Suberoylanilide Hydroxamic Acid, an Inhibitor of Histone Deacetylase, Suppresses the Growth of Prostate Cancer Cells in Vitro and in Vivo

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ABSTRACT

Suberoylanilide hydroxamic acid (SAHA) is the prototype of a family of hybrid polar compounds that induce growth arrest in transformed cells and show promise for the treatment of cancer. SAHA induces differentiation and/or apoptosis in certain transformed cells in culture and is a potent inhibitor of histone deacetylases. In this study, we examined the effects of SAHA on the growth of human prostate cancer cells in culture and on the growth of the CWR22 human prostate xenograft in nude mice. SAHA suppressed the growth of the LNCaP, PC-3, and TSU-Pr1 cell lines at micromolar concentrations (2.5–7.5 μM). SAHA induced dose-dependent cell death in the LNCaP cells. In mice with transplanted CWR22 human prostate tumors, SAHA (25, 50, and 100 mg/kg/day) caused significant suppression of tumor growth compared with mice receiving vehicle alone; treatment with 50 mg/kg/day resulted in a 97% reduction in the mean final tumor volume compared with controls. At this dose, there was no detectable toxicity as evaluated by weight gain and necropsy examination. Increased accumulation of acetylated core histones was detected in the CWR22 tumors within 6 h of SAHA administration. SAHA induced prostate-specific antigen mRNA expression in CWR22 prostate cancer cells, resulting in higher levels of serum prostate-specific antigen than predicted from tumor volume alone. The results suggest that hydroxamic acid-based hybrid polar compounds inhibit prostate cancer cell growth and may be useful, relatively nontoxic agents for the treatment of prostate carcinoma.

INTRODUCTION

Agents that induce differentiation and/or apoptosis of transformed cells have potential for the treatment of cancer (1, 2). SAHA is the prototype of a series of hydroxamic acid-based HPCs that are potent inducers of differentiation and/or apoptosis in murine erythroleukemia, bladder, and myeloma cell lines (3, 4) at micromolar concentrations. SAHA has growth-inhibitory effects on T/T5 rat prostate cancer cells (5). SAHA administered p.o. inhibits the development of N-methyl-N-nitrosourea-induced mammary tumors in rats (6) and lung tumors in mice induced by 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone, the carcinogen found in tobacco smoke (7).

SAHA and other hydroxamic acid-based HPCs are potent inhibitors of HDACs (4, 8). We have shown previously that SAHA inhibits partially purified HDACs 1 and 3 at concentrations as low as 100 nM and causes an accumulation of acetylated histones in cells in culture at low micromolar concentrations (4). The active catalytic site in HDACs is evolutionarily conserved and consists of a core pocket with a zinc atom at the base of the pocket (8). Co-crystals of SAHA and a HDAC-like protein have shown that SAHA fits into the core pocket of HDAC-like protein, and its hydroxamic acid moiety interacts directly with the zinc atom in the catalytic site (8). The acetylation of nucleosomal histones is regulated by the opposing activities of HDACs and histone acetyltransferases such as cAMP-responsive element binding protein-binding protein/p300, which are components of multigene complexes associated with gene promoters. It has been postulated that hypoacetylated histones maintain the repressed state of a gene by condensing the associated chromatin and restricting the access of transcription factors to the DNA, whereas acetylation of histones is generally associated with activation of gene expression (9, 10). Inhibition of HDACs by SAHA may contribute to the induction of differentiation or apoptosis in transformed cells by activating transcription of target genes.

Cancer of the prostate is a major health problem and is the second highest cause of deaths from cancer each year in males in the United States. The current treatment for most prostate cancers that have spread beyond the prostate gland is surgical or chemical castration, which causes regression of the tumor due to the dependence of prostatic epithelial cells on physiological levels of circulating androgens for growth and survival. Whereas this treatment may be initially successful, most tumors eventually recur due to the expansion of an androgen-insensitive population of tumor cells. Treatment of androgen-independent tumors with cytotoxic agents is generally unsatisfactory, and, at this stage, the disease is usually fatal to the patient (11).

