Antitumor Activity of Suberoylanilide Hydroxamic Acid against Thyroid Cancer Cell Lines In vitro and In vivo

Quang T. Luong, James O’Kelly, Glenn D. Braunstein, Jerome M. Hershman, and H. Phillip Koeffler

Abstract

Purpose: The histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), has multiple antitumor effects against a variety of human cancers. Experimental design: We treated several anaplastic and papillary thyroid cancer cell lines with SAHA to determine if it could inhibit the growth of these cells in vitro and in vivo. Results: SAHA effectively inhibited 50% clonal growth of the anaplastic thyroid cancer cell lines, ARO and FRO, and the papillary thyroid cancer cell line, BHP 7-13, at 1.3 × 10^{-7} to 5 × 10^{-7} mol/L, doses that are achievable in patients. In concert with growth inhibition, SAHA down-regulated the expression of cyclin D1 and up-regulated levels of p21WAF1. Annexin V and cleavage of poly(ADP)ribose polymerase were both increased by exposure of the thyroid cancer cells to SAHA. Expression of the death receptor 5 (DR5) gene was also increased by SAHA, but the combination of the DR5 ligand, tumor necrosis factor – related apoptosis-inducing ligand (TRAIL), with SAHA had little effect compared with SAHA alone. Of note, the combination of paclitaxel, doxorubicin, or paraplatin with SAHA enhanced cell killing of the thyroid cancer cells. In addition, murine studies showed that SAHA administered daily by i.p. injection at 100 mg/kg inhibited the growth of human thyroid tumor cells. Conclusion: Our data indicate that SAHA is a plausible adjuvant therapy for thyroid cancers.

Histone acetyltransferase and histone deacetylases (HDAC) have antagonistic actions on histones depending on the state of the cells (1, 2). Often, DNA methylation and histone deacetylation of tumor suppressor genes occur in many human cancers, leading to suppression of function of these genes and thereby conferring a growth advantage for the tumor cells (3, 4). HDAC inhibitors (HDACi), such as suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA), can directly interact with HDAC enzymes at the catalytic site and inhibit their functions (5, 6). This leads to acetylation of histones, which "opens up" the chromatin structure, allowing transcription of antigrowth and proapoptotic genes. In addition, the open chromatin structures provide additional target sites for DNA-damaging anticancer drugs, such as cisplatin and paraplatin.

In thyroid cancers, current therapy uses surgery to remove all, or nearly all, of the thyroid gland. Usually, surgery is followed by 131I therapy to ablate any remaining thyroid cells. Chemotherapy and external beam radiotherapy have had limited use (7, 8). In this study, we show that SAHA has antitumor activities against thyroid cancers both in vitro as well as those growing in immunodeficient mice. Furthermore, SAHA, when combined with chemotherapy agents, e.g., paclitaxel, doxorubicin, or paraplatin, had enhanced anticancer activity.

Materials and Methods

Cell culture and treatments. Papillary thyroid cancer cell lines BHP 2-7, BHP 7-13, BHP 10-3, and BHP 18-21 (9), and the anaplastic thyroid cancer cell lines, ARO and FRO (obtained from Dr. Guy Juillard, University of California at Los Angeles, Los Angeles, CA), were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were incubated at 37°C with 5% CO₂. Cultured cells were treated with the following agents either alone or in combinations as described in the text: SAHA (100 nmol/L-5 mmol/L), VPA (50 μmol/L-5 mmol/L), tumor necrosis factor – related apoptosis-inducing ligand (TRAIL, 10-40 ng/mL), paraplatin (10-100 ng/mL), doxorubicin (10-100 ng/mL), and paclitaxel (0.5-5 ng/mL). All treatments were done for the period of time indicated in the text.

Clonogenic soft agar assay. Cells were plated into 24-well, flat-bottomed plates using a two-layer soft agar system with 1 × 10³ per well in a volume of 400 μL/well as previously described (10). After 14 days...
of incubation, the colonies were counted. All of the experiments were done in triplicate wells on each plate and in triplicate plates per experimental point.

Cell proliferation (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were plated at $1 \times 10^5$ per well in 96-well plates in 100 µL medium and allowed to adhere to the plastic for 12 to 24 hours. Drugs were added as described and incubated at 37°C for the desired length of time. Ten microliters of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Aldrich, St. Louis, MO) were added and the cells were incubated at 37°C for 4 hours. Following this, the medium was removed and 50 µL DMSO were added to the cells to solubilize the MTT. Plates were read at wavelength of 540 nm on a plate reader. For each treatment, triplicate wells were used and the experiments were done thrice.

