcl-2 inhibited apoptosis induced by SBHA. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with SBHA (30 μg/ml) for 24 h before assay of apoptosis by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. **C,** Overexpression of Bcl-2 inhibited changes in the $\Delta\Psi_m$ induced by SBHA. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with SBHA (30 μg/ml) for 16 h before the $\Delta\Psi_m$ was measured by uptake of the MitoTracker Red CMXRos using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. **D,** overexpression of Bcl-2 inhibited caspase-3 activation induced by SBHA. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with SBHA (30 μg/ml) for 16 h before activated form of caspase-3 was measured in permeabilized cells using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. **E,** overexpression of Bcl-2 inhibited cleavage of PARP and ICAD induced by SBHA. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with SBHA (30 μg/ml) for 24 h before harvest. Whole cell lysates were subjected to Western blot analysis. Western blot analysis of β-actin levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments.

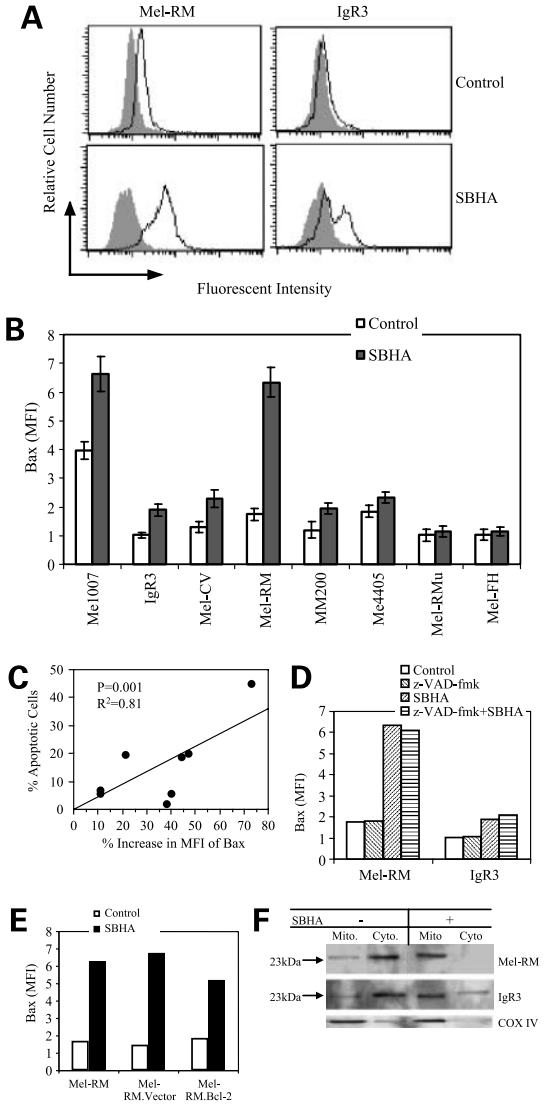

Figure 5. SBHA induced a conformational change of Bax and its relocation from the cytosol to mitochondria. **A,** representative flow cytometry histograms of assays of induction of a conformational change of Bax by SBHA. Mel-RM and IgR3 cells with or without treatment with SBHA (30 μg/ml) for 16 h were subjected to flow cytometry analyses using a Bax NH_2-terminal epitope-specific antibody in permeabilized cells. **B,** induction of the conformational change of Bax by SBHA in a panel of melanoma cell lines. Cells were treated and assays were carried out as described in **A**. *Columns,* mean of three individual experiments; *bars,* SE. **C,** correlation of the levels of increases in the conformational change of Bax and the levels of SBHA-induced apoptosis. Regression analyses were carried out in a Macintosh computer using the StatView software. **D,** induction of the conformational change of Bax is independent of caspases. Mel-RM and IgR3 cells were treated with z-VAD-fmk (20 μM) 1 h before adding SBHA (30 μg/ml) for another 16 h. The conformational change of Bax was measured as described in **A**. Data are representative of two individual experiments. **E,** overexpression of Bcl-2 did not inhibit the conformational change of Bax. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with SBHA (30 μg/ml) for 16 h before the Bax conformation change was measured as described in **A**. Data are representative of two individual experiments. **F,** relocation of Bax from the cytosol to mitochondria. Mel-RM and IgR3 cells were treated with SBHA (30 μg/ml) for 12 h before harvest. The mitochondrial and cytosolic fractions were subjected to Western blot analyses. Western blot analysis of COX IV levels was included to show relative purity of the mitochondrial fractions. Data are representative of two individual experiments.

up-regulated with expression peaking at 16 h after exposure to SBHA. By 6 h after treatment with SBHA, the levels of Bim were up-regulated by 165% for IgR3 cells and by 210% for Mel-RM cells. Figure 6A also shows that the expression of XIAP, an important inhibitor of apoptosis in melanoma cells (28), was markedly down-regulated in a time-dependent manner after treatment with SBHA. The expression levels of XIAP at 6 h after exposure to SBHA were about 25–50% less than those before treatment.

