

Continuous intracranial administration of suberoylanilide hydroxamic acid (SAHA) inhibits tumor growth in an orthotopic glioma model

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Abstract

Object Current treatments for malignant gliomas produce only a modest increase in survival time. New therapeutic approaches are desperately needed. Suberoylanilide hydroxamic acid (SAHA) is an effective inhibitor of the growth of many solid and hematological malignancies. Nevertheless, very few studies have investigated the effects of SAHA on glial tumors. The present study was designed to investigate the therapeutic effects of the intracranial local delivery of SAHA in an orthotopic glioma model.

Methods The antiproliferative effect of SAHA was examined in six glioblastoma and one endothelial cell lines *in vitro*. In addition, one glioblastoma cell line (U87MG) used in *in vivo* short term (14 days) and survival studies in an orthotopic human glioma athymic

mice model. Tumor volume, apoptosis rate, microvessel density, and proliferation index were determined by immunohistochemistry.

Results SAHA treatment inhibited the growth of all cell lines in concentrations ranging from 1 μM to 30 μM . For short-term studies, histological analysis showed an 80% reduction of tumor volume in the treatment group ($P < 0.001$). This reduction in tumor volume was associated with a significant increase in the apoptosis rate (31.9%, $P < 0.001$), a significant decrease in the proliferation (36.8%, $P < 0.001$) and angiogenesis rates (30%, $P < 0.05$). For survival studies, the mean survival time was 22 days in the control group, whereas it was 42 days in the treatment group.

Conclusions These results suggest that local delivery with SAHA inhibits intracranial glioma growth *in vitro* and *in vivo*. SAHA is a promising candidate for further preclinical and clinical studies on glial tumors.

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Introduction

Gliomas are the most common malignant primary brain tumors in adults, and their aggressive infiltration in the CNS typically produces progressive disability and eventually leads to death in nearly all cases. Despite the combination of modern microsurgical resection techniques with chemotherapy, radiotherapy and gene therapies expected survival time is

less than a year for patients with malignant gliomas [1–6]. New treatment strategies are desperately needed. Recently, histone deacetylase (HDAC) inhibitors have been identified as promising compounds for the treatment of various types of neoplasms, including lung [7], breast [8, 9], bladder [10], colon [11], prostate [12], ovary [13], endometrial [14] and hematological system [15]. However, there have only been only a few reports examining the effects of HDAC inhibitors on gliomas [16–19]. In addition, the Food and Drug Administration has approved the New Drug Application of SAHA (Zolinza-TM) for the treatment of advanced cutaneous T cell lymphoma in October, 2006.

HDACs and histone acetyl transferases (HAT) regulate the acetylation state of histones [15]. The reversible acetylation of the amino groups of specific histone lysine residues by histone deacetylases and histone acetyl transferases is an important regulatory mechanism of gene expression [16]. Histone acetylation is often associated with activated transcription and deacetylation correlates with transcriptional silencing. 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 such as *p21waf1* by epigenetic regulation [20–25]. SAHA selectively upregulates the expression of the pro-apoptotic members of the Bcl-2 family, and down regulates the expression of the antiapoptotic members [26]. It has also been shown that SAHA inhibits angiogenesis with modulation of angiogenesis related genes both in cancer cells and in endothelial cells [27–29]. Deletions or mutations inactivating HAT have been reported to be associated with tumor progression in humans [30, 31]

A well-established and steady pattern of drug exposure provided by osmotic minipumps for several weeks to months, has facilitated their use in several clinical conditions for direct intracranial delivery of substances [32]. In addition, the local continuous administration of inhibitors by osmotic minipumps has been shown to provide higher efficacy compared to daily systemic administration. Low quantities of drug, less toxicity and prolonged delivery are the advantages over systemic administration [33].

In this study, we elucidated inhibitory properties of the local delivery of the HDAC inhibitor SAHA, in an in vitro and in vivo glioma model. To our knowledge, this is the first study presenting the effect of the intracranial continuous local delivery of SAHA in an orthotopic glioma model.

Material and methods

Cell culture

The malignant human glioma cell lines U87MG, U343, LN229 (American Type Culture Collection, Manassas, VA), U251 (NCI-Frederick DCTD Tumor/Cell line repository Frederick, VA), G55 (gift from Manfred Westphal Hamburg, Germany), malignant mouse glioma cell line GL261, (NCI-Frederick DCTD Tumor/Cell line repository Frederick, VA) and porcine aortic endothelial cells (PAE) transfected with KDR (gift from Lena Claesson-Welsh, Ludwig Institute, Uppsala, Sweden) were used.