Additive model. The effect of combinations of drugs used in this study was assessed using an additive model (11, 12). The effect on cell proliferation by each drug alone is multiplied together to give the expected value ($E$) for the combination of drugs. The actual effect on cell proliferation when drugs are combined is the observed value ($O$). The additive model predicts whether the combined effects of two or more drugs are synergistic when the ratio $O/E < 0.8$; additive when $O/E = 0.8-1.2$; or subadditive when $O/E > 1.2$.

Annexin V staining. Cells were treated with the appropriate drugs as described and labeled with FITC-conjugated Annexin V antibody and propidium iodide using Annexin V–FITC Apoptosis Detection kit I (BD Biosciences, San Jose, CA) according to the instructions from the manufacturer. Positive cells were detected by fluorescence-activated cell sorting.

Western analysis. Cells were harvested and total cell lysates were prepared by lysing cells in radioimmunoprecipitation assay buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.5)] containing a mixture of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) as well as 1 mmol/L NaF and 1 mmol/L NaVO₄. Insoluble debris was removed by centrifugation at 13,000 rpm for 10 minutes at 4°C. Supernatants were collected, and the protein concentrations were determined spectrophotometrically. The lysates (30 µg) were denatured in the sample buffer [10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 62.5 mmol/L Tris-HCl (pH 6.8)] by boiling and then subjected to 4% to 15% SDS-PAGE followed by electrotransfer to polyvinylidene difluoride membrane. The immunocomplexes were visualized with either Supersignal West Pico Chemiluminescent Substrate or Supersignal West Dura extended duration substrate (Pierce, Rockford, IL) and normalized by internal control (actin). Antibodies were purchased from Santa Cruz Biotechnology, Inc. [Santa Cruz, CA; cyclin D1, p21WAF1, poly(ADP)ribose polymerase (PARP), β-actin] and Cell Signaling Technology (Danvers, MA; acetylated histone H4, 4E-BP1, and phosphorylated 4E-BP1).

Reverse transcription-PCR and quantitative real-time PCR. RNA was prepared from thyroid cancer cell lines and normal thyroid using Trizol reagent (Invitrogen). cDNA was prepared from 1 µg total RNA using Superscript II reverse transcription kit (Invitrogen). Levels of death receptor 5 (DR5) gene expression were normalized to β-actin expression. Real-time PCR primer sequences for DR5 and β-actin were as follows: DR5 5’ primer AGGTGGACACAATCCCTCTG; DR5 3’ primer AGTGAGCAAAATCCCTCTG; β-actin 5’ primer
CTACCCTGAAAGTACCCCATCG; β-actin 3’ primer CTGCTGATCCACACTCTGCTGG. A typical reaction contains 1× PlatinumTaq buffer (Invitrogen), 1.5 mmol/L MgCl2, 0.25 mmol/L deoxynucleotide triphosphates, 0.25 μmol/L primers and 1 unit PlatinumTaq (Invitrogen), 1× Sybr Green I dye, and 10 nmol/L fluorescein.

Murine studies. FRO human anaplastic thyroid cancer cells (1 × 10⁶) were resuspended in 100 μL Matrigel (BD Biosciences) for each tumor. Cells were injected s.c. on both flanks of immunodeficient BNX mice. SAHA was freshly prepared in DMSO at 200 mg/mL each day for injection. The treatment group (n = 8) received 100 mg SAHA per kilogram mass daily via i.p. injections. Control mice (n = 8) remained untreated. The sizes of the tumors were measured at regular intervals, and their volume was calculated by the following formula: A (length) × B (width) × C (height) × 0.5236. All measurements were made in millimeters. All mice were euthanized at the end of the study, and the final mass of the tumors was determined by weight after dissection. Data were analyzed by Student’s t test.

Results

HDACIs inhibit growth of thyroid cancer cell lines in vitro. The in vitro growth of anaplastic thyroid cancer cell lines, ARO and FRO, and papillary thyroid cancer cell line, BHP 7-13, was inhibited by both SAHA and VPA (Fig. 1 and Table 1). In clonogenic soft agar assays (Fig. 1A), BHP 7-13 showed the greatest inhibition (ED50: 1.3 × 10⁻⁷ mol/L SAHA) and FRO showed the least inhibition (ED50: 5 × 10⁻⁷ mol/L SAHA; Fig. 1B). Total inhibition of growth of each of the cell lines occurred at 2 × 10⁻⁶ mol/L SAHA. Similarly, VPA had antigrowth activity against thyroid cancer cell lines. However, the IC50 of VPA was much higher than those of SAHA (Fig. 1A). Using the less sensitive MTT assay, SAHA inhibited both the papillary and anaplastic thyroid cancer cell lines in a parallel fashion (Fig. 1C).