Agents that induce differentiation and/or apoptosis of prostate cancer cells may provide an alternative or additional approach to the treatment of this disease. Several classes of differentiating agents, including SAHA (5), butyrate and its analogues (12–15), retinoids (16–18), and vitamin D analogues (17–22), are reported to inhibit the growth of prostate cancer cells in culture and cause a shift in cellular phenotype toward a more differentiated epithelioid morphology. In this study, we tested the activity of SAHA in three prostate cancer cell lines (LNCaP, PC-3, and TSU-Pr1) and evaluated the antitumor effects of SAHA in vivo using the CWR22 human prostate cancer xenograft model in nude mice. CWR22 is an androgen-dependent tumor derived from a patient with metastatic prostate cancer (23, 24) that can be grown s.c. in nude mice. The tumors regress significantly after castration of the host mice, and this regression is preceded by a several thousand-fold reduction in serum PSA levels. The reduction in tumor volume is maintained for 80–400 days, after which androgen-independent CWR22 tumors emerge in the castrated hosts and grow until the animal must be sacrificed (25, 26).

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The abbreviations used are: SAHA, suberoylanilide hydroxamic acid; HDAC, histone deacetylase; HPC, hybrid polar compound; PSA, prostate-specific antigen.
suppresses the growth of CWR22 tumors in nude mice at doses that cause little evident toxicity.

MATERIALS AND METHODS

Cell Culture and Proliferation Assay. The LNCaP and PC-3 cell lines were purchased from the American Type Culture Collection. TSU-Pro1 prostate carcinoma cell lines were kindly provided by Dr. Neil Rosen (Memorial Sloan-Kettering Cancer Center, New York, NY). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

SAHA was synthesized as described previously (3) and dissolved and diluted in DMSO. The effect of SAHA on the viability of prostate cancer cells was determined by cell counting using a hemocytometer. Cells were seeded in triplicate in 24-well dishes at a density of 3 × 10^4 cells/well. Cells were allowed to attach overnight, and then the medium was replaced with medium containing the appropriate concentration of SAHA (0.25, 5, or 7.5 μM). Cells were counted at various times after the start of SAHA treatment, and cell viability was assessed by trypan blue dye exclusion.

Preparation of the CWR22 Prostate Cancer Xenografts. The CWR22 human prostate cancer xenograft was kindly provided by Dr. Thomas G. Pretlow (Case Western Reserve University, Cleveland, OH). Male BALB/c nude (nu/nu) mice were purchased at 4–6 weeks of age from the National Cancer Institute-Frederick Cancer Research & Development Center. Mice were housed under barrier conditions and maintained on a 12-h light/12-h dark cycle, with food and water supplied ad libitum. Two days before inoculation of the mice with tumor cells, a 90-day sustained-release 12.5-mg testosterone pellet (Innovative Research of America, Sarasota, FL) was implanted in the left flank. To inoculate mice with the CWR22 tumor cells, an established tumor was removed from a host mouse, minced finely through a mesh support, and suspended in RPMI 1640. An equal volume of the tumor cell suspension was injected s.c. in the right flank of each mouse. After 7 days, a palpable tumor of approximately 5 × 5 mm was detected in the inoculated animals.

SAHA Preparation and Administration. Mice with palpable CWR22 tumors were divided into four groups (eight or nine mice/group) for the treatment study. All mice in each treatment group had tumors of similar size (5 × 5 mm) at the start of treatment. For administration to mice, SAHA was dissolved and diluted in a vehicle of DMSO. Each group of mice received 25, 50, or 100 mg/kg SAHA daily by i.p. injection for 21 days. A control group of approximately 5 × 5 mm was detected in the inoculated animals.

RESULTS

SAHA Suppresses the Growth of Prostate Cancer Cells in Vitro

The growth-inhibitory activity of SAHA was assessed in three prostate cancer cell lines (LNCaP, PC-3, and TSU-Pro1) in culture. SAHA caused a dose-dependent inhibition of proliferation in all three cell lines at micromolar concentrations (2.5–7.5 μM), with LNCaP cells being the most sensitive to SAHA treatment, and TSU-Pro1 cells being the least sensitive to SAHA treatment (Fig. 1A). Culture with 2.5 μM SAHA caused complete growth suppression of the androgen-sensitive LNCaP cells but little evident induction of cell death as assayed by trypan blue dye exclusion. At concentrations of 5 and 7.5 μM, SAHA induced cell death in the LNCaP cells within 48 h of culture (Fig. 1A). Culture with SAHA at the indicated concentrations caused growth suppression of the androgen-insensitive cell lines PC-3 and TSU-Pro1 with little detectable cell death (Fig. 1A).