U87MG cells were cultured in α -MEM; U343, LN229, U251, G55, and GL261 in DMEM; and PAE/KDR cells in Ham's F-12 medium. All the media were supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml fungizone (Invitrogen, Grand Island, NY). For the intracranial implantation experiments, U87MG cells were dispersed with a 0.05% solution of trypsin/EDTA (Life Technologies, Inc.), and were adjusted to a final concentration of 1.2×10^5 cells/3 μ l in PBS. The cells were maintained in T-75 cm² tissue culture flasks in humidified atmosphere containing 5% CO₂ at 37°C. All the cell lines were used between passages 20 and 40.

Chemicals and osmotic pumps

SAHA (Fig. 1) was purchased from BioVision (Mountain View, CA) and stocks were prepared in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). Before experimental use, the drug was diluted in medium. The pumps were obtained from Alzet Corp. (Cupertino, CA).

In vitro cytotoxicity assay

To assess the cytotoxic effect of SAHA, cells (4×10^3 cells/well) were plated on 96-well plates

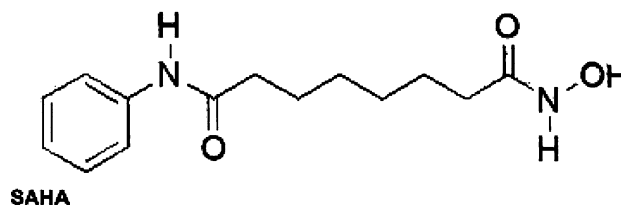


Fig. 1 A diagram of SAHA structure

(Corning, Inc., Acton, MA). Cells were cultured in the presence of increasing concentrations of SAHA (1, 3, 5, 8, 10, 15, 20, 25, 30 μ M). The same concentration of DMSO was added to the control wells. Cell viability was assessed by colorimetric assays using Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD). All the experiments were performed in quadruplicate and repeated three times. Viability determination was based on the bioconversion of the tetrazolium compound, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8), into formazan, as determined by absorbance at 450 nm using a multiwell scanning spectrophotometer (Labsystem multiskan MCC/340, Fisher). WST-8 is bio-reduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells. Cell viability was expressed as the mean \pm SD in percentage of the control viability (=100%).

Immunoblot analysis of acetylated-histones-H3 and H4

Subconfluent plates of U87MG cells were treated with varying concentrations of SAHA for 3 h at 37°C then washed in PBS, trypsinized, and resuspended in lysis buffer [0.02 M Tris (pH 7.4), 0.2 mM Triton X-100, and 0.02% B-mercaptoethanol] and disrupted by sonication for 2 min at 4°C. Protein was quantified using Protein Assay Reagent (Pierce, Rockford, IL) and 10 mg of each extract was separated by SDS-PAGE. Immunoblots were probed with α -acetylated-H3 or α -acetylated-H4 (Upstate Biotechnology, Lake Placid, NY), then incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA) and visualized by Western Blot Chemiluminescence Reagent (Cell Signaling Technology, Beverly, MA).

In vitro matrigel angiogenesis assay (tube formation)

PAE/KDR cells were cultured in Ham's F-12 media before being plated on 24-well plates (5×10^4 cells per well) previously coated with 300 μ L of growth factor-reduced Matrigel (BD Biosciences, Bedford, MA). The seeded cells were subjected to three different conditions: Condition 1 (negative control): cells grown in Ham's F-12; Condition 2 (positive control): cells incubated with U87MG-conditioned medium; and Condition 3: cells incubated in U87MG-conditioned medium plus SAHA at increasing concentrations (1, 5, 10, 20 μ M). The morphology of the capillary-like structures

formed by PAE/KDR cells was visualized using an inverted microscope (Nikon EclipseTE300) and photographed with a digital camera (Diagnostic Instruments, Inc., Houston, TX) 36 h after culturing. The number of microvessels were counted in five different fields at 200 \times magnification and recorded [34].