SAHA induces apoptosis in thyroid cancer cell lines. Apoptosis of ARO, FRO, and BHP 2-7 thyroid cancer cells was assessed

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>ED50 [M]</th>
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<tbody>
<tr>
<td>ARO</td>
<td>Anaplastic</td>
<td>3 × 10⁻⁷</td>
</tr>
<tr>
<td>FRO</td>
<td>Anaplastic</td>
<td>5 × 10⁻⁷</td>
</tr>
<tr>
<td>BPH 7-13</td>
<td>Papillary</td>
<td>1.3 × 10⁻⁷</td>
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NOTE: The ED50 values represent the dose of drug required to inhibit 50% clonogenic growth in soft agar compared with untreated cultures. These values were derived graphically from Fig. 1A.
by measurement of Annexin V by fluorescence-activated cell sorting after the cells were cultured with $5 \times 10^{-7}$ mol/L SAHA for 12, 24, and 48 hours. The percentage of apoptotic cells (Annexin V-positive/propidium iodide negative) present was 24% for ARO and 28% for FRO at 48 hours (Fig. 2). Earlier time points (12 and 24 hours) did not exhibit much apoptosis compared with untreated controls (Fig. 2). Because growth inhibition occurred in both anaplastic and papillary thyroid cancer cell lines in response to SAHA treatment, we decided to focus on anaplastic tumor cell lines as these are likely to be the least responsive to current therapy for thyroid cancer.

**Effect of SAHA on expression of proteins related to apoptosis and cell cycle regulation.** Treatment of ARO cells with 0, $1 \times 10^{-6}$, and $5 \times 10^{-6}$ mol/L SAHA for 12, 24, and 36 hours resulted in reduction of cyclin D1 expression at 24 and 36 hours (Western blot analysis; Fig. 3A). In addition, levels of cyclin-dependent kinase inhibitor, p21WAF1, were undetectable in the untreated cell lines and prominently increased at 12, 24, and 36 hours exposure to $5 \times 10^{-6}$ mol/L SAHA. Cleavage of PARP (activation of caspase-3) was seen at 24- and 36-hour exposure to $5 \times 10^{-6}$ mol/L SAHA. Increased acetylation of histone H4 was also observed in the SAHA-treated cells at each exposure time and each SAHA concentration. A study by Gao et al. (13) showed that SAHA treatment of leukemic and lymphoma cell lines inhibited phosphorylation of AKT. In this respect, we tested whether SAHA was able to block phosphorylation of 4E-BP1 (a component downstream of the AKT signaling pathway). In the ARO thyroid cancer cell line, 4E-BP1 expression increased over time. Similarly, phosphorylated 4E-BP1 also increased. Treatment with SAHA inhibited phosphorylation of 4E-BP1 at 12 hours compared with untreated control; and at longer time points, SAHA downregulated the expression of total 4E-BP1 (Fig. 3A).

**SAHA activates the death receptor pathway in anaplastic thyroid cancer cells.** Expression of the DR5 gene was measured by real-time PCR after treatment with either diluent (DMSO, control), $1 \times 10^{-7}$ or $1 \times 10^{-6}$ mol/L SAHA for 36 hours (Fig. 3B). Increased expression of DR5 was evident in both ARO and FRO anaplastic thyroid cancer cell lines. ARO cells displayed a 6-fold increase in DR5 expression with $5 \times 10^{-6}$ mol/L SAHA treatment, and FRO cells had >2-fold increase with both doses of SAHA.

The ligand for DR5 is TRAIL; we examined whether cell killing by TRAIL would be enhanced by the increased expression of DR5 mediated by SAHA. Cell viability was assessed by MTT assays at 48 hours. Treatment of anaplastic thyroid cancer cells with a relatively low dose of TRAIL resulted in 15% to 20% cell death of ARO and FRO anaplastic thyroid cancer cell lines (Fig. 4). The combination of SAHA ($5 \times 10^{-7}$, $1 \times 10^{-6}$, $2 \times 10^{-6}$, and $5 \times 10^{-6}$ mol/L) with either 10, 20, or 40 ng/mL TRAIL had nearly the same antiproliferative activity as SAHA alone (Fig. 4).

