Novel Histone Deacetylase Inhibitors in the Treatment of Thyroid Cancer

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Abstract

Histone deacetylases (HDAC) and histone acetyltransferases exert opposing enzymatic activities that modulate the degree of acetylation of histones and other intracellular molecular targets, thereby regulating gene expression, cellular differentiation, and survival. HDAC inhibition results in accumulation of acetylated histones and induces differentiation and/or apoptosis in transformed cells. In this study, we characterized the effect of two HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and m-carboxycinnamic acid bis-hydroxamide, on thyroid carcinoma cell lines, including lines originating from anaplastic and medullary carcinomas. In these models, both SAHA and m-carboxycinnamic acid bis-hydroxamide induced growth arrest and caspase-mediated apoptosis and increased p21 protein levels, retinoblastoma hypophosphorylation, BH3-interacting domain death agonist cleavage, Bax up-regulation, down-regulation of Bcl-2, A1, and Bcl-xL expression, and cleavage of poly(ADP-ribose) polymerase and caspase-8, -9, -3, -7, and -2. Transfection of Bcl-2 cDNA partially suppressed SAHA-induced cell death. SAHA down-regulated the expression of the apoptosis inhibitors FLIP and cIAP-2 and sensitized tumor cells to cytotoxic chemotherapy and death receptor activation. Our studies provide insight into the tumor type–specific mechanisms of antitumor effects of HDAC inhibitors and a framework for future clinical applications of HDAC inhibitors in patients with thyroid cancer, including histologic subtypes (e.g., anaplastic and medullary thyroid carcinomas) for which limited, if any, therapeutic options are available.

Thyroid cancer is the most prevalent endocrine neoplasia and is diagnosed each year in ~22,000 new cases (~16,300 in women and 5,700 in men) in the United States alone, and >1,500 patients are dying of thyroid cancer annually (1). For well-differentiated tumors, surgical resection and radioactive iodine can be an effective treatment. However, poorer prognosis is still associated with less differentiated histologic types, such as the tall-cell and the Hurthle variants. The even more undifferentiated anaplastic type carries an ominous prognosis (median overall survival of <6 months). Moreover, the histologically and phenotypically distinct medullary carcinomas, originating from the parafollicular C cells of the neural crest, also exhibit aggressive behavior and resist current therapeutic modalities.

A major mechanism controlling cellular differentiation and the biological behavior of cancer cells is the regulation of acetylation of lysine residues on their amino-terminal tails of histones. Histone acetylation modulates nucleosome and chromatin structure and regulates transcription factor accessibility and function (2–6). The turnover of histone acetylation is regulated by the opposing activities of histone acetyltransferases and histone deacetylases (HDAC). In general, chromatin composed of nucleosomes with underacetylated histones is transcriptionally silent. Importantly, dysregulated histone acetyltransferase or HDAC activity has been found in certain human cancers (7–10). In this manner, tumor cells are unable to undergo the normal cellular differentiation programs, which contributes to their neoplastic transformation.

These findings suggest that the histone acetyltransferase/HDAC function balance is a potential therapeutic target for human malignancy. Several compounds, such as butyrates, the anticonvulsant valproic acid and the antifungal agent trichostatin A, have been shown to act as HDAC inhibitors (11–14), but their clinical effectiveness has been limited by low potency, high toxicity, or poor stability. Depsipeptide (FR901228), a fermentation product isolated from Chromobacterium violaceum, has shown biological activity in a phase I trial in patients with refractory neoplasms (15). Recently, a class of
novel synthetic hybrid polar compounds with potent inhibitory effect on HDAC activity has been described. The prototypes of this class of compounds, hydroxamic acid–based suberoylanilide hydroxamic acid (SAHA) and m-carboxyaminoc acid bis-hydroxamid (CBHA), cause accumulation of acetylated histones in cultured cells, induce differentiation and/or apoptosis of transformed cells in culture (2–5, 10, 16, 17), and inhibit the growth of tumors in animals (14, 17). SAHA binds directly to the HDAC catalytic site, potently inhibiting enzymatic activity (18), and selectively induces the expression of specific genes, such as p21WAF1/CIP1 cyclin-dependent kinase inhibitor (19, 20). Induction of p21 mRNA by SAHA involves changes in promoter-associated proteins, including acetylation of core proteins, increased DNaSe I sensitivity, marked decrease in HDAC1 and Myc, and an increase in RNA polymerase II binding to the proximal portion of the p21 promoter (21). Because the growth-suppressive and apoptotic activity of these agents seems restricted to transformed cells (10), these novel HDAC inhibitors represent promising anticancer agents.