In vivo intracranial model

A pilot in vivo study was conducted to determine the lowest effective dose of SAHA. Alzet osmotic minipumps loaded with increasing concentrations of SAHA (10, 20 and 50 μ M in 100 μ l) were implanted into intracranial tumor bearing mice. Based on these pilot in vivo studies and our in vitro cytotoxicity assays we determined that the 20 μ M concentration of SAHA represented the best balance between the highest efficacy with no toxicity. Therefore, this dose was used in all subsequent in vivo studies. Twenty 4-week-old male athymic mice (Charles River, Wilmington, MA) were stereotaxically implanted with U87MG cells (1.2×10^5 cells/3 μ l in PBS) into the left forebrain at the following coordinates: 2.5 mm lateral and 1 mm anterior to bregma at a 2.5 mm depth from the skull surface under anesthesia. Five days after tumor cell injection, mice were implanted with type1002 Alzet osmotic minipumps. Animals were randomized into two groups of ten animals each, the control group (PBS containing 0.1% DMSO) or treatment group (SAHA, with a final volume of 100 μ l, 20 μ M concentration) to afford a constant pumping rate of 0.25 μ l/h for 14 days. All the pumps were weighed before and after filling. The pump reservoir was connected to an intracranial catheter placed into the tumor cell injection site in the same hemisphere. After 14 days of treatment, all the animals were sacrificed. The brains were removed after perfusion, placed in sucrose gradient solution, embedded in optimum cutting temperature compound (OCT-Tissue-Tek, Miles, Elkhart, IN), and stored at -80°C . Later, the brains were sectioned coronally using a cryostat into 10- μ m thick slices that were mounted on slides and then used for H&E staining and immunohistochemistry. Tumor volumes were calculated from brain sections by histological analysis using the formula ($V = \pi.L.H.W/6$) for ellipsoid tumors and expressed as mean \pm SD as previously described [35].

Immunohistochemistry

Immunohistochemistry was carried out using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The brain sections were fixed in cold acetone and endogenous peroxidase activity was

blocked with 0.3% hydrogen peroxide in methanol for 10 min at room temperature. Primary antibodies included anti-cleaved caspase-3 (1:100; Cell Signaling Technology, Beverly, MA) for the detection of apoptosis, anti-CD31 (1:100; BD Biosciences PharMingen, San Jose, CA) for blood vessel density, and anti-Ki67 nuclear antigen (1:100; DAKO, Carpinteria, CA) for proliferating cells. The sections were counterstained with hematoxylin, and the sections without primary antibody served as negative controls. The apoptotic and proliferation indices were defined as the percentage of positively stained cells of 100 nuclei from five randomly chosen high-power fields. Microvessels were counted in five different fields at 200 \times magnification and the vessel numbers were recorded [34, 36].

Survival study

The effects of a local infusion of SAHA on the survival of athymic mice with established U87MG human glioblastoma (18 animals) were assessed. U87MG cells were stereotaxically implanted (1.2×10^5 cells/ $3 \mu\text{l}$ in PBS) into the left forebrain. Five days after tumor cell injections, pumps were filled either with PBS containing 0.1% DMSO for the control animals or with SAHA ($n = 9$ per group) at a final volume of $100 \mu\text{l}$ ($20 \mu\text{M}$ concentration) to afford a constant pumping rate of $0.25 \mu\text{l/h}$. After 28 days, the pump reservoir and the intracranial catheter were replaced in surviving animals. Animals were sacrificed at the early onset of neurological signs. All the animals were examined for possible organ toxicity of SAHA. Animal studies were carried out in the animal facility at Brigham and Women's Hospital in accordance with federal, local, and institutional guidelines.

Statistics

All of the values were calculated as mean \pm SD or were expressed as percentage of control \pm SD. The P -values for cell viability, tumor volume, proliferation, apoptosis index, and microvessel density were determined using the Mann–Whitney U test. Values of $P < 0.05$ were considered significant. Kaplan Meier curve was used for evaluation of survival study. Survival for treated and control mice were compared with log-rank test.

Results

In vitro cytotoxicity assay

We examined the cytotoxic effects of the HDAC inhibitor SAHA on six gliomas cell lines. After 96 h

exposure to increasing concentrations of SAHA, cell viability was assessed by a colorimetric assay using a CCK-8 the kit. As shown in Fig. 2A, SAHA was a potent inhibitor of all glioma cell lines examined. Cytotoxicity was observed as evidenced by a sharp, significant decrease in cell viability. This dose-dependent response was observed for all tested glioma cell lines. PAE-KDR endothelial cells were also sensitive to SAHA treatment. The IC_{50} (50% inhibitory concentration) ranged from $2 \mu\text{M}$ to $9 \mu\text{M}$ (Fig. 2A). The histone deacetylase inhibitory activity of SAHA was evaluated in U87MG cells. Marked hyperacetylation of both histone H3 and H4 was observed following a 3-h incubation with $1\text{--}10 \mu\text{M}$ of SAHA (Fig. 2B).