To determine if the alterations in the protein expression levels of Bcl-2 family were related to changes in expression of their mRNA, Bcl-X_L and Bax mRNA expression levels in IgR3 and Mel-RM melanoma cells were quantitated by real-time RT-PCR before and after exposure of cells to SBHA for 6 h. Figure 6B shows that the level of Bcl-X_L mRNA expression was down-regulated by 83% in IgR3 and by 74% in Mel-RM cells at 6 h after treatment with SBHA. In contrast, Bax mRNA expression was up-regulated by 24% in IgR3 and by 37% in Mel-RM cells (Fig. 6C). These results suggest that alterations in Bcl-2 family protein expression may be due to the regulation of transcription by SBHA. Changes in Bcl-X_L and Bax mRNA expression levels could not be observed during induction of apoptosis by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor family that is known to induce apoptosis in Mel-RM and IgR3 cells (Refs. 27, 28; data not shown).

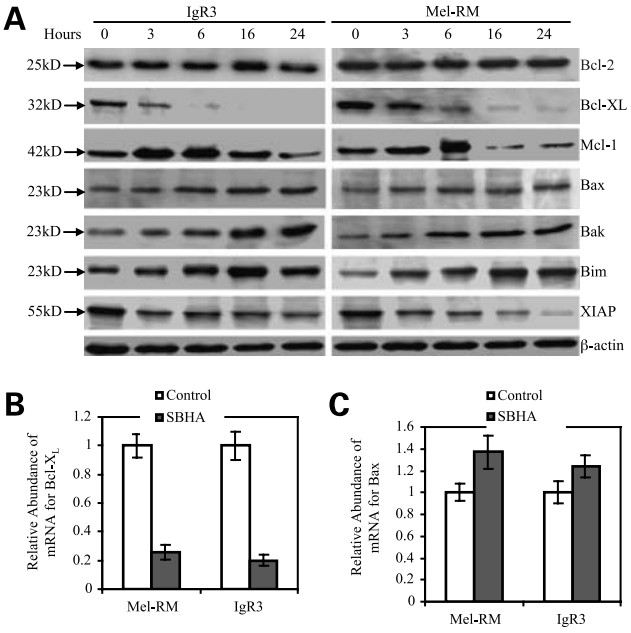

Figure 6. Regulation of the expression of Bcl-2 family members and XIAP in melanoma cells. **A,** whole cell lysates from Mel-RM and IgR3 cells with or without treatment with SBHA (30 μg/ml) at the indicated time periods were subjected to Western blot analyses. Data are representative of two individual experiments. **B** and **C,** SBHA down-regulates the Bcl-X_L mRNA levels but up-regulates the Bax mRNA levels. Mel-RM and IgR3 cells were treated with SBHA (30 μg/ml) for 6 h before total RNA was isolated and subjected to real-time PCR analyses. *Columns,* mean of three individual experiments; *bars,* SE.

SBHA Induces Translocation of Bim to Mitochondria and Its Association with Bcl-2

Up-regulation of the BH3-only protein Bim is of particular interest as it mediates apoptosis in response to a variety of apoptotic stimuli (34–39). Under normal condition, Bim_L and Bim_{EL} interact with the dynein light chain, thus being sequestered at microtubules away from mitochondria (34). However, certain apoptotic stimuli can free Bim from the dynein motor complex, allowing it to translocate to mitochondria and bind to Bcl-2 (34, 37). As shown in Fig. 7A, before treatment with SBHA, there was little, if any, Bim in mitochondria. In contrast, Bim was readily detected in mitochondrial fractions from both Mel-RM and IgR3 cells at 6, 12, and 16 h after treatment with SBHA but not with TRAIL (data not shown).

