Suberoylanilide Hydroxamic Acid, a Histone Deacetylase Inhibitor: Effects on Gene Expression and Growth of Glioma Cells

In vitro and In vivo

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Abstract

Purpose: Histone acetylation is one of the main mechanisms involved in regulation of gene expression. During carcinogenesis, tumor-suppressor genes can be silenced by aberrant histone deacetylation. This epigenetic modification has become an important target for tumor therapy. The histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), can induce growth arrest in transformed cells. The aim of this study is to examine the effects of SAHA on gene expression and growth of glioblastoma multiforme (GBM) cells in vitro and in vivo.

Experimental Design: The effect of SAHA on growth of GBM cell lines and explants was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Changes of the cell cycle and relative gene expression were detected by fluorescence-activated cell sorting, real-time reverse transcription-PCR, and Western blotting. After glioma cells were implanted in the brains of mice, the ability of SAHA to decrease tumor growth was studied.

Results: Proliferation of GBM cell lines and explants were inhibited in vitro by SAHA (ED50, 2 × 10^-6 to 2 × 10^-5 mol/L, 5 days). SAHA exposure of human U87 and T98G glioma cell lines, DA66 and JM94 GBM explants, as well as a murine GL26 GBM cell line resulted in an increased accumulation of cells in G2-M of the cell cycle. Many proapoptotic, antiproliferative genes increased in their expression (DR5, TNFs, p21WAF1, p27KIP1), and many antiapoptotic, progrowth genes decreased in their levels (CDK2, CDK4, cyclin D1, cyclin D2) as measured by real-time reverse transcription-PCR and/or Western blot after these GBM cells were cultured with SAHA (2.5 × 10^-6 mol/L, 1 day). Chromatin immunoprecipitation assay found that acetylation of histone 3 on the p27WAF1 promoter was markedly increased by SAHA. In vivo murine experiments suggested that SAHA (10 mg/kg, i.v., or 100 mg/kg, i.p.) could cross the blood-brain barrier as shown by prominent increased levels of acetyl-H3 and acetyl-H4 in the brain tissue. Furthermore, the drug significantly (P < 0.05) inhibited the proliferation of the GL26 glioma cells growing in the brains of mice and increased their survival.

Conclusions: Taken together, SAHA can slow the growth of GBM in vitro and intracranially in vivo. SAHA may be a welcome addition for the treatment of this devastating disease.

Histone acetylation is an important regulator of gene expression in higher eukaryotes. It is essential for the normal development of mammals and plays an important physiologic role (1, 2). Histone deacetylation is mediated by histone deacetylases (HDAC). HDACs catalyze the removal of acetyl groups, leading to chromatin condensation and transcriptional repression (3, 4). Histone acetyl transferases are enzymes involved in transferring acetyl groups to amino-terminal lysine residues in histones, which results in local expansion of chromatin and increases accessibility of regulatory proteins to DNA (5). These opposing activities between HDACs and histone acetyl transferases regulate gene expression through chromatin modification (6). In addition, methylated DNA-binding proteins can bind specifically to methylated DNA to cause transcriptional silencing (7), and once methylated DNA-binding proteins bind to DNA, HDACs are recruited by a separate transcriptional repressor domain (8–10), resulting in long-term silencing of the target gene (11, 12).
Histone deacetylation of tumor-suppressor genes occurs in a variety of human tumors (13–15). Suberoylanilide hydroxamic acid (SAHA) is an inhibitor of several members of the HDAC protein family (16). SAHA inhibits HDAC by direct interaction with the catalytic site of the enzyme (17). The loosening of chromatin by histone acetylation can increase the efficiency of anticancer drugs targeting DNA (18, 19).

Gliomas, particularly glioblastoma multiforme (GBM), are the most malignant, invasive, and difficult-to-treat primary brain tumors. These tumors have a rapid growth rate, being capable of doubling in size within 10 to 20 days. Successful treatment of GBM is rare, with a mean survival of only 10 to 12 months. In the present study, the antiglioma activity of SAHA was examined in vitro and in vivo using human GBM cell lines, primary GBM explants, and murine cell line. SAHA caused GBM cells to accumulate in the G2-M phase of the cell cycle, with an increased expression of p21WAF1 and p27KIP1, and decreased levels of CDK2, CDK4, cyclin D1, and cyclin D2. In vivo experiments strongly suggested that SAHA crossed the blood-brain barrier of mice to increase acetyl-H3 and acetyl-H4, and efficiently inhibited GBM growth in the brain. Taken together, SAHA regulates expression of genes associated with growth inhibition of glioma cells and may have a role in the therapy of this difficult-to-manage disease.

