Histone deacetylase inhibitor suberoylanilide hydroxamic acid induces apoptosis through both mitochondrial and Fas (Cd95) signaling in head and neck squamous carcinoma cells

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Abstract

Alterations in histone acetylation status have been implicated in carcinogenesis. Histone deacetylase inhibitors, such as suberoylanilide hydroxamic acid (SAHA), can potentially reactivate aberrantly silenced genes by restoring histone acetylation and allowing gene transcription. However, the mechanisms underlying the effects of SAHA on cell growth, differentiation, and death remain unclear. In this study, we assessed the activity of SAHA in modulating cell growth and apoptosis in head and neck squamous cell carcinoma (HNSCC) cells compared with premalignant leukoplakia and normal oral cells. SAHA induced growth inhibition, cell cycle changes, and apoptosis in HNSCC cell lines but had limited effects on premalignant and normal cells. Although SAHA triggered the mitochondrial pathway of apoptosis, including cytochrome *c* release, caspase-3 and caspase-9 activation, and poly(ADP-ribose) polymerase cleavage in HNSCC cells, specific inhibition of caspase-9 only partially blocked the induction of apoptosis induction. SAHA also activated the extrinsic apoptosis pathway, including increased Fas and Fas ligand (FasL) expression, activation of caspase-8, and cleavage of Bid. Interfering with Fas signaling blocked apoptosis induction and blunted growth inhibition by SAHA. Our results show for the first time that SAHA induces apoptosis in HNSCC cells through activation of the Fas/FasL death pathway in addition to the intrinsic mitochondrial pathway although having comparatively little activity against precancerous and normal oral cells with intrinsic Fas and FasL expression. [Mol Cancer Ther 2007;6(11):2967–75]

Introduction

Modulation of the acetylation status of histones and transcription factors is an important mechanism for regulating gene expression (1–3). Increased histone acetylation is associated with increased transcription, whereas hypoacetylation is linked to a repressed transcription (4–7). The levels of histone acetyltransferase and histone deacetylase (HDAC), which modulate histone acetylation, have been linked with malignancy (8–12). Many transcriptional activators and repressors involved in signaling pathways possess histone acetyltransferase or HDAC activity and can be acetylated themselves, implicating acetylation as a key mechanism of regulating cell signaling (13–18).

Elevated levels of HDAC activity have been observed in various cancer types (11). HDAC inhibitors (HDACI) induce growth arrest, differentiation, and apoptosis in various cancer cell lines *in vitro* (19, 20) and suppress tumor growth in animal xenograft models (20, 21). HDACIs also exhibit chemopreventive activity in animal models of epithelial tumorigenesis (22). As a result, HDACIs have received attention as a potential approach to cancer therapy (23–25). Several HDACIs, including suberoylanilide hydroxamic acid (SAHA), tributyrin, and depsipeptide, are being investigated in clinical trials as antineoplastic agents for patients with both hematologic and solid malignancies (26–28).

The ability of SAHA to inhibit HDAC activity and thereby regulate gene transcription has been linked to its ability to inhibit growth and induce apoptosis in several types of malignant cells *in vitro* (21, 29, 30). However, many of the mechanisms underlying the effects of this agent on cell growth, differentiation, and death have not been fully clarified. In this study, we evaluated the effects of SAHA on histone acetylation, cell growth, and apoptosis in head and neck squamous cell carcinoma (HNSCC) cells, premalignant leukoplakia cells, and normal oral keratinocytes (NOK). Our results show that SAHA has preferential activity against cancerous HNSCC cells compared with precancerous and normal oral cells. Furthermore, we showed that SAHA induces cell death via activation of the extrinsic apoptotic pathway involving Fas/FasL and the intrinsic mitochondrial pathway.

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Materials and Methods

Cell Culture and Reagents

The 10 human HNSCC cell lines used in this study were described previously (31). NOK cells derived from a cancer-free 68-year-old Caucasian female were purchased from Cambrex/Clonetics. MSKLeuk1 cells were derived from oral leukoplakia (32). SAHA (Midwest Research Institute) was dissolved in DMSO at a concentration of 10 mmol/L, and aliquots were stored at −70°C. All cells were seeded and allowed to adhere for 24 h before addition of SAHA to the medium.

Growth Inhibition Assay

Inhibition of cell growth was assessed using a crystal violet assay (33). All experiments were done at least twice in quadruplicate. Percentage of growth inhibition was determined using the equation $(1 - N_t/N_c) \times 100$, wherein N_t and N_c are the absorbencies of stain in treated and control cultures, respectively.

Protein Extraction and Western Blotting

Cells were harvested and proteins were extracted as previously described (33), except that histones were acid-extracted from cells according to an established procedure (34). Immunoreactive bands were detected using chemiluminescence (Amersham Pharmacia Biotech UK) according to the manufacturer's protocol. Antibodies against p21 were purchased from Oncogene; rabbit polyclonal antibodies against acetylated histones H3 or H4 were from Upstate Biotechnology; antibodies against caspase-9, caspase-8, caspase-3, poly(ADP-ribose) polymerase, and Bid were from Cell Signaling Technology; antibodies against cytochrome *c*, Fas, and FasL were from BD PharMingen.

Apoptosis Assays

Cells were incubated in medium with 4 μmol/L SAHA for various times, and apoptosis was analyzed by the terminal deoxynucleotidyltransferase-mediated dUTP biotin nick-end labeling assay using an ApoDIRECT kit (Phoenix Flow Systems) according to the manufacturer's instructions, as previously described (31). The ELISA Cell Death Detection kit (Roche Molecular Biochemicals) was used according to the manufacturer's instructions.