To test if Bim is physically associated with Bcl-2, we precipitated Bcl-2 in whole cell lysates from Mel-RM and Mel-RM Bcl-2 transfectants before and after treatment with SBHA. Resulting precipitates were analyzed by Western blot for Bcl-2 and Bim expression. Figure 7B shows that there was little association between Bim and Bcl-2 before treatment. In contrast, by 6 h after exposure to SBHA, they were clearly coprecipitated in both Mel-RM parental cells and Mel-RM cells overexpressing Bcl-2. The association of Bim with Bcl-2 in Bcl-2 transfectants after treatment with SBHA suggests that up-regulation and translocation of Bim is caspase independent in that Bcl-2 overexpression blocked caspase activation induced by SBHA. We confirmed this by coprecipitation of Bim and Bcl-2 in whole cell lysates from Mel-RM cells treated with SBHA in the presence of z-VAD-fmk, as shown in Fig. 7C. This suggests that activation and relocalization of Bim occurred upstream of caspase activation during the initiation of apoptosis. Bim could not be detected in precipitates from cells treated with TRAIL for the same time periods (data not shown).

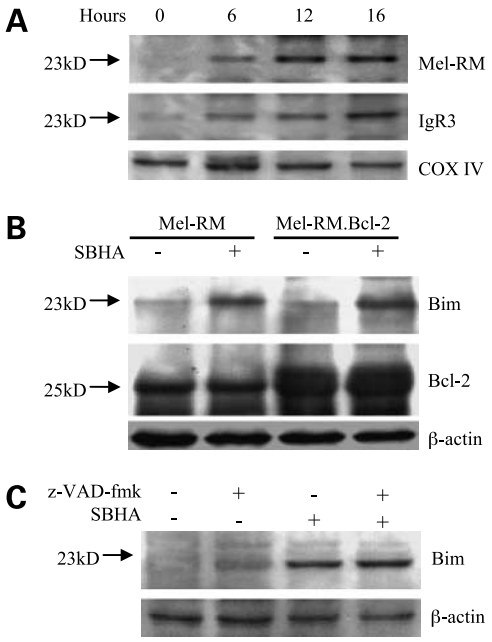

Figure 7. SBHA induced translocation of Bim to mitochondria and its association with Bcl-2. **A,** mitochondrial fractions from Mel-RM and IgR3 cells with or without treatment with SBHA at 30 μg/ml at the indicated time periods were subjected to Western blot analysis. Western blot analysis of COX IV levels was included to show relative purity of the mitochondrial fractions. Data are representative of two individual experiments. **B,** whole cell lysates from Mel-RM cells and Mel-RM Bcl-2 transfectants with or without treatment with SBHA at 30 μg/ml for 6 h were subjected to immunoprecipitation with a mouse mAb against Bcl-2. The precipitates were subjected to SDS-PAGE and probed with the mAb against Bcl-2 and an antibody against Bim, respectively. Western blot analysis of β-actin levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. **C,** Mel-RM cells with or without pretreatment with z-VAD-fmk (20 μM) for 1 h were treated with SBHA at 30 μg/ml for 6 h. Whole cell lysates were subjected to immunoprecipitation with a mouse mAb against Bcl-2. The precipitates were subjected to SDS-PAGE and probed with an antibody against Bim. Western blot analysis of β-actin levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments.

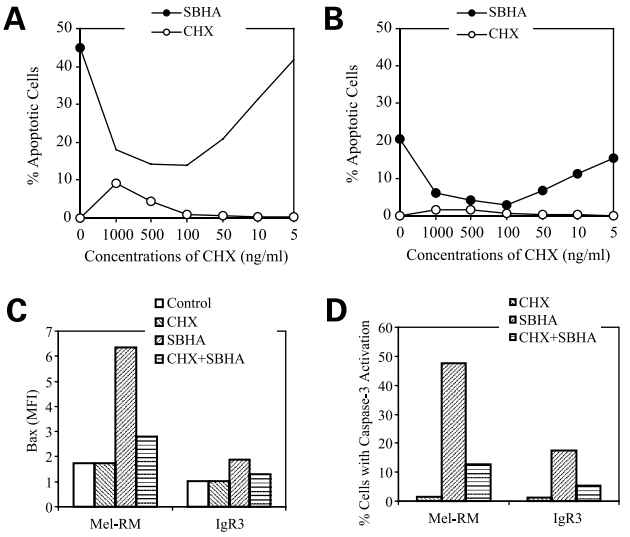

Figure 8. Induction of apoptosis by SBHA requires *de novo* protein synthesis. Mel-RM (**A**) and IgR3 (**B**) cells were treated with combinations of SBHA at 30 μg/ml and CHX at concentrations indicated for 24 h before assay of apoptosis by the propidium iodide method using flow cytometry. Data are representative of three individual experiments. **C,** Mel-RM and IgR3 cells treated with SBHA (30 μg/ml), CHX (100 ng/ml), or combination of both for 16 h were subjected to flow cytometry analyses using a Bax NH_2-terminal epitope-specific antibody in permeabilized cells. Data are representative of three individual experiments. **D,** Mel-RM and IgR3 cells treated with SBHA (30 μg/ml), CHX (100 ng/ml), or combination of both for 16 h were subjected to flow cytometry analyses using a mAb against the activated form of caspase-3 in permeabilized cells. Data are representative of three individual experiments.