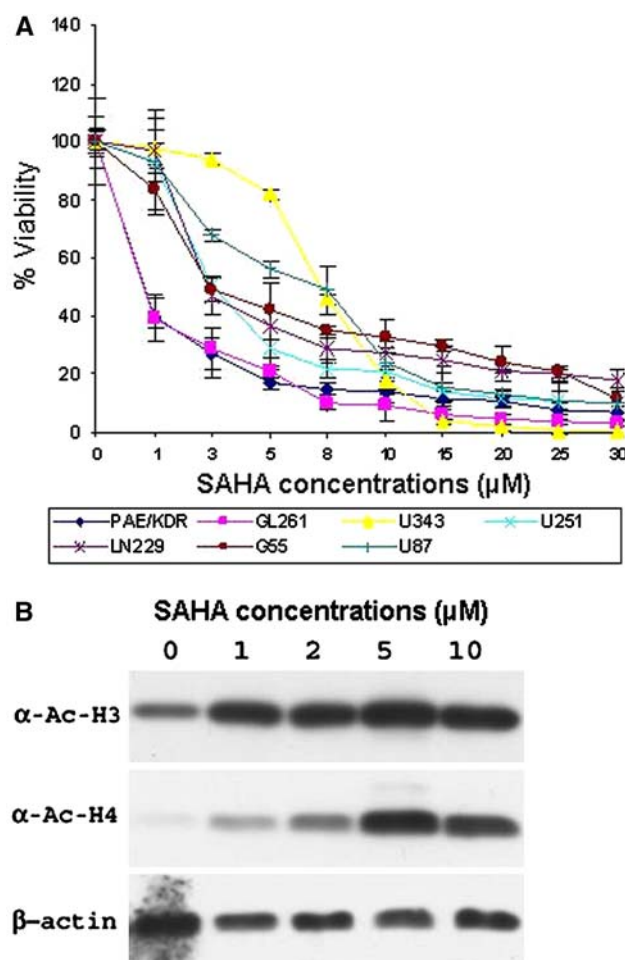


Fig. 2 (A) Cytotoxic effect of SAHA on endothelial and glioma cell lines. Cell viability was evaluated after 96 h exposure to SAHA. SAHA inhibited cell viability in a dose dependent manner. IC_{50} ranged from $2\text{--}9 \mu\text{M}$ for the cell lines used. (B) U87MG cells were treated at the indicated SAHA concentrations for 3 h. Cell extracts were prepared as described in “Materials and Methods”. Histone acetylation was detected by Western blot using an antibody against acetylated H3 or H4

Tube formation assay

Treatment with SAHA significantly inhibited vessel formation in a dose dependent manner (Fig. 3A, $*P < 0.05$). In the presence of U87MG conditioned media, endothelial cells form tubes and capillary-like structures on the surface of basement membrane matrices (Matrigel), through a process involving attachment, alignment, and migration. However, SAHA treatment disrupted the tube formation process even at the 1 μM concentration. Endothelial cells growing in Ham's F-12 were used as negative control (Fig. 3B).

In vivo therapeutic effect of SAHA on intracranial tumor growth

A pilot in vivo study showed a significant inhibition of tumor growth only for the 20 μM dose, but not for the 10 μM and 50 μM doses (data not shown). The viscosity of the 50 μM solution was high and therefore did not allow an even flow rate, which may account for the lack of inhibition of tumor growth observed at this higher dose.

Therapeutic effect on intracranial tumor growth

The *in vivo* therapeutic effect was assessed by intracranial local microinfusion of SAHA via osmotic-minipumps implanted at the tumor site 5 days after glioma cell injection. The release of a daily total dose of 0.25 $\mu\text{l/h}$ of SAHA for 14 days resulted in significant tumor growth inhibition (80%, $**P < 0.001$). A significant reduction of tumor volume was observed in the SAHA treated groups when compared with the

respective control group (control $33.46 \pm 25 \text{ mm}^3$ vs. treated $6.76 \pm 2.3 \text{ mm}^3$) (Fig. 4).