Measurement of Cytochrome *c* Release

Cells were exposed to 4 μmol/L SAHA for various times. Both floating and attached cells were then harvested for preparation of cytosolic extracts, and cytochrome *c* was detected by Western blotting using a mouse monoclonal antibody against cytochrome *c* as described previously (31).

Incubation with Caspase and Fas Inhibitors

17B and 22B HNSCC cells were pretreated with either the caspase inhibitor zVAD-fmk (150 μmol/L; Enzyme Systems Products) or inhibitor of caspase-8 Z-IETD-FMK, or caspase-9 Z-LEHD-FMK (75 or 150 μg/mL; R&D Systems), or soluble recombinant human Fas/Fc chimera/TNFRSF6 (5 μg/mL; R&D Systems) for 30 min before 2 μmol/L SAHA was added. We chose to use 2 μmol/L SAHA so that we could also use lower doses of the caspase inhibitors, which may have cytotoxic effects at high doses. Apoptosis was estimated using an ELISA assay.

Preparation of RNA and cDNA

Total cellular RNA was extracted from cells using the TRI reagent method (Molecular Research Center). For the reverse-transcription reaction, 10 μL of reaction mixture (1 μg of total RNA, 1 μL of random decamers, 1 μL of 10× RT buffer, 2 μL of deoxynucleotide triphosphate mix, 0.5 μL of RNase inhibitor, 0.5 μL of reverse transcriptase, and 4 μL of nuclease-free water) were prepared for each sample. The mixtures were incubated at 44°C for 1 h and then at 92°C for 10 min to inactivate the reverse transcriptase. The first-strand cDNAs were synthesized with a RETROscript kit (Ambion) and served as templates for the PCR.

PCR

A high-fidelity PCR master kit from Roche was used to perform PCR. The following primers were used: human tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; ref. 35) forward, 5′-CAACTCCGTCAGCTCGTTAGAAAG-3′; TRAIL reverse, 5′-TTAGACCAACAACTATTTCTAGCACT-3′; human death receptor 4 (DR4; ref. 36) forward, 5′-AGCATGTCAGTGCAAACCAGG-3′; DR4 reverse, 5′-TCCAGGGCGTACAATCCTTG-3′; human DR5 (36) forward, 5′-TGCATCTCCTGCAAATATGGAC-3′; DR5 reverse, 5′-TGCAGGGACTTAGCTCCACTTC-3′. Human β-actin was purchased from Ambion. PCR was done at 95°C for 4 min, at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 45 s, with 28 cycles for DR4 and DR5 and 32 cycles for TRAIL. PCR products were resolved by electrophoresis on a 1.8% agarose gel with 0.5 μg/mL ethidium bromide and photographed using an AlphaEase FC imaging system (Alpha Innotech).

Small Interfering RNA Transfection

Small interfering RNA (siRNA) duplex oligonucleotides targeting caspase-8, caspase-9, and Fas, as well as a nonspecific oligonucleotide, were purchased from Dharmacon. Approximately 1.5 million 22B cells per well were seeded in six-well plates overnight. One microgram of each siRNA was incubated with 3 μL of the transfection agent Lipofectamine RNAiMAX transfection reagent (Invitrogen) in serum-free medium for 45 min before being added to the six-well plates. After 24 h at 37°C, the cells received medium with or without 4 μmol/L SAHA and were incubated for another 24 h before harvesting. Total protein was extracted, and Western blot analysis was done as described above.

Clonogenic Assay

Cells transfected with siRNA, as described above, were trypsinized, washed, counted, and reseeded at low densities onto 100-mm tissue culture dishes. After 16 days of incubation in medium with or without 4 μmol/L SAHA, the adherent cell colonies were stained with crystal violet, washed, air-dried, and counted under a microscope.

Results

SAHA Inhibits the Growth of Human HNSCC Cells while Sparing Premalignant and Normal Oral Cells

We evaluated the effects of SAHA on cell growth in 10 HNSCC cell lines, an immortalized leukoplakia cell line (Leuk1), and NOK cells. As shown in Fig. 1A and B, SAHA