The effect of SAHA on histone acetylation in prostate cancer cells was assessed in LNCaP cells treated with SAHA (0.005–5 μM) for 3 h in culture (Fig. 1B). Cells treated without or with 0.005 or 0.05 μM SAHA had similar baseline levels of acetylated histones H3 and H4 (Fig. 1B). An incremental accumulation of acetylated histones H3 and H4 was observed in cells cultured with 0.5 and 2 μM SAHA. No further increase was observed after culture with 5 μM SAHA.

Efficacy and Toxicity of SAHA in the CWR22 Prostate Xenograft Model. The activity of SAHA against the growth of prostate cancer cells in vivo was examined in the androgen-dependent CWR22 human prostate cancer xenograft, which was grown s.c. in nude mice. Daily administration of SAHA caused significant suppression of the growth of established CWR22 tumors, such that doses of 25, 50, and 100 mg/kg/day caused reductions of 78%, 97%, and 97%, respectively, in the mean final tumor volume compared with vehicle-treated control animals (Fig. 2). Tumor regression (a reduction in tumor size relative to the size of the tumor at the initiation of treatment) was observed in one of eight mice receiving 25 mg/kg/day SAHA and in five of nine mice receiving 50 mg/kg/day SAHA. In the five animals receiving 100 mg/kg/day SAHA that survived for at least 20 days of treatment, three showed tumor regression.

The toxicity of SAHA was assessed by monitoring the body weight and survival of the mice and by the necropsy of one mouse in each group at the conclusion of the study (after 21 days of treatment).
Necropsy included histological examination of at least 26 different tissues or organs and a complete blood count and differential. No deaths were observed among the vehicle-treated controls or the mice treated with 25 mg/kg/day SAHA, and, on average, these mice gained 1.5 and 1.7 grams in body weight, respectively, relative to their weights at the initiation of treatment (Table 1). One of the nine mice from the 50 mg/kg/day SAHA treatment group died on the penultimate day of the study but had no preceding weight loss, suggesting that death may have been due to an injection injury. The mice in this group gained an average of 1.0 gram in body weight relative to their weight at the start of treatment (Table 1). Five of the nine mice receiving 100 mg/kg/day SAHA died during the study, and each of the mice that died experienced 1–2 days of rapid weight loss before death, suggesting that this is a toxic dose level. Surviving mice in this dose group had an average weight loss of 0.8 grams relative to their weight at the start of treatment.

Mice in all treatment groups, including the vehicle-treated control group, had evidence of inflammation and bacterial infection in the peritoneum, in the area of injection. The necropsied mouse that received vehicle alone showed no other abnormalities in the organs examined, and the hematology values were within normal limits. The necropsied mice receiving SAHA at each of the three dose levels had increased erythropoiesis in the spleen and bone marrow, but peripheral blood erythrocyte counts were within normal limits. The necropsied mouse from the 50 mg/kg/day SAHA treatment group had evidence of mild lymphoid hyperplasia in the spleen and bone marrow, but peripheral blood erythrocyte counts were within normal limits. The necropsied mouse from the 50 mg/kg/day treatment group had evidence of mild lymphoid hyperplasia in the spleen and bone marrow, but peripheral blood erythrocyte counts were within normal limits. The necropsied mouse receiving 100 mg/kg/day also had mild lymphoid hyperplasia in the spleen and showed evidence of neutrophilic leukocytosis. No other gross or histological abnormalities were found at necropsy.

Necropsies were also performed on two of the animals that died in the 100 mg/kg/day SAHA treatment group. Both mice showed in-
SAHA Suppresses Prostate Cancer Cell Growth

Acetylation of Histones in CWR22 Tumors. The effect of SAHA on the acetylation of histones in the CWR22 tumors in vivo was analyzed at two time points after the administration of the agent. Mice with established CWR22 tumors were injected with vehicle alone or with 25 or 50 mg/kg SAHA (four mice/group), and two mice from each group were sacrificed at 6 and 12 h after the injection. Tumors were removed from the animals, and histones were extracted for the detection of acetylated lysine residues by Western blotting (see “Materials and Methods”).