De Novo Protein Synthesis Is Required for Induction of Apoptosis by SBHA

To determine if macromolecular synthesis is required for induction of apoptosis by SBHA, we examined the effects of the protein synthesis inhibitor, CHX, on SBHA-induced apoptotic events. As shown in Fig. 8, A and B, CHX at concentrations ranging from 100 ng/ml to 1 μg/ml markedly inhibited SBHA-induced apoptosis, with maximal inhibitory effect and lowest toxicity when used at

100 ng/ml. CHX lost its inhibitory effects when used at concentrations lower than 100 ng/ml and had no impact on SBHA-induced apoptosis when used at 5 ng/ml. Figure 8, C and D, shows that the conformational change of Bax and caspase-3 activation induced by SBHA were inhibited by CHX at a concentration of 100 ng/ml.

Discussion

SBHA is a relatively new HDAC inhibitor that has been the subject of studies on cell division (40) and fetal globin gene expression (41), but relatively little is known about its ability to induce apoptosis in cancer cells or the mechanisms involved. In the present studies, we show that it induced varying degrees of apoptosis in eight of nine human melanoma lines but did not induce apoptosis in normal melanocytes and fibroblasts. Apoptosis occurred relatively late (24–48 h) after the addition of the drug and was dependent on induction of mitochondrial membrane permeability as shown by failure of SBHA to induce apoptosis in melanoma cells overexpressing Bcl-2. Apoptosis was caspase dependent and correlated with SBHA-induced conformational changes in the proapoptotic Bax protein. Of particular note, SBHA induced changes in expression of several proapoptotic and antiapoptotic proteins that are known to regulate the mitochondrial apoptotic pathway.

Involvement of the mitochondrial apoptotic pathway was shown by the changes in the $\Delta\Psi_m$ associated with release of cytochrome *c* and Smac/DIABLO and activation of caspase 9. These changes and the downstream events were inhibitable by overexpression of Bcl-2. The role of mitochondria in SBHA-induced apoptosis was further shown by the findings that a caspase-9 specific inhibitor blocked SBHA-induced apoptosis. It is now widely believed that apoptosis acting via the mitochondrial pathway is dependent on the balance between the proapoptotic BH3-only Bcl-2 family members and the antiapoptotic Bcl-2 family members. The proapoptotic BH3 proteins include Bid (activated by caspase 8), Bim or Bmf [transcriptionally regulated or released from damage to microtubules (34, 37) or the actin myoskeleton (42)], and Noxa or Puma [up-regulated by gene transcription resulting from activation of p53 (43, 44)]. The antiapoptotic Bcl-2 family members include Bcl-2, Bcl-X_L, and Mcl-1. Bcl-2 and, to a lesser extent, Bcl-X_L are bound to mitochondria and other membranes in the cells and bind any BH3-only proteins released into the cytosol (45). Should the concentration of BH3 proteins exceed that of the antiapoptotic proteins, Bax and Bak undergo changes in conformation that allow them to aggregate in mitochondrial membranes and induce release of apoptosis-inducing factors (46), such as cytochrome *c* and Smac/DIABLO.

Treatment with SBHA resulted in conformational changes in Bax and its relocation from the cytosol to mitochondria. This was not inhibited by a pan-caspase inhibitor, which suggests that the changes in Bax were upstream of changes in mitochondrial membrane permeability and activation of caspases. We examined which particular BH3 protein(s) may be involved in activation of Bax. There was no evidence that Bid was activated by SBHA and caspase-8 inhibitors did not block apoptosis. Noxa or Puma were not detected by Western blotting or in immunoprecipitation studies and Bmf was not detected by Western blot studies (data not shown). Changes in mRNA for Noxa/Puma or Bmf were also not detected (data not shown). In contrast, Bim was readily detected by Western blotting and treatment with SBHA was associated with an increase in its concentration. Moreover, SBHA induced relocation of Bim from cytosolic fractions to mitochondria and its association with Bcl-2. These changes were independent of caspase activation and appeared to precede changes in conformation of Bax and changes in mitochondrial membrane permeability. More definitive proof that Bim is the key initiator is the subject of ongoing studies.

