Histone Deacetylase Inhibitors Induce Growth Suppression and Cell Death in Human Rhabdomyosarcoma in Vitro

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ABSTRACT

Purpose: A group of histone deacetylase inhibitors has been shown to be effective in suppressing the growth of a variety of transformed cell lines in vitro and in vivo. The effects of two of these agents, suberoylanilide hydroxamic acid (SAHA) and suberoyl-3-aminopyridineamide hydroxamic acid (pyroxamide), were investigated for their growth-suppressive effects on rhabdomyosarcoma (RMS) cells.

Experimental Design and Results: Dose-response experiments of two RMS cell lines, RD (embryonal) and RH30B (alveolar), were performed with SAHA (0.25–3.0 μM) and pyroxamide (1.25–20.0 μM). Both agents caused a dose-dependent decrease in viable cell number and an increase in percentage of dead cells over time. Exposure of the RMS cells to SAHA and pyroxamide resulted in an accumulation of acetylated histones with increasing doses by Western blot analysis. Additionally, there was an induction of p21/WAF1 at 15 and 24 h when the cells were cultured with SAHA (2.0 μM) or pyroxamide (20.0 μM), concentrations that were tested because they successfully induced inhibition of cell growth and initiated cell death in both RMS cell lines. An increase in nuclei with hypodiploid or sub-G1 fraction was found by flow cytometry with increasing doses of both SAHA (0.25–3.0 μM) and pyroxamide (1.25–20.0 μM) over time. This finding is consistent with DNA fragmentation and cell death by apoptosis.

Conclusions: SAHA and pyroxamide induce growth suppression and cell death in human RMS in vitro. Accumulation of acetylated histones and induction of p21/WAF1 expression are observed in cells exposed to either agent.

INTRODUCTION

RMS9 is a malignant tumor derived from mesenchymal cells that are destined for skeletal muscle lineage. A multidisciplinary approach to treatment has resulted in improvement in outcomes (1). However, the overall 5-year survival rate of patients with metastatic RMS was reported to be 30% in the Intergroup RMS Study Group III pilot study (2). Preliminary data from the Intergroup RMS Study Group IV (3) indicate a failure-free survival rate of 23% in patients with metastatic disease. In the Intergroup RMS Study Group IV pilot study, those patients older than 10 years with embryonal RMS and patients with alveolar RMS and undifferentiated sarcoma (from infancy to age 20 years) had a 5-year survival rate of approximately 30% (4). These poor survival rates in high-risk patients necessitate investigation into new classes of therapeutic agents.

HDACs and histone acetyl transferases regulate the acetylation state of histones. The acetylation state of histones has been shown to have a role in transcriptional regulation (5). These enzymes exert their effects at the NH2-terminal lysine residues of core histones. Deacetylation of the lysine residues results in a positively charged histone tail, a polar interaction with DNA, and condensation of chromatin, resulting in repression of transcriptional activity. Inhibition of HDAC activity results in the accumulation of acetylated core histones, leading to a more open chromatin conformation and the transcriptional activation of a limited number of target genes (e.g., the cdk inhibitor p21/WAF1) in a variety of transformed cells (6–9).

A diverse group of compounds inhibits the activity of HDACs. The HDACIs include hydroxamic acids [e.g., trichostatin A, SAHA, suberoyl-3-aminopyridineamide hydroxamic acid (m-carboxycinnamic bishydroxamide; cdk, cyclin-dependent kinase).

9 The abbreviations used are: RMS, rhabdomyosarcoma; FACS, fluorescence-activated cell-sorting; HDAC, histone deacetylase; HDACI, histone deacetylase inhibitor; SAHA, suberoylanilide hydroxamic acid; CBHA, m-carboxycinnamic bishydroxamide; cdk, cyclin-dependent kinase.

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acid (pyroxamide), CBHA (6, 10), short-chain fatty acids [e.g., butyrates (11)], cyclic peptides [e.g., depsipeptide (12)], and benzamides [e.g., MS-27-275 (13)].