In this study, we characterized the effect of SAHA and CBHA on thyroid carcinoma cell lines. These novel HDAC inhibitors had activity against all cell lines tested and induced accumulation of p21 and caspase-mediated apoptosis associated with cleavage of BH3-interacting domain death agonist (Bid). These studies provide the framework for the clinical evaluation of HDAC inhibitors to overcome clinical drug resistance and improve clinical outcome in patients with thyroid carcinoma. Ongoing clinical evaluation in patients with solid tumors has revealed that SAHA is biologically active, as evidenced by histone acetylation in vivo, and well tolerated (22–24).

Materials and Methods

Cell lines. The SW579 cell line, derived from a poorly differentiated human thyroid adenocarcinoma (poorly differentiated carcinoma with nuclear features of papillary carcinoma and squamous differentiation), and the TT cell line, derived from a medullary thyroid carcinoma, were purchased from American Type Culture Collection (Manassas, VA). The anaplastic thyroid carcinoma cell lines FRO and ARO and the follicular carcinoma cell line WRO were a generous gift of Dr. James A. Fagin (University of Cincinnati School of Medicine, Cincinnati, OH; refs. 25, 26). All cells were grown in DMEM (BioWhittaker, Walkersville, MD) with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Life Technologies, Gaithersburg, MD), unless stated otherwise.

Reagents. SAHA and CBHA were obtained from Aton Pharma, Inc. (Tarrytown, NY). Administration of SAHA as an i.v. infusion at a dose of 300 mg/m² (which was determined to be the maximum tolerated dose for the hematology patients) resulted in an average plasma SAHA Cmax of 2,638 ng/ml (9.99 μM/L) in hematologic malignancies and as high as 3,298 ng/ml (12.46 μM/L) in solid malignancies. Intravenous infusion of SAHA at 600 mg/m² resulted in average Cmax as high as 10,815 mg/ml (40.96 μM/L), yet some toxicity was observed at this dose (22). These data suggest that the in vitro use of SAHA concentrations in the range of 1 to 10 μM/L is physiologically relevant and clinically achievable without prohibiting toxicity. Most of our studies were done with 1 to 5 μM/L HDAC concentrations.

The anti-Fas antibody CH-11 was from Panvera (Madison, WI) and the anti-CD3 antibody isTAC from Immunotech (Marseille, France). The anaplastic thyroid carcinoma cell lines FRO and ARO and the follicular carcinoma cell line WRO were from American Type Culture Collection (Manassas, VA). The anti-CD3 antibody isTAC from Immunotech (Marseille, France).

Immunoblotting analysis. Immunoblotting analysis was done as described previously (28). The antibodies used were as follows: mouse monoclonal antibodies for Bcl-2, Bcl-xL, A1, Bax, and tubulin and polyclonal antibodies for caspase-3 and -9 (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal antibody for caspase-8 inhibitor IETD-FMK, the caspase-9 inhibitor LEHD-FMK, or the caspase-3/7 inhibitor DEVD-FMK (all used at 20 μM/L and all from Oncogene Research) for 1 hour before exposure to SAHA (5 μM/L for 36 hours). Quantification of cell death was done by measuring the activity of lactate dehydrogenase released from the cytosol of damaged cells into the culture supernatant using the Cytotoxicity Detection kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the instructions of the manufacturer.