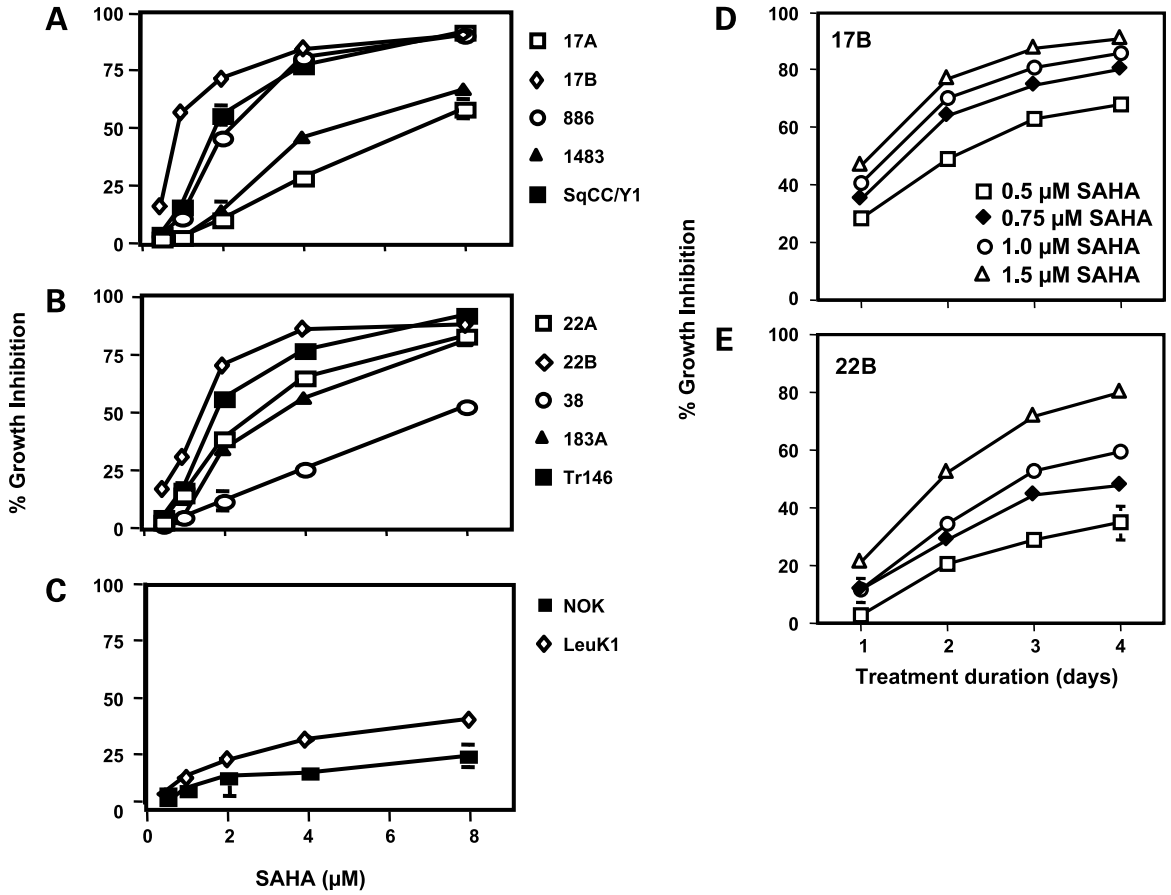

Figure 1. Effects of SAHA on growth of HNSCC cells compared with Leuk1 and NOK cells. Concentration-dependent growth inhibition in HNSCC cells (**A** and **B**) and, to a limited extent, in Leuk1 and NOK cells (**C**) was seen after incubation with SAHA for 3 d. Time- and dose-dependent growth inhibition was also seen in 17B (**D**) and 22B (**E**) HNSCC cells. *Points,* means of three replicate experiments.

inhibited the growth of all 10 cancer cell lines in a concentration-dependent manner, with an estimated IC_{50} ranging from 1.35 to 7.7 μmol/L after a 3-day treatment. In contrast, SAHA had a more limited effect on the growth of Leuk1 and NOK cells (Fig. 1C). The inhibition of the growth of HNSCC cell lines 17B and 22B was both dose- and time-dependent between 1 and 4 days (Fig. 1D and E). These results suggest that SAHA selectively inhibits growth of HNSCC cells compared with immortalized precancerous or normal oral cells.

SAHA Selectively Induces Apoptosis in HNSCC Cells

Treatment of 17B and 22B cells for 24 h with 4 μmol/L SAHA induced ~60% apoptosis compared with <10% in Leuk1 and NOK cells (Fig. 2A). SAHA also induced DNA fragmentation, a hallmark of apoptosis, in both HNSCC cancer lines (Fig. 2C). These results indicate that SAHA induced apoptosis preferentially in HNSCC cancer cells.

SAHA Increases Acetylation of Histones H3 and H4 in HNSCC, Leuk1, and NOK Cells

At concentrations that induce growth inhibition and apoptosis, SAHA induced a time-dependent increase in acetylated H3 and H4 in all four cell lines, beginning at 3 h (Fig. 3A and B). Thus, SAHA actively modulated chromatin by inhibiting HDAC in normal, precancerous, and cancerous cells, but the increased acetylation of histones in and of itself was not sufficient to inhibit growth or induce apoptosis in Leuk1 or NOK cells.

SAHA Induces Expression of $p21^{WAF1}$ mRNA and Protein

The cell cycle kinase inhibitor WAF1 is induced in various transformed cells by HDACIs, such as butyrate, trichostatin, and SAHA (29, 37, 38), and may play a role in the growth inhibition observed after *in vitro* treatment with these agents. We detected increased expression of $p21^{WAF1}$ mRNA (Fig. 3C) and protein (Fig. 3D) in HNSCC cell lines 17B and 22B after treatment with 4 μmol/L SAHA. In the 17B cells, the level of $p21^{WAF1}$ protein peaked at 15 h and then declined, whereas in the 22B cells, maximal $p21^{WAF1}$ protein expression was seen at 24 h. In contrast, both Leuk1 and NOK cells had higher constitutive levels of WAF1 mRNA than did the malignant cells (Fig. 3E), yet their $p21^{WAF1}$ protein levels were low (Fig. 3F). Nonetheless, incubation with 4 μmol/L SAHA had little effect on $p21^{WAF1}$ mRNA but increased the level of $p21^{WAF1}$ protein

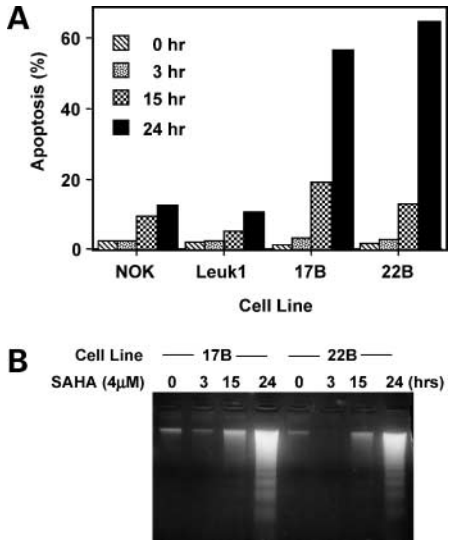

Figure 2. Effects of SAHA on apoptosis. SAHA induced a time-dependent increase in apoptotic HNSCC cells, as measured by terminal deoxynucleotidyltransferase-mediated dUTP biotin nick-end labeling assay (**A**) and DNA fragmentation (**B**). In contrast, the effect of SAHA on apoptosis in Leuk1 and NOK cells was limited (**A**).

in Leuk1 by 24 h and in NOK cells by 3 h. Thus, the increase in p21 alone could not explain the differential inhibition of the HNSCC cells by SAHA.