In this study, we have examined the activity of two HDACs: SAHA and pyroxamide, on human RMS in vitro. These compounds have growth-suppressive activity in a variety of tumor models (6, 14–17) and are currently under investigation in Phase I and Phase II clinical trials for the treatment of solid and hematological tumors in adults (18, 19). This study shows the growth-suppressive effects of both SAHA and pyroxamide on embryonal and alveolar RMS cell lines in a dose-dependent fashion at micromolar concentrations. We also demonstrate the induction of cell death by both SAHA and pyroxamide in RMS cells by accumulation of sub-G1 nuclei in cell cycle analysis. The accumulation of acetylated core histones and the induction of p21/WAF1 expression are observed in response to SAHA and pyroxamide in both cell lines, serving as possible biological markers for the activity of these agents. These findings suggest a potential role for these agents in the treatment of RMS.

MATERIALS AND METHODS

Cell Culture. The RD cell line was obtained from the American Type Culture Collection (Manassas, VA). The RH30B cell line was provided by Dr. David Lyden (Memorial Sloan Kettering Cancer Center). Cells were maintained in a logarithmic growth phase at 37°C and 5% CO2 in either DMEM supplemented with high glucose and 10% FCS (RD) or RPMI 1640 containing 10% FCS (RH30B). Both SAHA and pyroxamide were maintained as 100 mM stock solutions in 100% DMSO. Cells (5.0 × 104) were seeded in 6-well plates in triplicate and allowed to attach for at least 12 h before the addition of either SAHA (0–3 μM) or pyroxamide (0–20 μM). Cells were harvested with 0.05% trypsin/0.02% EDTA in HBSS after 24, 48, and 72 h of exposure to each compound. Cells were counted using a hemacytometer, and cell viability was assessed by trypan blue exclusion.

Flow Cytometry. Cells were cultured without or with either SAHA (0.25–3.0 μM) or pyroxamide (1.25–20.0 μM) as indicated for 12, 24, or 48 h. Cells were harvested and washed in ice-cold PBS. The cells were then fixed in 66% methanol, and DNA was stained with propidium iodide (50 μg/ml) containing 5 mg/ml RNase (Boehringer Mannheim) in a 1:100 dilution. Cell cycle analysis was performed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and sub-G1 values were obtained by standard histogram analysis using CellQuest software (Becton Dickinson) as described previously (15).

Western Blot Analysis of Isolated Histones. Cells were cultured as described above. After 24 h of culture without or with either SAHA (0.25–3.0 μM) or pyroxamide (1.25–20.0 μM), cells were harvested and washed in ice-cold PBS. Nuclei were isolated by Dounce homogenization of cells in 1 ml of buffer containing 8.6% sucrose, 1% Triton X-100, 50 mM sodium bisulfite, 10 mM Tris-HCl (pH 6.5), and 10 mM MgCl2. Histones were isolated from the nuclear fraction by acid extraction as described previously (10). Histones (1 μg) were separated on 15% SDS-PAGE minigels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Amerham, Buckinghamshire, United Kingdom). Hyperacetylated H3 histones were detected using a rabbit anti-acetylated H3 antibody at a dilution of 1:1000 (Upstate Biotechnology, Lake Placid, NY) as described previously (15). The primary signal was detected using a peroxidase-labeled donkey antirabbit secondary antibody (Amersham) at a dilution of 1:5000. Antibodies were diluted in 3% milk in PBS, and signals were visualized by chemiluminescence (Pierce Chemical Co., Rockford, IL). Coomassie Blue-stained gels were used as controls for protein loading.

Western Blot Analysis of p21/WAF1. Cells were cultured as described above. After culture with either 2.0 μM SAHA or 20.0 μM pyroxamide, cells were harvested and washed in ice-cold PBS at 6, 15, and 24 h. The cells were then lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 5 mM EDTA, and 0.5% NP40 with protease inhibitors (Boehringer Mannheim). Proteins (25 μg) were subjected to PAGE through 10% gels (RH30B; Bio-Rad) or 15% gels (RD; Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (Amerham). The p21/WAF1 protein was detected using the Santa Cruz Biotechnology H164 rabbit antibody at a concentration of 1 μg/ml. The secondary signal was detected using a peroxidase-labeled donkey antirabbit secondary antibody. The level of cdk2 protein was used as a loading control, as described previously (17).