Immunoblotting analysis. Immunoblotting analysis was done as described previously (28). The antibodies used were as follows: mouse monoclonal antibodies for Bcl-2, Bcl-xL, A1, Bax, and tubulin and polyclonal antibodies for caspase-3 and -9 (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal antibody for caspase-8 and polyclonal antibodies for FLIP and phospho-retinoblastoma (Rb; Upstate Biotechnology); monoclonal antibody for poly(ADP-ribose) polymerase (Biomol); polyclonal antisera against cIAP-2 (R&D Systems, Inc., Minneapolis, MN); monoclonal antibody for p21 (Oncogene Research); polyclonal antisera against Bid and caspase-2 and -7 (Cell Signaling, Beverly, MA); Complete mixture of proteinase inhibitors and SDS (Life Technologies); and enhanced chemiluminescence kit, which includes the peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies (Amersham, Arlington Heights, IL).

Effect of suberoylanilide hydroxamic acid in thyroid carcinoma cells overexpressing Bcl-2. To evaluate the role of the antiapoptotic

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molecule Bcl-2 in SAHA-induced cell death, anaplastic carcinoma FRO cells were stably transfected with a vector carrying the Bcl-2 cDNA (Upstate Biotechnology) or the empty (neo) vector using LipofectAMINE 2000 according to the instructions of the manufacturer. Forty-eight hours later, the cells were incubated in growth medium containing G418 (500 μg/mL, Life Technologies) to select pools of stable clones that were subsequently treated with SAHA (2.5-10 μmol/L for 36 hours). The overexpression of Bcl-2 in transfected cells has been confirmed by immunoblotting.

Statistical analysis. Statistical significance was examined by two-way ANOVA followed by Duncan’s post hoc test. In all analyses, P < 0.05 was considered statistically significant.

Results

Suberoylanilide hydroxamic acid induces accumulation of acetylated histones in thyroid carcinoma cells. We first investigated the effect of SAHA and CBHA on histone acetylation status in anaplastic thyroid carcinoma FRO cells. FRO cells treated with SAHA or CBHA for 8 hours exhibited increased acetylation of histones H3 and H4 compared with controls (Fig. 1A). Protein loading was monitored by Coomassie blue staining. A dose-response experiment for levels of acetylated H3 in FRO cells treated with SAHA (1-10 μmol/L) is shown in Fig. 1B.

Suberoylanilide hydroxamic acid induces growth arrest and apoptosis in thyroid carcinoma cells. We next investigated the effect of SAHA and CBHA on growth and survival of thyroid carcinoma cells. Treatment of papillary (SW579), follicular (WRO), anaplastic (FRO and ARO), and medullary (TT) thyroid carcinoma cells with SAHA or CBHA (2.5, 5, and 10 μmol/L) for 48 hours potently suppressed cellular proliferation as quantified by bromodeoxyuridine incorporation (Fig. 2).

Cell cycle analysis by propidium iodide of FRO cells treated with SAHA or CBHA (1 μmol/L for 24-72 hours) revealed inhibition of proliferation and induction of apoptosis (Fig. 3). Treatment of FRO, TT, and ARO cells with SAHA at the concentration determined to inhibit 50% of cell proliferation (bromodeoxyuridine incorporation in Fig. 2), which was 3.85, 1.7, and 2 μmol/L, respectively, resulted in 97 ± 0.5%, 85 ± 2%, and 55 ± 3% decrease in colony formation, respectively, suggesting that SAHA severely impairs the long-term growth potential of thyroid carcinoma cells.

Suberoylanilide hydroxamic acid increases p21 protein levels and decreases phosphorylation of retinoblastoma. To detect the mediators of SAHA-induced growth arrest, we evaluated the levels of p21 and p53 by immunoblotting analysis. SAHA rapidly increased p21 protein levels in anaplastic thyroid carcinoma FRO cells (Fig. 4). Because p21 induces growth arrest by inhibiting the ability of the cyclin E/cyclin-dependent kinase2 complex to phosphorylate the cell cycle
We investigated the effect of SAHA on the phosphorylation status of Rb. We found that SAHA profoundly and rapidly decreased the phosphorylation of Rb. These data suggest that SAHA-induced growth arrest occurs via p21-mediated inhibition of Rb phosphorylation. In addition, SAHA transiently stimulated the expression of the proapoptotic Bax.