Materials and Methods

Glioma cell lines and primary cell cultures. Human GBM cell lines U87 [p53 wild-type (wt)], T98G [p53 mutant (mt, M237I)], and U118 [p53 mt (R273H)] were maintained in DMEM (Life Technologies, Gaithersburg, MD) with 10% FCS (Gemini Bio-Products, Calabasas, CA), 10 units/mL penicillin G, and 10 mg/mL streptomycin (Gemini Bio-Products). GL26 (murine GBM line) was maintained in RPMI 1640 containing 10% FCS, 10 units/mL penicillin G, and 10 mg/mL streptomycin. Primary GBM explants DA66 (p53 wt), JM94 (p53 mt, R237C), and MM156 (p53 wt) were established from patients with GBM undergoing surgery. Written informed consent for research use of tumor tissue was obtained from each patient before surgery, according to a protocol approved by the institutional ethics committee. Tumor specimens were immediately transported to the laboratory, finely minced to a single-cell suspension, and cultured in complete medium (Ham's F-12/DME high glucose medium (Irvine Scientific, Santa Ana, CA) containing 10% FCS (Gemini Bio-Products, Calabasas, CA), 10 units/mL penicillin G, and 10 mg/mL streptomycin (Gemini Bio-Products), and 2 mmol/L glutamax-I (Invitrogen, Carlsbad, CA)) into 100-mm tissue culture plastic dishes. Cells were harvested, and aliquots were cryopreserved in liquid nitrogen. One aliquot of cells was kept in culture and grown to confluence. Cells used in these experiments were subcultured for no more than 10 additional passages. All cells were incubated at 37°C in 5% CO2.

Chemicals. Histone acetylation inhibitor SAHA was generously provided by Dr. V.M. Richon (Merck & Co., Inc., Rahway, NJ) and was dissolved in DMSO at a stock concentration of 100 mg/mL and stored at −20°C. Fresh dilutions in medium were made for each experiment.

Real-time reverse transcription-PCR. GBM cell lines and explants were cultured in either control medium or SAHA (2.5 × 10−5 mol/L) for 24 h. Cells were harvested and total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. DNA was removed by DNase. Two micrograms of RNA were reverse transcribed with random primers and Superscript II reverse transcriptase (Invitrogen). The cDNA was used for real-time PCR with Platinum Taq (Invitrogen) and SYBR Green I (Molecular Probes, Eugene, OR) in triplicates in an iCycler iQ system (Bio-Rad, Hercules, CA). PCR conditions were as follows: 2 min at 94°C followed by 45 cycles of 94°C for 15 seconds, 60°C for 15 seconds, 72°C for 15 seconds, and fluorescence determination at the melting temperature of the product for 20 seconds. The specificity of PCR products was checked on agarose gel. The expression of β-actin was used as an endogenous reference. A comparative threshold cycle was used to determine targeting genes and β-actin gene expression relative to the no-sample control (calibrator). The mRNA expression level was normalized by β-actin expression. The relative expression level of target genes in control

<table>
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<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Melting temperature (°C)</th>
</tr>
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<tbody>
<tr>
<td>Human DR5</td>
<td>Forward</td>
<td>5'-AACGACCTTGTGCTCTTGTG</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGGTTGCAACATCCCTCTG</td>
<td>86</td>
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<tr>
<td>p21WAF1</td>
<td>Forward</td>
<td>5'-GCAGACACCATGACAGATTT</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AAAGTGTAGAGCGGCTTT</td>
<td>81</td>
</tr>
<tr>
<td>TNFα</td>
<td>Forward</td>
<td>5'-CCAGCGACTGACATTCCTTC</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TGAGGTAACACCTGCTGAT</td>
<td>82</td>
</tr>
<tr>
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<td>5'-CAGCTGAGAGGAGTGAGA</td>
<td>82</td>
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<tr>
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<td>5'-TGCTCACTCCGGATACCTT</td>
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<td>82</td>
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<td>Reverse</td>
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<td>β-actin</td>
<td>Forward</td>
<td>5'-ATCCACATGCTGCTGTGAC</td>
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sample was normalized to a relative value of 1. Final results in treated samples were expressed as n-fold difference to the control samples. The sequences of primers are shown in Table 1.