In addition to Bim, the multidomain Bax and Bak proteins were also up-regulated. At the same time, there was a marked decrease in the antiapoptotic Bcl-X_L, Mcl-1, and XIAP proteins. These changes and the relatively slow onset of apoptosis induced by SBHA lead us to hypothesize that SBHA may induce apoptosis by transcriptional regulation of genes rather than the more rapid direct effects on existing caspases or post-translational effects on existing proapoptotic proteins. This was supported by changes in mRNA expression studied by real-time PCR, showing down-regulation of mRNA for Bcl-X_L and up-regulation of mRNA for Bax. The changes in the expression of multiple apoptotic mediators did not appear to be the consequences of the apoptotic cascade itself because they occurred prior to the onset of changes in mitochondria, caspase-3 activation, and apoptosis. Studies with the protein synthesis inhibitor CHX also suggested that new protein synthesis was required in that activation of caspase-3, changes in Bax, and mitochondrial membrane permeability were inhibited by treatment with CHX.

SBHA-induced excess of BH3 proteins (Bim) over Bcl-2 antiapoptotic proteins (Bcl-X_L and Mcl-1) may provide the conditions needed for induction of a conformational change in Bax and consequent changes in mitochondrial membrane permeability and apoptosis. Although Bim can be released from microtubules by apoptotic initiators such as UV irradiation (34), it is also transcriptionally regulated [*e.g.*, by the forkhead transcription factor FKHR-L1 (47, 48) and FoxO (49)]. The increase in transcriptional up-regulation of Bim might therefore be sufficient to induce apoptosis in cells with low levels of certain Bcl-2 family proteins. Down-regulation of Bcl-X_L gene expression has been reported to lead to apoptosis in mesothelioma cells by another HDAC inhibitor, sodium butyrate (21). Similarly, transcriptional down-regulation of Bcl-2 by sodium butyrate appeared to be responsible for apoptosis induction in MCF-7 breast cancer, mesothelioma, and prostate cancer cells (19, 20, 22). However, down-regulation of Bcl-X_L observed in the present study is not likely to be the initiating factor in induction of apoptosis in melanoma cells by SBHA because new protein synthesis is clearly required for apoptosis induction as evidenced by studies using CHX. Up-regulation

of the multidomain proapoptotic proteins of the Bcl-2 family Bax and Bak by SBHA may facilitate apoptosis induction as they are absolutely required to induce mitochondrial dysfunction by BH3-only proteins. Moreover, down-regulation of XIAP in melanoma cells by SBHA may accelerate the activation of caspase-9 and enhance caspase-3 activity, thus promoting apoptosis induction (28).

Another hydroxamic acid HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), was reported to induce apoptosis by direct cleavage of Bid (50). In the present study, Bid was slightly up-regulated, but its cleavage was not detected following treatment with SBHA. Apoptosis induced by SAHA appeared to be caspase independent, whereas apoptosis induced by SBHA was dependent on activation of caspases (50). The apoptotic pathway induced by SBHA may therefore be novel and complementary to other apoptosis-inducing agents. It is however noteworthy that the differences between SBHA and other HDACs in induction of apoptosis may be merely due to the differences in cell types used in the different studies (19–22, 50). Considering the importance of BH3-only proteins in initiating apoptosis, it would be of interest to examine in future studies if up-regulation and activation of Bim is involved in induction of apoptosis in melanoma cells by other HDACs such as sodium butyrate and SAHA.

The mechanism by which SBHA regulates such a diverse array of genes involved in apoptosis is not clear. The balance between histone acetylation and deacetylation plays an important role in transcriptional regulation of genes (1–5). Likely, SBHA targets HDACs associated with transcriptional factors that are involved in regulation of apoptosis such as p53 or c-Myc. The forkhead transcription factors FKHR-L1 and FoxO have both been reported to regulate transcription of Bim (47–49). Down-regulation or up-regulation of other genes by HDAC inhibitors has been reported by others (6, 25, 51). SBHA up-regulates the expression levels of p21 in melanoma cells as do other HDACs (data not shown). However, a complete profile of its target enzyme specificities and its effects on gene expression remain to be elucidated. Further studies are in progress to explore the effects of SBHA on gene transcription.

The complementary proapoptotic changes induced in several different proteins involved in apoptosis makes SBHA potentially important as an anticancer agent (*e.g.*, in combination with chemotherapeutic agents that induce other proapoptotic BH3-only proteins such as Noxa/Puma or Bmf or in combination with TRAIL to induce activation of Bid). Melanocytes and fibroblasts were not killed by SBHA, but its effects on other normal cells need to be tested. Not all melanoma cells were sensitive to SBHA and further study is needed to understand why melanoma lines such as MM200 were resistant to SBHA but very sensitive to other apoptosis-inducing agents such as TRAIL (27, 28).

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