**Involvement of caspases, but not p53, in suberoylanilide hydroxamic acid–induced apoptosis.** We subsequently assessed the functional role of caspases in SAHA-induced apoptosis in thyroid carcinoma cells. We found by immunoblotting that SAHA induced cleavage of caspase-8 and -9 followed by caspase-3, -7, and -2 in our model (Fig. 5A). We also found that SAHA down-regulated the expression of Bcl-2, A1, and Bcl-xL, thus shifting the balance toward the proapoptotic members of the Bcl-2 family. Therefore, we hypothesized that inhibition of Bid-induced mitochondrial events would protect from SAHA-induced cell death. Indeed, overexpression of Bcl-2 in thyroid carcinoma cells partially protected them from SAHA-induced cell death (Fig. 6B). These data suggest a role for mitochondria and the Bcl-2 family members in SAHA-induced apoptotic signaling.

**Suberoylanilide hydroxamic acid sensitizes thyroid carcinoma cells to death receptor–induced cell death.** We also investigated the effect of SAHA on cell death induced by cell surface death receptors. SAHA sensitized thyroid carcinoma cells to cell death mediated by cross-linking Fas with the CH-11 antibody and to cell death induced by TRAIL/Apo2L (Fig. 7A). This sensitizing effect was associated with decreased expression of the anti-apoptotic proteins FLIP and cIAP-2 (Fig. 7B).

**Suberoylanilide hydroxamic acid sensitizes thyroid carcinoma cells to cytotoxic chemotherapy.** Finally, we studied the effect of SAHA on the sensitivity of thyroid carcinoma cells to DNA damage using as a model the chemotherapeutic agent doxorubicin. We treated SW579, FRO, and TT cells concurrently with doxorubicin (0.25 μg/mL) and SAHA (at indicated concentrations) for 48 hours. Thyroid carcinoma cells are relatively resistant to conventional chemotherapy, but cotreatment with SAHA had a strong synergistic effect.
(Fig. 8). The LD$_{50}$ for SAHA alone in this experiment was 7.66, 2.38, and 0.72 μmol/L for SW579, FRO, and TT cells, respectively, compared with 1.68, 0.39, and 0.38 μmol/L, respectively, for treatment with SAHA in the presence of doxorubicin.

**Discussion**

From a clinical and biological standpoint, thyroid cancers represent a broad spectrum of neoplastic disorders that include histologic subtypes, such as poorly differentiated, anaplastic and medullary thyroid carcinomas, which are refractory to most conventional systemic anticancer therapeutic strategies, highlighting the need for novel therapeutic strategies. In this study, we evaluated the effects of the novel, potent HDAC inhibitors SAHA and CBHA in a panel of thyroid carcinoma cell lines and found that they potently suppress proliferation and induce apoptosis. Prior studies have shown that the HDAC inhibitor depsipeptide enhances apoptotic killing by $p$53 gene therapy in thyroid carcinoma cells (31) and increases expression of the Na$^+$/I$^-$ symporter and iodine accumulation in poorly differentiated thyroid carcinoma cells (32). Moreover, the HDAC inhibitors sodium butyrate and trichostatin A induce cell cycle arrest and promote apoptosis in anaplastic thyroid cancer cells (33). The novel synthetic hybrid polar compounds, such as SAHA and CBHA, potently inhibit HDAC activity and induce accumulation of acetylated histones, differentiation, and/or apoptosis of...
transformed cells in vitro (2–5, 10, 16, 17). SAHA suppresses the growth of prostate carcinoma cell lines at micromolar concentrations (2.5-7.5 μmol/L) in vitro (17) and induces accumulation of acetylated histones in tumor tissue from xenografts of human prostate carcinomas and neuroblastomas (34) in nude mice. In this study, we show potent activity of SAHA and CBHA against a panel of thyroid carcinoma cell lines that includes lines originating from papillary, follicular, anaplastic, and medullary carcinomas. The activity of these novel agents against medullary carcinoma cells is particularly impressive, because this type of tumor resists current chemotherapeutic approaches.