CWR22 tumors from mice treated with vehicle alone had low baseline levels of acetylated histones H3 and H4 (Fig. 4). At 6 h after injection of SAHA, an increased accumulation of acetylated histones H3 and H4 was present in tumors from one animal receiving 25 mg/kg SAHA and in both animals receiving 50 mg/kg SAHA (Fig. 4). By 12 h after injection of SAHA, the accumulation of acetylated histones

Fig. 2. s.c. growth of the CWR22 prostate cancer xenograft in nude mice treated with daily i.p. injection of vehicle alone or SAHA (25, 50, or 100 mg/kg). Data are presented as the mean tumor volume ± SE of the surviving animals in each treatment group. All groups contained eight or nine mice at each time point, except for the group receiving 100 mg/kg/day SAHA, in which five animals died by the end of the treatment period. Treatment commenced 7 days after implantation of the tumors, when palpable tumors (mean volume, 65 mm³) were evident in the animals (designated as day 0).

Fig. 3. Effect of SAHA treatment on the peripheral blood PSA levels in mice bearing CWR22 prostate xenografts and PSA mRNA expression in the tumors. A, levels of PSA protein in the serum (shown as mean ± SE) were measured in all mice treated daily with vehicle or SAHA (25, 50, or 100 mg/kg) for 21 days. PSA was detected by RIA in serum taken from each mouse by tail-bleed on the first day of treatment (day 0), day 9 of treatment, and at the end of the experimental period (day 21). B, the expression of PSA mRNA was determined by Northern blot using total RNA extracted from two tumors in each treatment group. The top panel shows the PSA mRNA levels in the tumors detected using a 32P-labeled 510-bp cDNA probe corresponding to a region within the open reading frame of the human PSA protein. The ethidium bromide-stained 18S rRNA on the blot is shown in the bottom panel to indicate RNA loading.

Table 1 Effect of SAHA administration on the body weights and survival of the CWR22-bearing mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Deaths [days (D) of treatment]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (DMSO)</td>
<td>26.2 ± 0.5 (n = 8)</td>
<td>27.7 ± 0.6 (n = 8)</td>
<td>0</td>
</tr>
<tr>
<td>25 mg/kg SAHA</td>
<td>23.8 ± 0.7 (n = 8)</td>
<td>25.5 ± 1.3 (n = 8)</td>
<td>0</td>
</tr>
<tr>
<td>50 mg/kg SAHA</td>
<td>24.8 ± 0.7 (n = 9)</td>
<td>25.8 ± 0.7 (n = 8)</td>
<td>1 (D20)</td>
</tr>
<tr>
<td>100 mg/kg SAHA</td>
<td>24.8 ± 0.7 (n = 9)</td>
<td>24.0 ± 0.8 (n = 4)</td>
<td>5 (D6, D9, D14, D16, and D21)</td>
</tr>
</tbody>
</table>

*All values are presented as the mean ± SE for the surviving animals (n) in each treatment group. Initial and final body weights refer to the weight of the mice at the start of SAHA treatment and the end of the experiment, respectively.

* Mice that died in this dose group showed marked weight loss for 1–2 days before death.

SAHA suppresses prostate cancer cell growth

Materials and Methods. All animal experiments were conducted in accordance with the guidelines of the American Association for Cancer Research. Mice were housed in a pathogen-free environment, and appropriate precautions were taken to minimize discomfort and pain. The animals were housed in individual cages with food and water ad libitum. The experimental design and statistical methods are described in detail in the “Materials and Methods” section.

Fig. 1. A, experimental design. Mice were injected s.c. with CWR22 prostate cancer cells and then treated daily i.p. with vehicle alone or SAHA (25, 50, or 100 mg/kg). Treatment commenced 7 days after implantation of the tumors, when palpable tumors (mean volume, 65 mm³) were evident in the animals (designated as day 0).

Fig. 2. s.c. growth of the CWR22 prostate cancer xenograft in nude mice treated with daily i.p. injection of vehicle alone or SAHA (25, 50, or 100 mg/kg). Data are presented as the mean tumor volume ± SE of the surviving animals in each treatment group. All groups contained eight or nine mice at each time point, except for the group receiving 100 mg/kg/day SAHA, in which five animals died by the end of the treatment period. Treatment commenced 7 days after implantation of the tumors, when palpable tumors (mean volume, 65 mm³) were evident in the animals (designated as day 0).