SAHA Induces Apoptosis via the Mitochondrial (Intrinsic) Pathway in HNSCC Cells

Apoptosis can be initiated via the mitochondrial (intrinsic) pathway, which functions through caspase-9 (39–41), or via the death receptor (extrinsic) pathway, which acts through caspase-8 (42). We next examined the effects of SAHA on several key aspects of apoptosis initiation via the intrinsic pathway, such as cytochrome *c* release, activation of caspase-9 and caspase-3, and cleavage of poly(ADP-ribose) polymerase. As shown in Fig. 4A, 4 μmol/L SAHA triggered cytochrome *c* release into the cytosol within 3 h in 17B and 22B HNSCC cells. This was followed by activation of caspase-9 within 15 h (Fig. 4B). Similarly, caspase-3 was activated within 15 h and cleaved its substrate poly(ADP-ribose) polymerase (Fig. 4B). Low levels of cleaved poly(ADP-ribose) polymerase were present in untreated 22B cells, but those levels increased after 3 h in 22B cells compared with 15 h in 17B cells, possibly indicating a higher level of spontaneous apoptosis among the 22B cells. Taken together, these results show that SAHA targets the mitochondria and activates the intrinsic cytochrome *c*–mediated apoptotic pathway in HNSCC cells.

Activation of the Caspase Cascade Is Required for SAHA-Induced Apoptosis

The pan-caspase inhibitor zVAD-fmk suppressed SAHA-induced apoptosis in 17B and 22B cells (Fig. 4C), indicating that activation of the caspase cascade is indeed required for SAHA to induce apoptosis in these cells. A specific caspase-9 inhibitor alone and a specific caspase-8 inhibitor alone only partially blocked the induction of apoptosis (Fig. 4D), indicating that both intrinsic and extrinsic apoptosis pathways are involved.

SAHA Activates the Death Receptor (Extrinsic) Apoptosis Pathway

The extrinsic apoptosis pathway involves binding of a ligand [e.g., TRAIL and FasL (CD95L)] to one of the tumor necrosis factor family of death receptors [Fas (CD95), DR4, DR5], followed by activation of caspase-8 and caspase-3. The intrinsic and extrinsic pathways are linked through the ability of caspase-8 to cleave Bid, which in turn leads to release of cytochrome *c* from the mitochondria. As shown in Fig. 5A, there was little expression of Fas and FasL in untreated 17B and 22B cells, but SAHA induced in both HNSCC cell lines the expression of Fas and FasL which increased by 3 h, peaking at 15 h in 17B cells and increasing up to 24 h in 22B cells. SAHA also activated caspase-8 (Fig. 5B) and cleaved Bid (Fig. 5C) by 15 h in both HNSCC cell lines, demonstrating activation of the extrinsic apoptosis pathway by this agent. In contrast, untreated Leuk1 and NOK cells showed constitutive expression of both Fas and FasL that was not affected by SAHA (Fig. 5A). SAHA did not alter the expression of DR4 or DR5 and increased TRAIL expression only marginally after 24 h (Fig. 5D).

Incubation of 17B and 22B cells with SAHA in the presence of soluble Fas, which competes for secreted FasL, blocked the induction of apoptosis by SAHA (Fig. 5E). This suggests that activation of Fas is required for SAHA to induce apoptosis in HNSCC cells.

Partial Silencing of Caspase-8 and Caspase-9 Reduces SAHA-Induced Apoptosis

We blocked the expression of caspase-9, caspase-8, and Fas by transfecting 22B HNSCC cells with specific siRNAs and then analyzing whether SAHA could still induce apoptosis in these cells. siRNA transfection effectively blocked activation of caspase-9 (Fig. 6A) and caspase-8 (Fig. 6B) in SAHA-treated cells. As shown in Fig. 6C, blocking these two molecules decreased the ability of SAHA to induce apoptosis in 22B cells by 84% and 78%, respectively. Transfection of Fas siRNA alone also reduced the ability of SAHA to induce apoptosis in 22B cells by 63% (Fig. 6C). These results indicate the importance of both the mitochondrial and death receptor–mediated signaling pathways in the induction of programmed cell death by SAHA.

Inhibition of Fas Activation Limits SAHA-Induced Inhibition of Anchorage-Dependent Colony Formation

To determine whether blockade of SAHA-induced apoptosis by the siRNAs described above could also protect cells from growth inhibition, we analyzed the ability of 22B cells to form colonies in the presence or absence of SAHA after siRNA transfection. As shown in Fig. 6D, SAHA inhibited colony formation by ~98% in untransfected cells, whereas transfection with siRNA targeting caspase-9 or caspase-8 decreased the inhibitory effect of SAHA to 45% and 43%, respectively. Transfection of Fas siRNA also decreased the ability of SAHA to suppress colony formation in 22B cells to 61%, whereas transfection with nonspecific siRNA decreased the effect of SAHA by <10%. These findings further supported the contributions of both apoptotic

pathways in mediating the effects of SAHA on growth inhibition and apoptosis in HNSCC cells.

Discussion

In the present study, we showed for the first time that SAHA preferentially inhibits growth and induces apoptosis in HNSCC cells, compared with its limited activity against Leuk1 (derived from a premalignant oral lesion) and normal oral gingival cells. This finding has important clinical implications, as it suggests that SAHA is a good candidate for therapy in patients with these malignancies.