RESULTS

Effects of HDACIs on RMS Cells in Vitro. The effects of both agents on the growth of embryonal (RD) and alveolar (RH30B) RMS cells in vitro were determined. Cells were cultured without or with SAHA (0.25–3.0 μM) or pyroxamide (1.25–20.0 μM) for 24, 48, and 72 h. For both cell lines, there was a concentration-dependent decrease in viable cell number over time with increasing doses of SAHA (Fig. 1, A and B). There was complete growth suppression of both RD and RH30B cells at 2.0 μM SAHA at 72 h of culture as compared with control. The percentage of dead cells in culture increased with increasing concentrations of SAHA. After 72 h of culture with 3.0 μM SAHA, 34% of the RD cells were nonviable (Fig. 1C). At this same concentration of SAHA, 68% of the RH30B cells were nonviable after 72 h of culture (Fig. 1D). Similar results were found in cells cultured with pyroxamide (1.25–20.0 μM). There was complete growth suppression of both RD and RH30B cells after 72 h of culture with increasing concentrations of pyroxamide (Fig. 2, A and B). Both the RD and RH30B cell lines showed significant decreases in viable cell number as compared with control. Culture of RD cells with 20.0 μM pyroxamide for 72 h resulted in 44% dead cells (Fig. 2C), whereas culture of RH30B cells with 20.0 μM pyroxamide for 72 h resulted in 86% dead cells (Fig. 2D).

HDACIs Cause Accumulation of Sub-G1 Nuclei. RD and RH30B cells were cultured with SAHA (0, 2.0, and 3.0 μM) and pyroxamide (0, 10.0, and 20.0 μM), concentrations that induced growth suppression and cell death in both cell lines. There was an increase in the hypodiploid or sub-G1 fraction of nuclei in the RD cell line after culture with increasing concentrations of both SAHA and pyroxamide at 48 h. This finding is consistent with DNA fragmentation and cell death. The control (no SAHA) sample of RD showed a sub-G1 fraction of 3.8% as
compared with cells cultured with 2.0 and 3.0 μM SAHA, which showed sub-G₀ fractions of 27.7% and 54.1%, respectively. RD cells that were cultured with pyroxamide similarly showed an accumulation of sub-G₀ nuclei. Samples cultured with either 10.0 or 20.0 μM pyroxamide had sub-G₀ fractions of 21.6% and 56.0%, respectively (Fig. 3A). Sub-G₁ fractions of RH30B cells cultured with SAHA and pyroxamide were also increased when compared with control samples at 48 h. At 2.0 and 3.0 μM SAHA, the sub-G₁ fractions were 39.0% and 51.7%, respectively, whereas cells cultured with 10.0 and 20.0 μM pyroxamide had sub-G₁ fractions of 45.0% and 72.3%, respectively (Fig. 3B).

**DISCUSSION**

HDACIs have previously been shown to have potent antitumor effects (13, 20, 21). These agents induce transformed cell differentiation, growth suppression, and apoptosis in multiple cell lines including murine erythroleukemia and human bladder, breast, myeloma, and neuroblastoma. These effects have been seen both in vitro (6, 14–17, 22) and in vivo (16, 23). We have shown previously that HDACIs induce apoptosis in human neuroblastoma cells in vitro (15). Additionally, we have demonstrated growth-suppressive effects of the HDACI CBHA.

**HDACIs Cause Accumulation of Acetylated Histones.**

After 24 h of culture of RD and RH30B cells with either SAHA (0.25–3.0 μM) or pyroxamide (1.25–20.0 μM), histones were isolated via acid extraction and subjected to Western blot analysis with anti-acetylated histone H3 antibody. A concentration-dependent accumulation of acetylated histones was found at all concentrations of SAHA and pyroxamide tested in both the RD cell line (Fig. 4A) and RH30B cell line (Fig. 4B). The Coomassie Blue-stained gels are shown as controls for protein loading.