Immunohistochemistry

A 3.1-fold increase in the apoptotic index (31.9%) ($**P < 0.001$), a 30% decrease in microvessel density ($*P < 0.05$), and 36.8% inhibition in the proliferation index ($**P < 0.001$) were observed in the SAHA treated group compared to the PBS controls (Fig. 5).

Survival study

To assess the effect of intracranial microinfusion of SAHA on survival time of athymic mice with established orthotopic human glioblastoma xenografts, minipumps were implanted in the brain 5 days after tumor injection. The average survival of the control group was 23 days (19–26) after tumor implantation; while it was 42 days (33–53) for the SAHA treated mice (Fig. 6). SAHA treatment resulted in a significant increase in survival time (91%, $P < 0.001$). No signs of toxicity were observed in any of the animals.

Discussion

In our study, we examined the inhibitory properties of the local delivery of the HDAC inhibitor SAHA, in an *in vitro* and *in vivo* glioma model. Malignant gliomas are aggressive and highly vascularized tumors, which usually recur within 2 cm of the original tumor and are rarely metastatic. Even with recent advances in surgical techniques, chemotherapy and radiation, the survival of patients with malignant gliomas has not

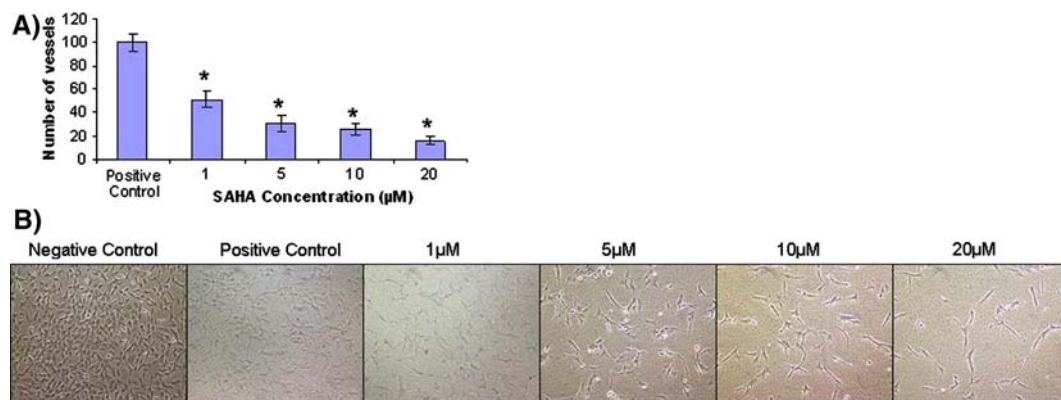


Fig. 3 The *in vitro* tube formation assay. **(A)** Five random fields were counted from control and treated wells, under the microscope after 36 h ($*P < 0.05$, Mann–Whitney *U* test). SAHA inhibited vessel formation in dose-dependent manner.

(B) PAE/KDR cells in normal growth media (negative control). PAE/KDR cells in U87MG conditioned media alone (positive control) or increasing concentrations of SAHA (1, 5, 10, and 20 μM , respectively)

Fig. 4 Tumor volumes were calculated 20 days (15 days after pump implantation) after tumor inoculation ($n = 20$) (H&E staining, original magnification—1 \times). Comparing to control group (A) treated group (B) showed 80% reduction in tumor volume. (C) Tumor volumes were calculated from histological analysis; (SD bars, $**P < 0.001$, Mann–Whitney U test)