The inhibition of growth and induction of apoptosis in HNSCC cells by SAHA occurred at time periods and concentrations that also resulted in the accumulation of acetylated histones H3 and H4 and increased expression of $p21^{WAF1}$ in HNSCC, Leuk1, and NOK cells. However, SAHA was much less active in inhibiting cell growth and inducing apoptosis in Leuk1 and NOK cells than in HNSCC cells. The mechanisms accounting for this selective growth inhibition in malignant cells have not yet been elucidated, but it is unlikely to result from less-effective uptake by the nonmalignant cells, as the presence of acetylated histones was detected at similar drug concentrations and time periods in all cell lines. Similar selectivity of SAHA-induced cytotoxicity in breast cancer cells compared with normal breast epithelial cells and fibroblasts has been shown (30). It seems that the ability to inhibit HDAC is not, in and of itself, sufficient to cause the demise of the cell, rather SAHA may facilitate transcription of death-activating factors or repress transcription of death inhibitory factors present in transformed cells but not in normal cells.

Further analysis of 17B and 22B, the two HNSCC cell lines sensitive to the growth inhibitory– and apoptosis-inducing effects of SAHA, was done to better clarify the

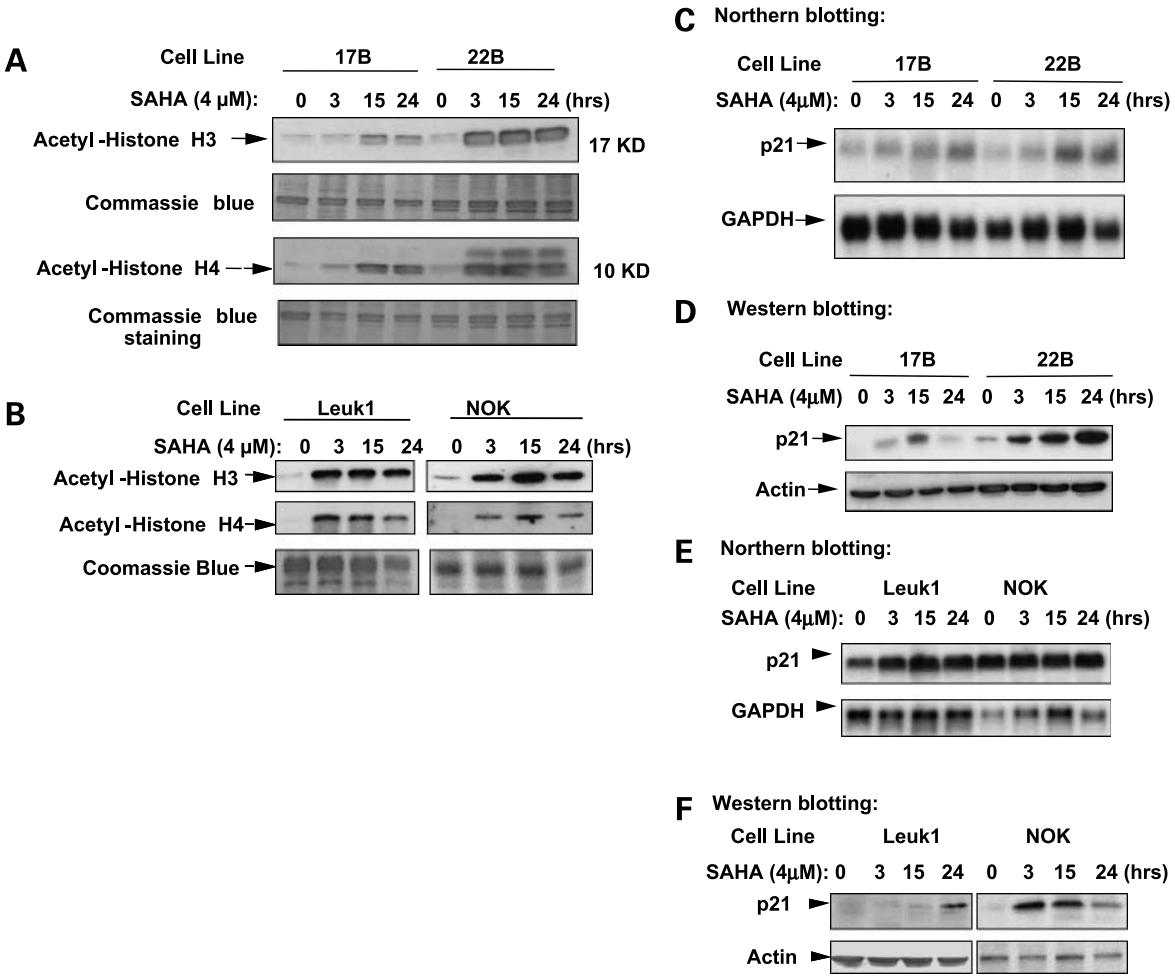

Figure 3. SAHA induces accumulation of acetylated histones and increased p21 expression in HNSCC, Leuk1, and NOK cells. HNSCC cells (**A, C, D**) and Leuk1 and NOK cells (**B, E, F**) were cultured with 4 μmol/L SAHA for the indicated time periods. Acetylation of acid-extracted histones was detected by Western blotting using specific antibodies against acetylated H3 and H4 (**A** and **B**). Coomassie Blue staining was used as a loading control. Expression of p21 mRNA and protein was detected by Northern and Western blotting (**C – F**).