**HDACIs Induce p21/WAF1 Expression.**

RD and RH30B cells were cultured with SAHA (2.0 μM) or pyroxamide (20.0 μM), concentrations that cause complete suppression of cell growth as well as cell death in both cell lines. Cells were harvested, and protein lysates were isolated at 6, 15, and 24 h as described above. Protein extracts (25 μg) were subjected to Western blot analysis with antibody to p21/WAF1. Induction of p21/WAF1 expression was seen at 15 and 24 h in SAHA-cultured RD cells (Fig. 5A) and SAHA-cultured RH30B cells (Fig. 5B). There was also an induction of p21/WAF1 expression seen at 15 and 24 h in pyroxamide-cultured RD cells (Fig. 5C) and pyroxamide-cultured RH30B cells (Fig. 5D). Western blots with antibody to cdk2 are shown as controls for protein loading because the expression of cdk2 is not known to be affected by HDACIs.

**Fig. 1** Induction of growth inhibition and cell death in the embryonal RMS cell line RD and the alveolar RMS cell line RH30B with increasing doses of SAHA. Cells were treated with SAHA at 0 ( ), 0.25 ( ), 0.5 ( ), 1.0 ( ), 2.0 ( ), and 3.0 μM ( ). Cells were harvested at 24, 48, and 72 h, and viability was assessed by trypan blue exclusion. Each dose and time point was repeated in triplicate. The data shown are the means and their SDs. Error bars are not shown for some data points because the numeric values are too small to be graphically depicted. A and B show the effects of increasing doses of SAHA on the viable cell number over time of the RD (A) and RH30B (B) cell lines. C and D depict percentage of dead cells of the RD (C) and RH30B (D) cell lines with increasing doses of SAHA over time.
neuroblastoma alone and synergistically with all-trans retinoic acid in vivo in mice (23). A chemopreventative effect of SAHA on the carcinogen-induced rat mammary and murine lung tumors has also been observed (24, 25). These studies lay the groundwork for testing the potential efficacy of the HDACIs in the treatment of RMS.

The effects of SAHA and pyroxamide on cell growth and cell death were tested in two RMS cell lines, RD (embryonal cell line) and RH30B (alveolar cell line). These types of RMS differ in histology, genetic markers, current treatment protocols, and prognosis. Embryonal RMS generally occurs in the head and neck areas or genitourinary tract of younger children. It is associated with loss of heterozygosity at the 11p15 locus, as well as RAS mutations. The 11p15 locus is the site of six imprinted genes: (a) IGF-II gene, an important growth factor in a variety of malignancies; (b) H19, a growth suppressor gene that regulates the IGF-II gene; (c) p57kip2, a cdk inhibitor that causes G1 arrest; (d) K_LQT1, a voltage gated potassium channel; (e) TSSC3, a gene involved in Fas-mediated apoptosis; and (f) TSSC5, a transmembrane protein-encoding gene (26). Alveolar RMS usually occurs on the extremities of adolescents, is associated with a PAX3/FHRK or PAX7/FHRK translocation, and generally has a poor prognosis. The PAX3, PAX7, and FHRK genes encode proteins that are transcriptional regulators. The PAX3/FHRK and PAX7/FHRK translocations result in the formation of chimeric proteins that are thought to alter transcription and promote tumorigenesis (27, 28).

Both SAHA and pyroxamide had significant growth-suppressive effects in both RMS cell lines tested. SAHA was effective in causing complete growth suppression in both cell lines at a concentration of 2.0 μM. A similar effect was seen in both cell lines at a pyroxamide concentration of 20.0 μM. The percentage of dead cells also increased with increasing concentrations of both SAHA and pyroxamide. The RH30B cell line grew more rapidly than the RD cell line, resulting in higher initial viable cell counts. Exposure of RH30B to SAHA or pyroxamide resulted in a greater absolute reduction in cell number, but it is unclear whether RH30B is actually more sensitive to the HDACIs or whether this observation was related to its faster growth rate.

Previous studies have shown the induction of cell death by HDACIs in various transformed cell lines (6, 15). FACS analysis of propidium iodide-stained nuclei of both the RD and RH30B cells after exposure to SAHA and pyroxamide revealed an accumulation of sub-G1 nuclei with increasing doses and...
length of exposure to each agent, a finding that is consistent with DNA fragmentation and cell death.