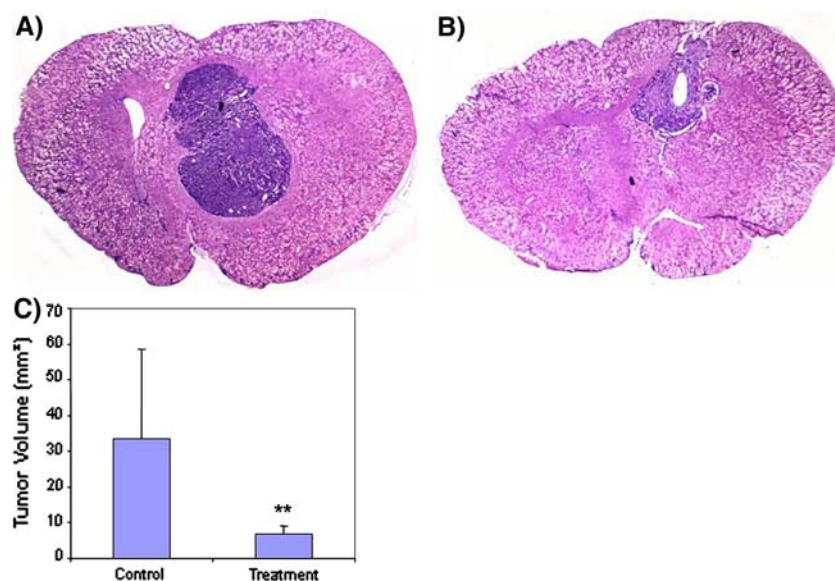
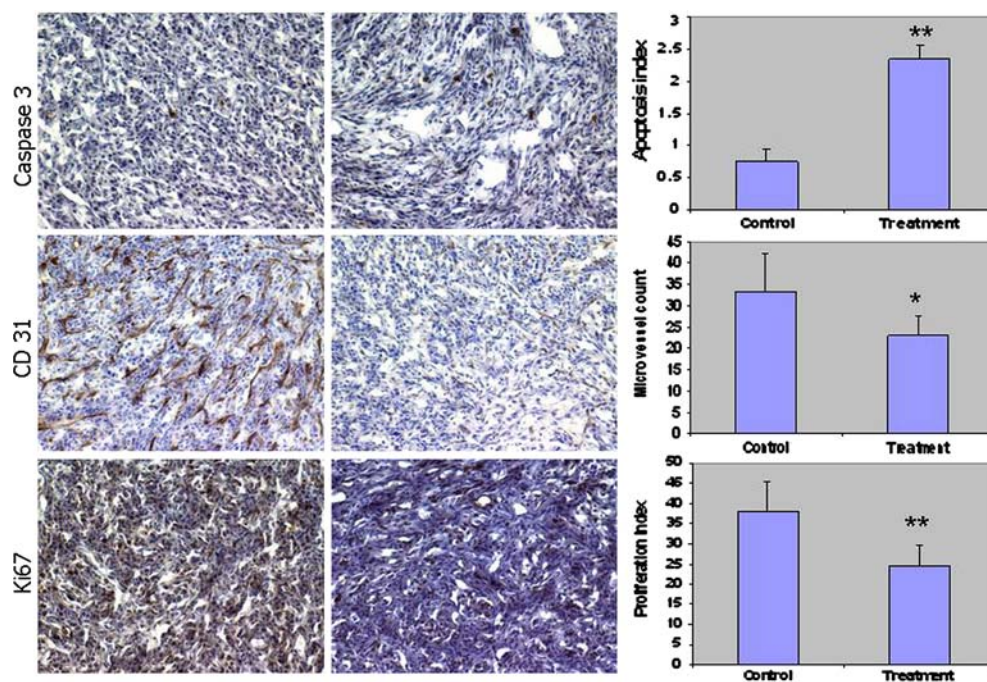


Fig. 5 Treatment effects assessed by immunohistochemistry ($n = 20$). Primary antibodies included anti-cleaved caspase-3 for the detection of apoptosis, anti-CD31 for blood vessels, and anti-Ki67 nuclear antigen for proliferating cells. Sections were counterstained with H&E (original magnification, $\times 200$). (SD bars - apoptosis index and proliferation index, $**P < 0.001$; microvessel count $*P < 0.05$, Mann–Whitney U test)



increased. Therefore, new treatment strategies are desperately needed for malignant glioma. Histone deacetylase inhibitors are a promising class of antineoplastic drugs. Modulation of the acetylation status of histones is a critical mechanism involved in the regulation of transcriptional activity of genes vital to tumorigenesis and treatment resistance [23, 30, 37]. The acetylation and deacetylation of histones is a posttranslational modification of core nucleosomal histones that alters chromatin structure resulting in modulation of gene expression. While a variety of

studies have demonstrated the anti-glioma properties for SAHA in vitro, there is only one study reporting the effect of SAHA in an intracranial glioma model. Eyupoglu et al. [17] report that a single dose of SAHA can increase the life span of intracranial glioma bearing mice ($n = 3$). We extended this study by using a larger number of animals and delivering the SAHA continuously via intracranial osmotic minipumps. In addition, we studied not only survival, but also tumor volume, apoptotic and angiogenic indexes and cell proliferation.