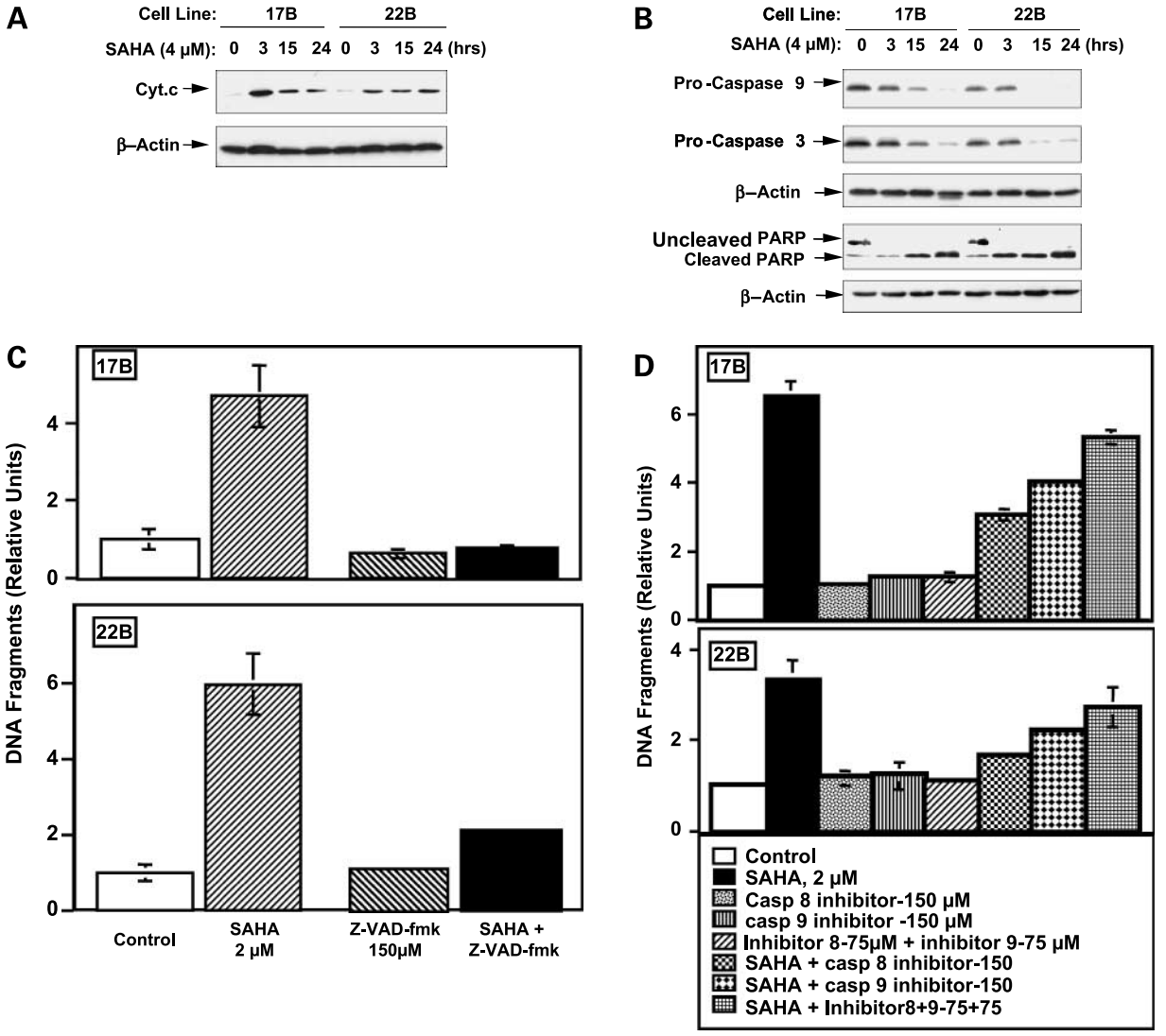

Figure 4. SAHA activates the intrinsic cell death pathway in human HNSCC cells. **A,** SAHA increased cytochrome *c* release from the mitochondria into the cytosol of 17B and 22B cells. **B,** SAHA activated caspase-9 and caspase-3 and increased cleavage of poly(ADP-ribose) polymerase (*PARP*) in 17B and 22 B cells. **C** and **D,** the pan-caspase inhibitor zVAD-fmk effectively blocked SAHA-induced apoptosis in 17B and 22B cells (**C**), whereas selective inhibition of caspase-8 or caspase-9 had only a partial effect (**D**).

mechanisms of SAHA-induced apoptosis in these cells. The apoptotic signaling pathways are generally divided into two types: the extrinsic, or death receptor, pathway and the intrinsic, or mitochondrial, pathway. The extrinsic pathway involves cell surface death receptors, such as tumor necrosis factor receptor 1, Fas, DR-3, DR-4, and DR-5, which upon binding their ligands initiate signaling that activates caspase-8. Caspase-8 can then cleave Bid and induce cytochrome *c* release or directly activate caspase-3. The intrinsic pathway is triggered by various signals, including cytotoxic chemotherapy agents, radiation, and DNA damage, that perturb the mitochondrial membrane potential and trigger the release of cytochrome *c*. Cytochrome *c* binds to Apaf-1, which activates caspase-9 and then caspase-3 (43). Our results initially revealed that SAHA activated the intrinsic pathway in HNSCC cells, either by direct activity of SAHA on the mitochondria or through cleavage of Bid and subsequent initiation of cytochrome *c* release. Similar results indicating activation of the mitochondrial apoptotic pathway by SAHA in U937 leukemia cells (38) and other cell types (44) have been reported.