There was a dose-dependent accumulation of acetylated histones after culture of both RD and RH30B cells with all concentrations tested of SAHA and pyroxamide for 24 h. This serves as a biological marker for the HDAC inhibitory activity of these agents. In one Phase I trial, i.v. doses of 300–900 mg/m² SAHA resulted in plasma levels that exceeded 2.5 μM at all dose levels. Accumulation of acetylated histones was consistently seen at doses of 600–900 mg/m² in the same study (19).

The cdk inhibitor p21/WAF1 has previously been shown to be induced by several HDACIs including trichostatin A, phenylbutyrate, and SAHA in multiple transformed cell lines.

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**Fig. 3** FACS analysis profiles of RD (A) and RH30B (B) cells treated with SAHA and pyroxamide. Cells were treated with SAHA doses of 0, 2.0, and 3.0 μM and pyroxamide doses of 0, 10.0, and 20.0 μM. Data shown are from cells harvested after 48 h of exposure to the respective agent.

**Fig. 4** Accumulation of hyperacetylated core histones extracted from RD cells (A) and RH30B cells (B) after treatment with either SAHA (0–3.0 μM) or pyroxamide (0–20 μM) for 24 h. Hyperacetylated core histones were detected by Western blot analysis with anti-acetylated histone H3 antibody. The Coomassie Blue-stained gels are shown as controls for protein loading.
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20 RH30B cells after treatment with 20 μM pyroxamide and Phase II clinical trials in adults with both hematological and solid tumors (18). The effectiveness of SAHA and pyroxamide and Phase II clinical trials in adults with both hematological and solid tumors (18). The effectiveness of SAHA and pyroxamide. These agents are currently in Phase I induction of cell death in RMS cells exposed to two HDACIs, but the relative sensitivities of the different HDACs to these agents is not established (6, 9, 18).

Thus it is reasonable to develop both agents for clinical use. The exposure of both RMS cell lines to SAHA (at least in part) for the arrest in cell growth seen after exposure to this agent (17). The induction of both HDACs in control cells that were exposed to DMSO for 6, 15, and 24 h, respectively. Lanes 4–6 represent protein extracts from the RD and RH30B cells treated with 2 μM SAHA for 6, 15, and 24 h. A similar induction is seen in RD and RH30B cells after treatment with 20 μM pyroxamide. C and D, Lanes 1–3 represent control cells that were exposed to DMSO for 6, 15, and 24 h, respectively. Lanes 4–6 represent protein extracts from the RD and RH30B cells treated with 20 μM pyroxamide for 6, 15, and 24 h. Western blots of cdk2 expression are shown as controls for protein loading.

on RMS cells in vitro suggests a potential role for these agents in the treatment of RMS.

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both in vitro and in vivo (14, 17, 20, 29). This induction is associated with hyperacetylation of the chromatin in the promoter region of the p21/WAF1 gene (7, 30) and also occurs in cells that lack p53 (31, 32). Two Sp1 binding sites in the promoter region of the p21/WAF1 gene are required for induction by the HDACs (31). The induction of p21/WAF1 by pyroxamide has also been associated with a G1 arrest in transformed prostate and bladder cells in vitro, possibly accounting (at least in part) for the arrest in cell growth seen after exposure to this agent (17). The exposure of both RMS cell lines to SAHA and to pyroxamide resulted in an induction of p21/WAF1 at 15 and 24 h that serves as an additional marker for the activity of these agents. SAHA and pyroxamide have different aqueous solubilities and pharmacokinetic properties. Both agents inhibit class I and class II HDACs, but the relative sensitivities of the different HDACs to these agents is not established (6, 9, 18). Thus it is reasonable to develop both agents for clinical use.

RMS portends a poor prognosis in patients with metastatic disease. We have shown both growth-suppressive effects and induction of cell death in RMS cells exposed to two HDACIs, SAHA and pyroxamide. These agents are currently in Phase I and Phase II clinical trials in adults with both hematological and solid tumors (18). The effectiveness of SAHA and pyroxamide...


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