**SAHA effectively enhances cell killing by chemotherapeutic drugs.** We next tested whether SAHA can enhance cell killing by chemotherapy drugs using the anaplastic thyroid cancer cell line, ARO. Both paraplatin and doxorubicin were efficient at killing ARO cells. Paraplatin treatment resulted in 60% cell death at 50 ng/mL and >80% cell death at 100 ng/mL (Fig. 5A); doxorubicin produced >50% growth inhibition at 100 ng/mL (Fig. 5B). Paclitaxel was highly potent, showing over 80% growth inhibition of ARO cells at 5 ng/mL but only 10% inhibition of growth at 0.5 to 1 ng/mL (Fig. 5C).

We observed mostly additive killing by the combination of SAHA and chemotherapeutic agents (Fig. 5D). However, synergistic interactions occurred between paraplatin (50 ng/mL) and SAHA ($5 \times 10^{-7}$ - $1 \times 10^{-6}$ mol/L; O/E = 0.7 and 0.7, respectively). The combination of doxorubicin (100 ng/mL) with SAHA ($1 \times 10^{-6}$ - $2 \times 10^{-6}$ mol/L; O/E = 0.7 and 0.7, respectively) or paclitaxel (1 ng/mL) with SAHA ($5 \times 10^{-7}$ - $2 \times 10^{-6}$ mol/L; O/E = 0.6 and 0.6, respectively) was also synergistic (Fig. 5D). Thus, the combination of SAHA with either of these chemotherapeutic drugs can enhance the effectiveness of each drug alone.

**SAHA inhibits thyroid cancer cell growth in vivo.** We tested the ability of SAHA to kill human thyroid cancer cells growing in vivo by treatment of athymic nu/nu mice bearing thyroid cancer xenografts with SAHA at 0, 1, and 10 mg/kg for 5 days. There was more than a 10-fold increase in the growth delay of the SAHA-treated tumors compared with those of control tumors (Fig. 5E). The combination of SAHA and chemotherapy drugs was also synergistic in vivo (Fig. 5F). These results suggest that SAHA is a promising agent for the treatment of thyroid cancer.

Fig. 3. Actions of SAHA on the expression of cell cycle- and apoptosis-regulated proteins, histone acetylation, and DR5. A, Western blot analysis of thyroid cancer cell line, ARO, treated with diluent control and $1 \times 10^{-6}$ and $5 \times 10^{-6}$ mol/L SAHA for 12, 24, and 36 hours. Cleavage of PARP (activation of caspase-3) was seen at 24- and 36-hour exposure to $5 \times 10^{-6}$ mol/L SAHA. Increased acetylation of histone H4 was also observed in the SAHA-treated cells at each exposure time and each SAHA concentration. B, quantitative real time reverse transcription-PCR analysis of DR5 mRNA expression in thyroid cancer cell lines following treatment with SAHA (36 hours).
in vivo. Immunodeficient mice were injected with FRO anaplastic thyroid cancer cells on both flanks (two tumors per mouse) and subjected to i.p. injections of SAHA at 100 mg/kg (eight mice), 5 d/wk, for 7 weeks. The control group (eight mice) was not treated. Figure 6A shows two representative mice from each group at the end of the experiment. Control mice had obvious large tumors, whereas mice from the SAHA-treated group possessed much smaller tumors. The growth of the tumors was measured at regular intervals during this period, and the volume was calculated by the formula described in Materials and Methods. Figure 6B shows the mean increase in tumor size for each group. Tumors from control mice were approximately thrice the size of those in the SAHA-treated mice at the end of the experiment. Similarly, the difference in tumor mass was evident at the end of the experiment with control tumors weighing on average 650 mg, whereas those from SAHA-treated mice weighed a mean 200 mg (Fig. 6C).

Following the final SAHA injection, one mouse was sacrificed at each of the specified time points as indicated in Fig. 6D. Tumors were harvested and proteins were extracted for Western blot analysis. In concordance with our in vitro thyroid cancer cell line data, SAHA increased acetylation of histone H4 (24 hours after SAHA injection) and blocked phosphorylation of 4E-BP1 (24 and 48 hours after SAHA injection) in tumors resected from the experimental mice (Fig. 6D).

Discussion

HDACIs inhibit acetylation of histones H3 and H4 and other key proteins, resulting in up-regulation of tumor suppressor, proapoptotic, and growth-inhibitory genes in various types of cancers (14–16). Studies by our laboratory have shown that SAHA has profound antigrowth activity
against human lymphoma cells (17), as well as endometrial and ovarian cancer cells (18, 19). Others have shown antiproliferative effects of SAHA against other human cancer cells [breast cancer (20) and non–small cell lung carcinoma (21)]. These studies highlight the observation that the inhibitory activity of SAHA on cancer cell growth spans many tissue types, suggesting it can be a useful agent for the treatment of a wide variety of malignancies. In addition, we and others have shown that SAHA blocks signaling through the AKT pathway (13). This pathway is key for efficient cellular proliferation (22).