The inability of specific caspase-9 inhibition to completely block induction of apoptosis by SAHA suggested that activation of the intrinsic pathway alone is not sufficient to explain the apoptotic effects of SAHA on HNSCC cells. Further investigation revealed that SAHA also initiated the extrinsic apoptotic pathway in 17B and 22B cells through activation of Fas (CD95). In contrast, expression of Fas and FasL was unaffected by SAHA in Leuk1 and NOK cells,

which did not undergo apoptosis after exposure to SAHA. It is possible that the cancer cells developed epigenetically regulated silencing of Fas and FasL as a survival mechanism, so that reexpression of these molecules after SAHA treatment rendered the cancer cells more susceptible to apoptosis. Loss of Fas expression is seen during carcinogenesis of various tumors (45, 46) and has been linked to tumor progression and resistance to chemotherapy (47). Interestingly, specific polymorphisms in the Fas gene have been correlated to risk of developing HNSCC (48). Competitive inhibition of Fas by soluble Fas blunted the SAHA-induced apoptosis of HNSCC cells, suggesting that activation of the Fas signaling pathway is required for apoptosis. Transfection with anti-Fas siRNA markedly reduced the ability of SAHA to induce apoptosis and growth inhibition in HNSCC cells, further supporting the importance of this pathway in mediating the apoptosis-inducing effects of SAHA in HNSCC cells. Thus, our results show for the first time that activation of the extrinsic apoptotic pathway by Fas is instrumental for SAHA-mediated effects on apoptosis and growth inhibition.

Our findings that SAHA-induced apoptosis was inhibited both by caspase inhibitors and by competitive inhibition of the death receptor pathway are in contrast with the findings of Ruefli et al. (44). This group evaluated the effects of SAHA in the leukemia cell line CEM and noted that transfection with CrmA to inactivate caspase-8 and caspase-10 and incubation with the caspase inhibitor zVAD-fmk failed to inhibit SAHA-induced cell death. A similar investigation showed that SAHA-induced apoptosis in malignant B-cell lines was not associated with activation of caspase-9 or caspase-93 and could not be blocked by pan-caspase inhibitors (49). In contrast, in our study, coincubation of HNSCC cells with SAHA and zVAD-fmk, as well as siRNA-mediated inhibition of caspase function, significantly blocked induction of apoptosis. This may reflect a redundant signaling pathway around caspases in CEM and other leukemia cells or tissue-type selectivity of SAHA in activation of death signaling pathways. Activation of Fas by other HDACIs, such as apicidin in acute promyelocytic leukemia cells and *m*-carboxycinnamic acid *bis*-hydroxamide in neuroblastoma cells, has been shown (50, 51). The HDACIs trichostatin and valproic acid were recently shown to selectively increase the levels of Fas, FasL, DR5, and TRAIL in leukemia cells (52). In contrast, we found that SAHA did not up-regulate DR4 or DR5 and increased TRAIL only slightly in 22B HNSCC cells. Taken together, these findings suggest that the induction of apoptosis by SAHA involves different signaling pathways in different cell types. Further investigation of the downstream signaling factors activated by this agent is warranted.