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Fig. 4. Western blot analysis of acetylated histones H3 and H4 in tumors from mice treated with vehicle or SAHA (25, 50, or 100 mg/kg) for 21 days. A, acetylation of histones was detected using Western blotting with antibodies specific for acetylated lysine residues. B, inhibition of histone deacetylase activity, which results in increased histone acetylation, was observed in tumors from mice receiving SAHA. The ethidium bromide-stained 18S rRNA on the blot is shown in the bottom panel to indicate RNA loading.

Fig. 5. Immunohistochemical staining of acetylated histones H3 and H4 in tumors from mice treated with vehicle or SAHA (25, 50, or 100 mg/kg) for 21 days. A, acetylation of histones was detected using immunohistochemistry with antibodies specific for acetylated lysine residues. B, inhibition of histone deacetylase activity, which results in increased histone acetylation, was observed in tumors from mice receiving SAHA. The ethidium bromide-stained 18S rRNA on the blot is shown in the bottom panel to indicate RNA loading.

Fig. 6. Flow cytometry analysis of cell proliferation and apoptosis in tumors from mice treated with vehicle or SAHA (25, 50, or 100 mg/kg) for 21 days. A, cell proliferation was detected using a BrdU incorporation assay. B, apoptosis was detected using Annexin V staining. The ethidium bromide-stained 18S rRNA on the blot is shown in the bottom panel to indicate RNA loading.

Fig. 7. Mass spectrometry analysis of protein expression in tumors from mice treated with vehicle or SAHA (25, 50, or 100 mg/kg) for 21 days. A, protein expression was detected using mass spectrometry with a MSD Orbitrap instrument. B, the expression of protein A was determined using liquid chromatography-mass spectrometry (LC-MS/MS). The ethidium bromide-stained 18S rRNA on the blot is shown in the bottom panel to indicate RNA loading.

Fig. 8. RNA sequencing analysis of gene expression in tumors from mice treated with vehicle or SAHA (25, 50, or 100 mg/kg) for 21 days. A, gene expression was detected using RNA sequencing with a 454 Sequencer. B, the expression of gene A was determined using digital gene expression analysis. The ethidium bromide-stained 18S rRNA on the blot is shown in the bottom panel to indicate RNA loading.
was reduced to the level seen in vehicle-treated controls. These findings are consistent with the relatively short half-life of SAHA (15–20 min) that we have determined in a mouse, dogs, and humans (data not shown).

DISCUSSION

We have reported previously that hydroxamic acid-based HPCs, such as SAHA, are potent inducers of differentiation and/or apoptosis in transformed cells in culture (3). Here we demonstrate that SAHA can suppress the growth of both androgen-sensitive and -insensitive prostate cancer cells in culture at micromolar concentrations, and administration of the agent to nude mice bearing human androgen-dependent CWR22 prostate tumors can arrest tumor growth at doses that cause little detectable toxicity. A dose of 50 mg/kg/day caused a reduction of 97% in the mean final tumor volume compared with that of tumor-bearing mice receiving vehicle alone, with no detectable toxicity as evaluated by weight loss and extensive necropsy studies. Our findings suggest that this novel class of agents may be effective for the treatment of human prostate cancers and other neoplasms.

s.c. growth of the CWR22 xenograft in nude mice is usually associated with an increase in serum levels of PSA that correlates with tumor burden (24). We had similar findings in the prostate tumor-bearing animals not receiving SAHA. In animals treated with SAHA (50 mg/kg), despite an almost complete suppression of CWR22 tumor growth, serum PSA levels rose 12-fold. Northern blot analysis to evaluate gene expression in prostate tumors revealed that the rise in serum PSA levels in SAHA-treated mice was correlated with an induction of PSA mRNA. These findings indicate that serum PSA levels may not be a good surrogate marker of tumor burden or disease progression after treatment with SAHA. Induction of PSA expression in prostate cancer cells has also been observed with other agents that suppress the growth of transformed cell lines (31, 32), although both the normal and transformed cells show similar increases in histone acetylation (31).

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