SAHA and CBHA induced accumulation of acetylated histones in our model, early up-regulation of the cyclin-dependent kinase inhibitor p21, decrease in phosphorylation of the cyclin-dependent kinase substrate Rb, followed by growth arrest and apoptosis. It should be noted that the SW579, ARO, and WRO cell lines carry mutant p53 (26, 35) and that the FRO line expresses very low levels of p53 mRNA (26), which did not increase with SAHA treatment. Therefore, p21 up-regulation seems to be p53 independent, consistent with studies by Richon et al. (19), Huang et al. (20), and Vrana et al. (36), showing that

SAHA-induced up-regulation of p21 is mediated by Sp1 sites in the p21 promoter and is p53 independent. Therefore, HDAC inhibitors, such as SAHA and CBHA, are expected to be active even against malignant cells with defects in the p53 pathway.
This is of particular clinical importance because p53 mutations are among the most common genetic aberration in human cancer in general and because they are also very frequent in poorly differentiated and anaplastic thyroid cancer (26).

We then investigated the mechanism of SAHA-induced apoptosis in our model and detected cleavage of caspase-8 and -9 and (later) caspase-3, -7, and -2. Moreover, poly(ADP-ribose) polymerase was cleaved into an 85-kDa fragment, indicating caspase activation. In support, the pan-caspase inhibitor ZVAD-FMK completely abolished SAHA-induced apoptosis in FRO cells, and the specific caspase-8, -9, and -3/2 inhibitors, respectively, exerted a significant protective effect. Therefore, in this model, SAHA-induced apoptosis seems to be caspase mediated. The role of caspases in HDAC inhibitor–induced apoptosis seems to be tumor type or tissue specific. We and others have reported cleavage of Bid and caspase-independent cell death in SAHA-treated malignant cells of hematopoietic origin (37, 38), whereas, in solid tumor models, HDAC inhibitor–induced cell death is caspase mediated (39, 40).

SAHA treatment of multiple myeloma cells irreversibly commits them to apoptosis within 8 hours as well, as documented by experiments where the drug was washed away after brief exposures and the cells were further incubated in drug-free medium to detect the resulting apoptosis (41). In agreement, SAHA treatment of thyroid carcinoma cells in the present study resulted in evidence of caspase cleavage as early as within 8 hours of incubation, suggesting early commitment to apoptosis.

The induction by SAHA of a dual apoptotic cascade mediated by activation of both caspase-8 and -9 prompted us to further investigate the involvement of death receptor–mediated and mitochondrial regulators of apoptosis. Activation of caspase-8 is the initiator of death receptor (e.g., Fas or TRAIL receptor)–mediated apoptosis (42, 43), whereas caspase-9 activation is a nodal point for mitochondrial-induced apoptotic signaling (44). There is also cross-talk between the two apoptotic pathways, because caspase-8 can cleave the Bcl-2 family member Bid, which then translocates to the mitochondria and results in an allosteric activation of Bak and Bax, inducing their intramembranous oligomerization that leads to mitochondrial dysfunction (30).

Cleavage of Bid was also detected in our model together with transient up-regulation of Bax on SAHA treatment. Moreover, SAHA down-regulated the levels of Bcl-2, A1, and Bcl-xL, suggesting that SAHA-induced apoptosis can be related to a shift in the balance between proapoptotic and antiapoptotic members of the Bcl-2 family toward the proapoptotic side. In support of these findings, overexpression of Bcl-2 in thyroid carcinoma cells partially attenuated SAHA-induced cell death. The degree of antiapoptotic protective effect of Bcl-2 overexpression varies between experimental models and proapoptotic stimuli (45, 46), ranging between complete suppression of apoptosis and no effect. In experimental models where the proapoptotic agent efficiently activates caspase-8, apoptosis proceeds in a mitochondrial-independent manner and overexpression of Bcl-2 has no protective effect (47). On the contrary, overexpression of Bcl-2 completely abrogates apoptosis in models where caspase-8 is inefficiently activated and the apoptotic pathway requires mitochondrial involvement (47). Our data suggest that SAHA-induced apoptosis in thyroid carcinoma cells is at least partially mediated by the mitochondria. Multiple myeloma cells, on the other hand, are completely protected against SAHA-induced cell death by overexpression of Bcl-2 (37), suggesting a greater importance of mitochondrial-dependent signaling in that model.