**Chromatin immunoprecipitation assay.** U87 cells (1 × 10⁶) were cultured with SAHA (2.5 × 10⁻⁵ mol/L) for 1 day. Untreated cells were used as controls. Formaldehyde was added to the cells to a final concentration of 1%, and the cells were incubated at 37°C for 10 min. The cells were collected and subjected to chromatin immunoprecipitation assay using a chromatin immunoprecipitation kit (Upstate, Lake Placid, NY), according to the manufacturer’s protocol. Anti-acetylated histone H3 antibody (Upstate) or normal rabbit serum were used for immunoprecipitation. Immunoprecipitated DNA was recovered and used as a template for PCR. The reaction conditions for the PCR were as follows: denaturing at 95°C for 1 min and annealing at 60°C for 1 min, elongating at 72°C for 1 min, 30 cycles. The PCR products were run on 2% agarose gel to visualize them. The primers for p21⁰⁷⁻⁰⁴ were as follows: forward, 5'-GGTGTCTAGGTGCTCCAGGT-3'; reverse, 5'-CCACCTCTCGAGGAGGACA-3'.

**Cell proliferation.** Cells were placed onto 96-well plates at 2.0 × 10³ per well, and proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the protocol provided by Roche Molecular Biochemicals (Basel, Switzerland).

**Western blot.** Cells were harvested for total cell lysates with 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. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were collected, and the protein concentrations were measured. The lysates (30 μg) were denatured in the sample buffer [10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 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 antibodies were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Cell cycle analysis.** GBM cultures (U87, T98G, DA66, JM94, and GL26) were placed in six-well dishes and cultured with SAHA. After being trypsinized and washed twice with ice-cold PBS, cells were fixed in 70% ice-cold ethanol overnight. The samples were treated with RNase A and exposed to propidium iodide, then analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

**Preparation of the GL26 brain tumor xenografts.** Male BALB/c nude (nu/nu) mice and C57BL/6 mice were purchased at 4 to 6 weeks of age. Mice were housed under barrier conditions and maintained on a 12-h light/12-h dark cycle, with food and water supplied ad libitum. For brain implantation, the bregma was identified and a small burr hole was made 2.0 mm lateral to it. A tumor cell suspension (5 × 10⁵) was implanted via a Hamilton syringe exactly 3.5-mm deep to the dura of the brain of each mouse. Wild-type GL26 and GL26 cells stably transfected with the luciferin gene cells were implanted into BALB/c nude mice and C57BL/6 mice, respectively.

**SAHA preparation and administration.** Mice with GL26 glioma cells implanted in their brains were randomly divided into two groups, experimental and control. For administration to BALB/c nude mice by i.v. injection (10 mg/kg), the stock SAHA (100 mg/mL in DMSO) was first diluted in polyethylene glycol 400, then water was added to a final SAHA concentration of 2 mg/mL with 30% polyethylene glycol 400 and 4% DMSO. The freshly diluted SAHA solution was maintained at 50°C until injection. For i.p. administration to C57BL/6 mice, SAHA (100 mg/kg) was diluted in DMSO (10 mg/mL). The mice were injected from the 2nd day after implantation of the GL26 cells and then 5 d/wk. The control group was injected with vehicle only (30% polyethylene glycol 400 and 4% DMSO for BALB/c nude mice by i.v. injection; DMSO for C57BL/6 by i.p. injection). The tumor volume was measured in nude mice and calculated using the following formula: \( V = 0.526 \times S \times L \), where \( S \) is the largest tumor area in the serial sections and \( L \) is the depth of tumor invasion. In C57BL/6 mice, the luciferin from the intracranial GL26 was detected by the Xenogen IVIS 200 imaging system (Xenogen, Hopkinton, MA) after treatment for 2 weeks with SAHA.

**Immunofluorescence microscopy.** Frozen section samples were fixed in 4% paraformaldehyde. After nonspecific sites were blocked with 3% bovine serum albumin in TBS (PBS with 1% Triton-100) for 60 min at room temperature, the sections were incubated with the primary antibody [anti-human acetyl-H3 (Upstate) and anti–acetyl-H4 (Cell Signaling, Danvers, MA)] dilution overnight at 4°C in a...
moist chamber. Slides were washed twice with TBS and incubated with the TRITC-conjugated secondary antibodies (Sigma, St. Louis, MO; 1:400 diluted) for 60 min at 37°C. Cells were examined with a laser scan microscope. The immunohistochemical specificity of the antibody was confirmed by two types of negative controls: substituting rabbit nonimmune IgG for the primary antibody and omitting the primary antibody in the staining protocol.