In an initial phase I clinical trial of orally administered SAHA, 73 patients with a variety of hematologic and solid tumors received the drug at a variety of doses. Twenty-two

Fig. 5. Effect of SAHA and chemotherapeutic drugs on growth of thyroid cancer cell line ARO. SAHA, combined with paraplatin (A), doxorubicin (B), or paclitaxel (C) for 48 hours. ***, Synergistic interactions are indicated (see below). D, synergistic, additive, and subadditive effects of combined SAHA and chemotherapy drugs are represented by the O/E ratio [O/E < 0.8, synergistic (**); O/E = 0.8-1.2, additive; O/E > 1.2, subadditive].
individuals had a clinical response including four of six patients with thyroid cancer. These results prompted us to look more closely at the effects of SAHA on a variety of thyroid cancer cell lines both in vitro and in vivo.

Our study used papillary and anaplastic thyroid cancer cell lines and shows that SAHA causes histone acetylation, induces apoptosis, and inhibits tumor cell growth in vitro and in vivo. These global changes were associated with down-regulation of the cell cycle regulator, cyclin D1, and up-regulation of the cyclin-dependent kinase inhibitor, p21 WAF1. SAHA also increased cell surface Annexin V and induced PARP cleavage. Each of these actions is consistent with induction of apoptosis by SAHA.

In a recently published, independent study by Mitsiades et al. (24), SAHA and CBHA (another novel hydroxamic acid-derived HDACI) were investigated in anaplastic, follicular, and medullary thyroid cancer cell lines. They found that SAHA caused histone H3 and H4 acetylation, down-regulation of p21 and several antiapoptotic genes including Bcl2, and cleavage of PARP and caspase-8 and caspase-9. Our data are consistent with these studies. Also, Mitsiades et al. (24) showed enhanced cell killing when SAHA was combined with either TRAIL or FasL in the FRO anaplastic thyroid cancer cell line. We found that SAHA increased expression of DR5, the receptor for TRAIL, but we were unable to detect enhanced cell killing when SAHA and TRAIL were combined. However, we did find that SAHA enhanced the cytotoxicity mediated by either doxorubicin, paraplatin, or paclitaxel.

Our clonogenic assays found that 50% inhibition of clonal growth of anaplastic thyroid cancer cell lines occurred in a range of $3 \times 10^{-7}$ to $5 \times 10^{-7}$ mol/L SAHA. Clinical studies have shown that these levels can be achieved in individuals receiving the drug (23). Our studies suggest that the antitumor actions of SAHA were not merely an in vitro phenomenon. SAHA also had a profound effect on growth of anaplastic human thyroid cancer cells, FRO, in immunodeficient mice. During the trial, these mice had no signs or symptoms of toxicity from the drug (data not shown). The serum half-life of SAHA is fairly short. Nevertheless, we found that phosphorylated 4E-BP1 was at its lowest level, and acetylated histones was at its highest expression in thyroid cancer cells growing in nude mice at 48 hours after the last injection of SAHA in these animals. This suggests that SAHA can have prolonged effects in vivo, which may permit decreasing the frequency of dosage of the drug in patients.

In summary, Mitsiades et al. (24) and we provide evidence that SAHA has antithyroid cancer activity. SAHA is not a chemotherapeutic drug and is nonradioactive; thus, it does not have cross-reactive toxicity with the two mainstays of

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**Fig. 6.** SAHA inhibits growth of human thyroid cancer cells in vivo. Immunodeficient mice with FRO anaplastic thyroid cancer cells were treated with SAHA (100 mg/kg) for 7 weeks (5 d/wk) by i.p. injections. *A*, representative mice from control and SAHA-treated cohorts. *B*, growth of tumors during course of experiment as measured by increases in calculated volume size of individual tumors. *C*, final mass of resected tumors at the end of the experiment. *P* < 0.001 between control tumors (n = 16) and SAHA-treated tumors (n = 16) by Student’s t test. *D*, protein (whole cell lysates) was prepared from tumors resected from control (untreated: C1, C2, C3) mice. Lysates were also prepared from tumors of SAHA-treated mice at 12 hours (72), 24 hours (24), 48 hours (48), 72 hours (72), and 96 hours (96) after the final dose of SAHA was administered.
therapy for progressive disease. SAHA may, therefore, be useful as either an adjuvant therapy when occult disease is likely to be present or combined with other mainstay therapies for metastatic disease.

Acknowledgments

We thank Dr. Victoria M. Richon (Merck & Co., Inc., Whitehouse Station, NJ) for kindly providing SAHA.

References

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