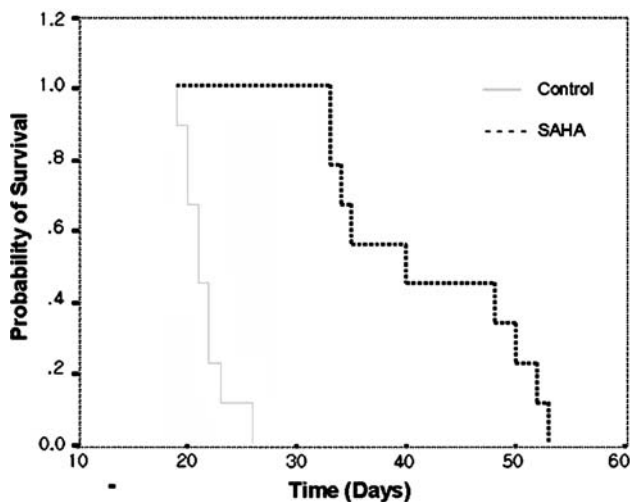


Fig. 6 Local delivery of SAHA increased survival in orthotopic tumor bearing mice. Kaplan–Meier curves illustrating the survival of mice treated with PBS ($n = 9$) or SAHA ($n = 9$) after implantation of intracranial tumor

High local concentrations of SAHA are required to achieve effective histone deacetylase inhibition. We found that 5–10 μM of SAHA was required to inhibit deacetylation of histones H3 and H4 *in vitro*. Previous studies have demonstrated that in addition to high local concentrations, extended exposure to SAHA is required to down-regulate target mRNA expression [38]. A major obstacle to effective therapy for malignant gliomas has been the inability to achieve high intratumoral drug levels due to the blood-brain barrier. In our study, SAHA was administered via continuous intracranial delivery by osmotic minipumps. By continuous drug delivery, high local levels can be achieved at the tumor site, which would be expected to optimize its anti-tumor activity. Future preclinical studies could combine intracranial SAHA with other agents delivered either systemically or by pump as well.

In our *in vitro* study, SAHA was cytotoxic to tumor and endothelial cells. Furthermore, SAHA was also shown to inhibit vessel formation of endothelial cells at micromolar doses. In addition, immunohistochemical staining with Caspase-3, CD31, and Ki67 revealed that the apoptotic index of the tumor tissue was significantly increased while the vascularization and proliferation of the tumor tissue was decreased compared to the control group. SAHA treatment resulted in decrease in tumor volume in short term *in vivo* studies, and significantly increased the survival time of athymic mice with intracranial U87MG tumors. Future studies should evaluate the effect of SAHA on other glioma cell lines including conduct pharmacokinetics.

Phase 1 clinical trials have shown that even in advanced cancers, SAHA can be used with some side

effects like nausea, diarrhea, fatigue and thrombocytopenia [37, 39, 40, 41]. SAHA crosses the blood-brain barrier; however, to provide maximum drug concentration and efficacy in the tumor bed for prolonged exposure, we preferred local continuous delivery. In a model of organotypic glioma invasion, SAHA inhibited tumor associated cytotoxicity of adjacent brain parenchyma [20]. In the organotypic brain environment, no significant toxicity or neuronal damage was observed even at 80 μM dose of SAHA (17). Likewise, we did not encounter any toxic effects during post mortem organ evaluation at 20 μM concentration of SAHA when given locally. SAHA increases the cytotoxicity of anti-cancer drugs that target DNA such as cyclophosphamide, VP-16, 5-FU, and doxorubicin [42]. SAHA also appears to sensitize cells to radiation by suppression of DNA repair mechanisms [16, 43]. The ability of SAHA to sensitize to a wide variety of cytotoxic therapies and radiation may facilitate its usefulness in combined modality treatment of glioma. In summary, SAHA is effective in inhibiting the growth of malignant glioma and endothelial cell lines *in vitro*. SAHA also reduced glioma growth *in vivo* and increased survival when administered by continuous local intracranial delivery through osmotic minipumps. SAHA therapy resulted in pro-apoptotic, anti-angiogenic and anti-proliferative effects *in vivo*. These results suggest that with local delivery, SAHA may serve as an effective therapy for glioma. Future studies should include pharmacokinetic data and expand the number of cell lines used for the *in vivo* experiments.

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