**Statistical analysis.** Differences between the results of experimental treatments were evaluated by the t test. Survival was analyzed by Wilcoxon test. Differences were considered significant at values of \( P < 0.05 \).
**Results**

**SAHA inhibits cell proliferation of glioma cells in vitro.** We initially examined the effect of SAHA on the proliferation of the U87 GBM cell line (p53, wt) and the GBM explant MM156 (p53, wt) at different concentrations of SAHA (2 × 10⁻⁸ to 2 × 10⁻⁴ mol/L) and different lengths of exposure (2-7 days) in liquid cell culture using the MTT assay (Fig. 1A and B). SAHA inhibited growth of both, in a time- and concentration-dependent manner. For the U87 GBM cells, the ED₅₀ was 2 × 10⁻⁶ mol/L at 5 to 6 days. For the MM156 GBM explant cells, the ED₅₀ was 2 × 10⁻⁶ mol/L at day 7 (Fig. 1A and B). Furthermore, we examined the effect of SAHA (2 × 10⁻⁸ to 2 × 10⁻⁴ mol/L, 5 days) on additional three glioma cell lines, T98G (p53, mt, M237I), U118 (p53, mt, R273H), and murine GL26, as well as, two glioma explants, JM94 (p53, mt, R273C) and DA66 (p53, wt; Fig. 1C). The ED₅₀ (2 × 10⁻⁶ to 2 × 10⁻⁵ mol/L) of SAHA was comparable for each of these cell lines, suggesting that it could inhibit growth of GBM cells independent of their p53 status. Of interest, a clinical study in humans found that the mean serum level of SAHA ranged between 2 × 10⁻⁶ and 4 × 10⁻⁵ mol/L at 2 h after administration of the drug (75-900 mg/m², i.v.; ref. 20).

**SAHA increases the percentage of GBM cells in the G₂-M phase and alters their expression of cell cycle–related transcripts and proteins.** To explore the mechanism of action of SAHA, changes in the cell cycle distribution of GBM cell lines (U87, T98G), GBM explants (JM94, DA66), and a murine glioma cell line (GL26) were examined before and after their exposure to either control medium or SAHA (2.5 × 10⁻⁶ mol/L, 1 day). For all five populations, the percentage cells in the G₂-M phase increased at least 2-fold (Fig. 2).

**Fig. 4.** SAHA affects the level of acetyl-H3 in the brain. SAHA (100 mg/kg) was injected i.p. into C57BL/6 mice, followed 2, 6, and 24 h later with sacrifice of the mice, and lysates of the brains were made for Western blotting, which was probed with antibody against acetyl-H3 and glyceraldehyde-3-phosphate dehydrogenase (control for equal loading).

Gioma cell lines U87 and T98G, explants DA66 and JM94, and murine cell line GL26 were exposed to either diluent control or SAHA (2.5 × 10⁻⁶ mol/L) for 24 h. mRNA was extracted and reverse transcribed, and real-time reverse transcription-PCR was done. The genes involved in cell apoptosis...
Fig. 6. SAHA inhibits growth of intracranial glioma cells in BALB/c nude mice. GL26 glioma cells (5 × 10⁴) were injected into the brains of nude mice, and the mice were treated with SAHA (10 mg/kg, i.v.) or diluent control as described in Materials and Methods. After 19 d, mice were sacrificed and their intracranial GBMs were studied.

A, immunofluorescence examination of expression of acetyl-H3 and acetyl-H4. Sections of the murine brain were fixed with paraformaldehyde and blotted with primary antibody (anti-acetyl-H3 and acetyl-H4) followed by second antibody. Left column, background fluorescence of GBM cells (top two panels) and normal brain cells (bottom two panels). Middle column, acetyl-H3 expressed in GBM cells (top two panels) and normal cells (lower two panels). Right column, acetyl-H4 expressed in the GBM cells (top two panels) and normal brain cells (bottom two panels; ×B00). B, multiple sections of the brains were fixed and stained with H&E (HE). The largest tumor areas in the serial sections were identified and the volumes were determined. Representative diluent controls (top) and experimental SAHA-treated mice (bottom); the tumor areas are outlined. C, GBM size (volume) of each tumor and the mean of each group (dashed line).
and growth arrest were increased (DR5, TNFα, p21WAF1), and genes promoting cell cycle progression and growth were decreased (CDK2, CDK4; Fig. 3A).

Western blot results showed that SAHA (2.5 × 10⁻⁶ mol/L) caused the accumulation of the cyclin-dependent kinase inhibitors p21WAF1 and p27KIP1 (U87, T98G, DA66, JM94, GL26; Fig. 3B). At the same time, expression of proteins associated with enhanced cell growth, including CDK2, CDK4, cyclin D2, and cyclin D1, decreased. In addition, acetyl-H3 was markedly increased by SAHA in all of the cells exposed to SAHA (Fig. 3B).