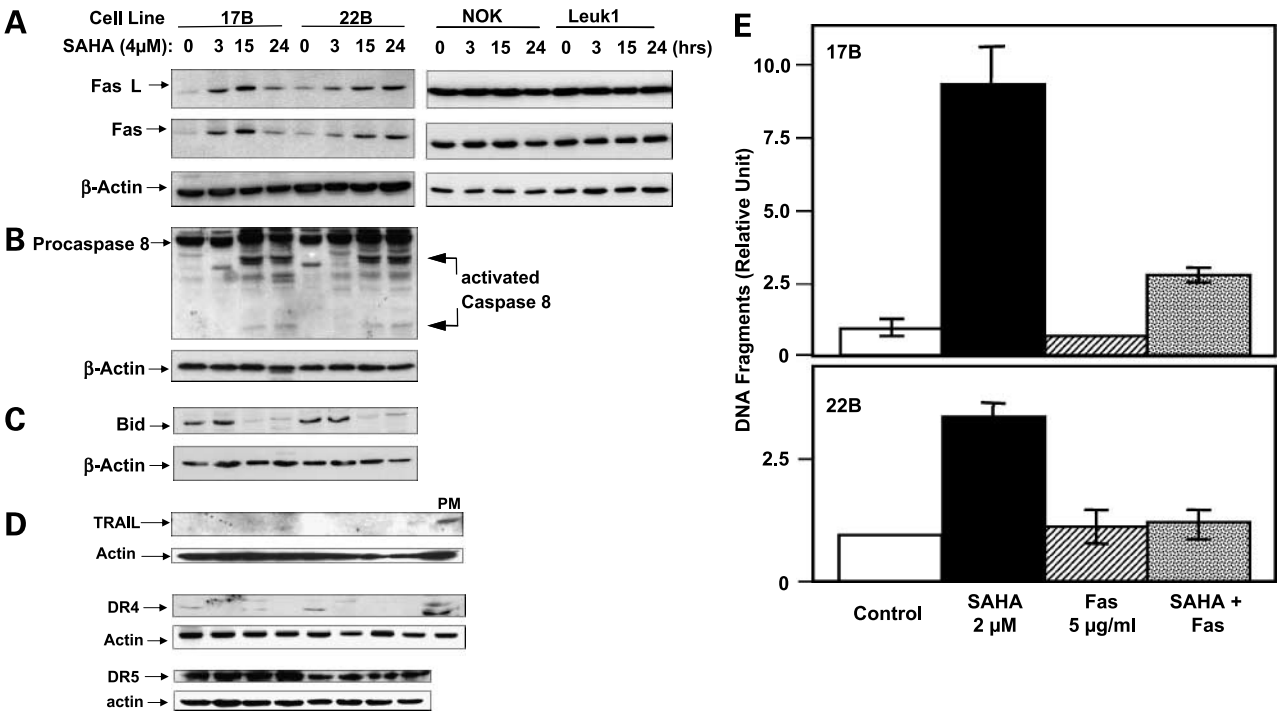

Figure 5. SAHA activates the extrinsic death pathway, with increased expression of Fas and FasL, in HNSCC cells. **A,** Fas and FasL protein levels were increased in both 17B and 22B cells after 3 h of exposure to SAHA. In contrast, there was no change in Fas and FasL expression in Leuk1 and NOK cells. Procaspase-8 was activated (**B**), and its substrate Bid was cleaved (**C**) after 3 h in 17B and 22B cells. **D,** SAHA did not affect the mRNA levels of the death receptors DR4 and DR5 and only slightly increased the level of their ligand, TRAIL, by 24 h, as detected by reverse-transcription PCR. *PM,* positive marker. **E,** incubation of 17B and 22B cells with soluble Fas, which competes for FasL, blocked SAHA-induced apoptosis. *Columns,* means of triplicate determinations; *bars,* SD.

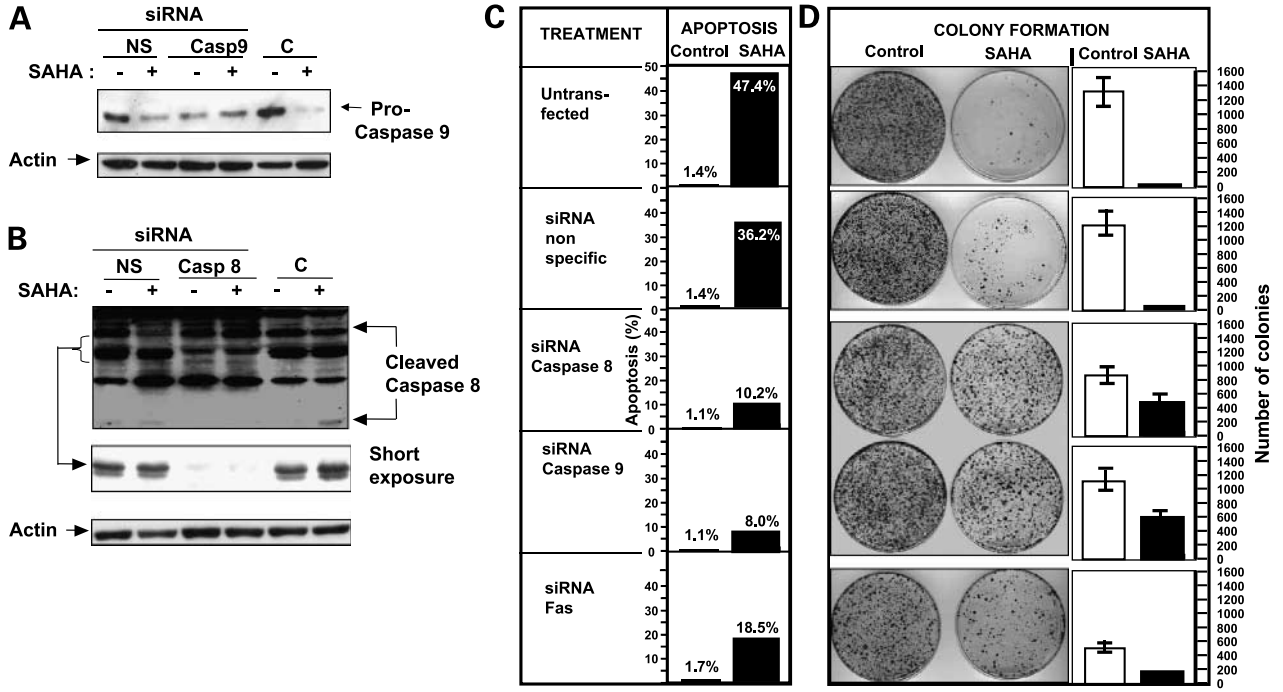

Figure 6. Inhibitory effects of siRNAs targeting caspase-8, caspase-9, and Fas on SAHA activity in HNSCC 22B cells. **A** and **B,** specific siRNA effectively blocked activation of caspase-9 (**A**) and caspase-8 (**B**) in SAHA-treated cells compared with nonspecific siRNA (*NS*) and control. **C** and **D,** siRNA targeting caspase-9, caspase-8, and Fas greatly reduced the ability of SAHA to induce apoptosis in 22B cells, as indicated by terminal deoxynucleotidyltransferase-mediated dUTP biotin nick-end labeling assay (**C**) and diminished the inhibition of anchorage-dependent colony formation by SAHA (**D**).

In conclusion, our results indicate that SAHA has growth inhibitory– and apoptosis-inducing effects in HNSCC cells *in vitro*. Furthermore, these results show for the first time that SAHA induces apoptosis in HNSCC cells through activation of the Fas/FasL death pathway in cancer cells although having comparatively little activity against pre-cancerous and normal oral cells with intrinsic Fas and FasL expression. A phase I clinical trial to assess the tolerability of oral SAHA in patients with HNSCC may shed light on the effects of this agent *in vivo*. Reports of synergistic antineoplastic activity of SAHA in combination with other molecularly targeted agents, such as imatinib (53) and flavopiridol (54), raise the possibility that combinations of SAHA with chemotherapeutic or biological agents may increase its activity against HNSCC. Translational investigations to determine whether Fas and FasL are induced by SAHA in HNSCC tumors *in vivo* and whether the reexpression of these death factors correlates with clinical response should further elucidate the mechanisms of action of this new HDAC agent in HNSCC.

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