We next investigated the effect of SAHA on apoptosis induced by the cell surface death ligands FasL and TRAIL via their respective receptors Fas and TRAIL-R1(DR4)/TRAIL-R2(DR5) (ref. 48). FasL and TRAIL are death ligands expressed by activated immune effector cells and participate in cell-mediated cytotoxicity and antitumor surveillance (48). Modulation of cancer cell sensitivity to the Fas and TRAIL apoptotic pathways could affect their response to immune-based therapeutic approaches. This is of particular importance in thyroid carcinomas that are frequently associated with an intense inflammatory reaction (’peritumoral thyroiditis’; ref. 49). Thyroid carcinoma cells are sensitive to apoptosis induced by TRAIL and resistant to FasL (50). We now found that SAHA sensitized thyroid carcinoma cells to the Fas-activating monoclonal antibody CH-11 and to a low, subtoxic concentration of recombinant TRAIL. Therefore, HDAC inhibitors may modulate immune responsiveness of tumor cells and could be useful to overcome refractoriness to immune-based therapies. Furthermore, these data indicate that HDAC inhibitors can influence the interaction of the tumor cells with their local microenvironment. This sensitizing effect was associated with decreased expression of the antiapoptotic proteins FLIP and cIAP-2 as well as up-regulation of TRAIL-R1 and TRAIL-R2 and, to a lesser extent, Fas expression. FLIP inhibits death receptor–induced apoptosis in thyroid carcinomas (51) and the protein synthesis inhibitor cycloheximide, the protein kinase C inhibitor bisindolylmaleimide, and a FLIP antisense oligonucleotide sensitize thyroid carcinoma cells to death receptor–mediated apoptosis by down-regulating FLIP (51). However, none of these reagents represents a currently available clinical approach for the treatment of aggressive thyroid cancer.

Our study identifies SAHA as the first clinically applicable pharmacologic agent that can down-regulate FLIP and increase sensitivity to TRAIL and, more importantly, restore sensitivity to Fas-mediated apoptosis.

Thyroid carcinomas are generally poorly responsive to cytotoxic chemotherapy, which could be attributed to the presence of intracellular inhibitors of the apoptotic signaling cascade. Having shown the ability of HDAC inhibitors to down-regulate the expression of antiapoptotic proteins, such as Bcl-2, Bcl-xL, cIAP-2, and FLIP, and to up-regulate expression of proapoptotic Bax, we investigated its effect on doxorubicin-induced cell death and found that HDAC inhibition confers to thyroid cancer cells a potent chemosensitizing effect. This finding suggests that novel therapies combining SAHA with conventional chemotherapy could improve the outcome in aggressive thyroid cancer.

The clinical applicability of SAHA administration in cancer patients is highlighted by the early experience generated from phase I clinical trials, where SAHA, despite a relatively short plasma half-life after i.v. infusion, was found to be biologically active (inducing accumulation of acetylated histones in vivo for at least 4 hours after infusion), with an acceptable safety profile (22). There was also early evidence of antitumor activity (22–24), including reduction in measurable disease in refractory papillary thyroid cancer (52). Early clinical studies of an oral SAHA formulation are currently under way (52).

In conclusion, we have evaluated the effects of HDAC inhibition in a panel of thyroid carcinoma cell lines using...
the novel HDAC inhibitors SAHA and CBHA. We found that these HDAC inhibitors suppressed growth in all cell lines tested, including those with defects in the p53 pathway. SAHA up-regulated p21 expression, promoted cleavage of Bid, down-regulated Bcl-2, A1, and Bcl-xL, and induced caspase-mediated apoptosis. SAHA sensitized thyroid cancer cells to apoptosis mediated by death receptors and cytotoxic chemotherapy. These findings provide the preclinical rationale for clinical studies of SAHA, either as a monotherapy or in combination with other anticancer therapies, in an effort to improve the clinical outcome of patients with aggressive cases of thyroid cancer.

References

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