Because the cyclin-dependent kinase inhibitor p21WAF1 was increased in GBM cells by SAHA, we examined if histones in the region of the gene were modified in these cells. SAHA increased the acetylation of histone 3 (H3) ~6-fold in the promoter of p21WAF1, as shown by chromatin immunoprecipitation (Fig. 3C).

**SAHA inhibits growth of glioma cells in vivo and increases survival of mice.** Because SAHA inhibited the growth of glioma cells in vitro, we wanted to examine if SAHA could inhibit GBM growth in vivo. Initially, SAHA was injected i.p. (100 mg/kg) into mice, and the brains were examined for levels of acetyl-H3; it increased by 6 h after injection, providing indirect evidence that SAHA crossed the blood-brain barrier (Fig. 4). Also, in a previous study, we found that SAHA (100 mg/kg, i.p., 5 d/wk) did not have noticeable side effects in mice, including their body weight, appearance, and behavior (21).

C57BL/6 mice were implanted with GL26 GBM cells stably transfected with the luciferin gene. After treatment by SAHA (i.p. 100 mg/kg) for 2 weeks, the β-luciferin firefly was injected i.p. (150 mg/kg) and tumors were imaged. Significantly lower luciferin activity was measured in the brains of the SAHA-treated mice (P < 0.05; Fig. 5A and B). Furthermore, these SAHA-treated mice lived 28 days, whereas the diluent-treated control group lived 20 days (median survival; Fig. 5C), which was significantly (P < 0.001) longer than the control.

To provide more evidence for the in vivo efficacy of SAHA, GL26 glioma cells were implanted into the brains of BALB/c nude mice, and a low dose of SAHA was injected i.v. into them (10 mg/kg, 5 d/wk). On day 19, the nude mice were euthanized, and frozen-sections were made of their brains. Immunofluorescence showed that the levels of acetyl-H3 and acetyl-H4 were markedly increased in both the normal brains and the intracranial tumors after treatment of the mice with SAHA, again consistent with the agent crossing the blood-brain barrier (Fig. 6A). Sections of these brains were fixed and stained with H&E and tumor volumes were calculated. Tumors from the SAHA-treated mice were a mean 4.9 ± 2.4 mm³ and those of the diluent control mice were 11.8 ± 3.5 mm³ (P < 0.05, t test; Fig. 6B and C).

**Discussion**

Our in vitro studies showed that SAHA inhibited the growth of GBM cells, with accumulation of these cells in G2-M. Both p53 mutant (T98G, JM94) and p53 wild-type (U87, MM156, DA66) glioma cells were equally growth-inhibited by SAHA (Fig. 1). Two other studies are consistent with our results, showing that proliferation of leukemic and breast cancer cells was inhibited by SAHA in a p53-independent manner (22, 23); in contrast, another study showed that transformed fibroblast cell lines were killed by the HDAC inhibitors trichostatin A and SAHA in a p53-dependent manner (24). Taken together, the ability of HDAC inhibitors to mediate growth arrest may be dependent on tissue type and/or the genetic lesion present in the cell. In GBM cells, the antiproliferative effect of SAHA is independent of p53 activity.

We found that SAHA significantly increased expression of anti-growth genes (p21WAF1, p27KIP1, DR5, TNFα) and decreased expression of pro-growth genes (CDK2, CDK4, cyclin D1, cyclin D2). CHIP analysis showed that SAHA acetylated the histones in the promoter region of p21WAF1 concurrent with enhanced expression of the gene.

A previous murine in vivo study administered doses of SAHA up to 100 mg/kg i.p. without apparent toxicity (25). We also gave either 100 mg/kg i.p. or 10 mg/kg i.v. without any noticeable ill effects (data not shown). Also, we found that SAHA caused an increase of histone acetylation of the brain and brain tumor in vivo after we administered SAHA (10 mg/kg, i.v.), providing evidence that the drug could probably cross the blood-brain barrier. Another study modeling Huntington’s disease also showed that SAHA could cross the blood-brain barrier (26). Our data showed that SAHA could retard the growth of intracranial GBM and increase the survival of these mice. The mode of action of this agent is different than chemotherapy and radiation therapy; therefore, clinical trials combining all three modalities of therapy may show increased efficacy.

**References**

increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 2003;63:7291–300.


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