J Neurooncol (2006) 79: 259–270
DOI 10.1007/s11060-006-9142-0

LABORATORY INVESTIGATION

Suberoylanilide hydroxamic acid is effective in preclinical studies of medulloblastoma

Susan E. Spiller · Ali C. Ravanpay · Andrew W. Hahn · James M. Olson

Received: 8 May 2005 / Accepted: 6 March 2006 / Published online: 28 April 2006

Abstract
Purpose Suberoylanilide hydroxamic acid (SAHA) has been studied in adult solid and hematologic malignancies. However, little information has been reported on the effects of SAHA on central nervous system (CNS) tumors including medulloblastoma, the most common malignant brain tumor in children. We investigated SAHA in preclinical medulloblastoma models to determine its anti-cancer efficacy as well as its ability to affect intracranial lesions when administered systemically.
Experimental design and results Tissue culture studies were performed treating primary human fibroblasts, established medulloblastoma cell lines, and primary human medulloblastoma tumors with SAHA. At 10 μM concentration, SAHA had little effect on normal fibroblasts but caused >90% apoptosis in cultured medulloblastoma cells. Primary medulloblastomas from patients were sensitive to SAHA compared to vehicle alone in ex vivo studies. In athymic mice with medulloblastoma xenograft tumors, oral SAHA resulted in apoptosis of tumor tissue and significantly slowed tumor growth. In the ND2:Smo transgenic mouse medulloblastoma model, SAHA treatment caused significant apoptosis in these cerebellar tumors.
Conclusions SAHA effectively induces cell death in established medulloblastoma cell lines, human patient primary tumor cultures, medulloblastoma xenografts and intracranial spontaneous medulloblastomas. Fibroblasts in culture and mice treated with SAHA did not reveal prohibitive toxicity profiles. These findings support the advancement of SAHA to pediatric clinical trials.

Keywords HDI · Brain tumor · Pediatric · Medulloblastoma · SAHA · ND2:Smo

Abbreviation

SAHA	suberoylanilide hydroxamic acid
HDI	histone deacetylase inhibitor

Introduction

The most common malignant brain tumor in children is medulloblastoma**.** Event-free survival in children with medulloblastoma is approximately 79% at 5 years [1]. However, high-risk features of young age at diagnosis, incomplete resection, or metastatic disease are each associated with a much worse prognosis. Survivors often suffer substantial deficits caused from therapy [2].

Finding chemotherapeutic agents that are capable of effectively entering and eliminating intracranial tumors without causing severe systemic toxicity is one of the many challenges faced by adult and pediatric neurooncologists. The issues in pediatric neurooncology include

S. E. Spiller · A. C. Ravanpay · A. W. Hahn · J. M. Olson (✉)
Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Seattle, WA 98109, USA
E-mail: jolson@fhcrc.org
Tel.: +1-206-667-7955
Fax: +1-206-667-2917

S. E. Spiller · J. M. Olson
Department of Pediatrics, University of Washington and Children's Hospital and Regional Medical Center, Seattle, WA 98105, USA

A. C. Ravanpay
Program in Neurobiology and Behavior, University of Washington, Seattle, WA 98195, USA

not only curing the tumor and decreasing acute toxicities of therapy, but also preserving the child's ability to acquire and retain knowledge and memory, and development or refinement of motor skills. In addition, the hypothalamic–pituitary axis needs to be preserved in order to maintain normal hormone levels needed for growth velocity changes and entry and progression through puberty. The majority of cognitive and endocrine side-effects sustained during treatment are secondary to radiation therapy. Clinically, in an attempt to protect CNS function, cooperative group trials are focusing on maintaining survival benchmarks while lowering the dose of radiation. It is crucial that new chemotherapeutic candidates do not have neurotoxic or other systemic effects that may exacerbate the damage done by radiation.

Histone deacetylase inhibitors (HDIs) are an exciting class of drugs that show promise of successfully treating a variety of hematologic and solid tumors both in preclinical and clinical studies. They have a more tolerable side-effect profile than traditional chemotherapy, making them attractive candidates for the pediatric brain tumor population. It is generally accepted that HDIs kill tumors of various cell types by providing increased opportunity for transcription of cell cycle regulation, apoptotic pathways and tumor surveillance genes. Histone modifications include phosphorylation, methylation, ubiquitination and acetylation. There are well-balanced systems in place to allow transcriptional activation or repression based on these modifications. Histone acetyl transferases apply acetyl groups to lysine residues on histone tails, and histone deacetylases remove the acetyl groups. Although HDIs globally alter gene transcription [3], toxicity to normal tissue appears to be minimal. The positive charges on histones that keep them tightly wrapped in negatively charged DNA is neutralized by acetylation of histone tail lysine residues, allowing DNA relaxation. This relaxation allows transcriptional machinery more access to DNA promoter regions. The mechanism of action common to all HDIs is that they bind the catalytic site of histone deacetylase enzymes, preventing histone deacetylation. Preventing deacetylation keeps DNA in 'open' transcriptional conformation, allowing for enhanced opportunity for genes such as tumor suppressors to be transcribed [3]. Whether this accounts for the therapeutic window between the effects on cancer cells compared with normal tissues remains to be established.

SAHA has been formulated in both oral and intravenous forms, with good bioavailability in both routes of administration [4]. Microarray analysis of the genetically engineered ND2:Smo mouse model for medulloblastoma indicates that these tumors overexpress HDAC2, one of the histone deacetylases (data not shown). This information coupled with the success of SAHA anti-tumor activity reported in many other tumors suggested that it could be active against medulloblastoma.

Thus far, little evidence has been presented to suggest that SAHA will be as successful in intracranial tumors as HDIs are in cancers outside of the central nervous system. Hockly et al. [5] reported histone hyperacetylation in whole brain extracts of wild-type mice and R6/2 mice, a Huntington Disease mouse model, after subcutaneous injection of SAHA formulated to be water-soluble, suggesting that SAHA is able to cross the blood–brain barrier. However, the cancer therapeutic mechanism of HDIs was not addressed, so anticancer activity within the brain is still not known. Eyupoglu et al. [6] inoculated rat brains with F98 glioma cells and a week later SAHA was administered locally to the area of glioma cell transplantation. In this experiment, rats receiving SAHA injections at the site of glioma insertion survived about twice as long as those receiving vehicle injections alone. No other reports were found that describe more clinically applicable evidence of SAHA activity in intracranial tumors. One goal of the studies reported here was to recapitulate the clinical scenario of administering SAHA systemically to treat an intracranial tumor.

We present a set of experiments to show the effect of SAHA on medulloblastoma cells in vitro and in vivo. In addition, SAHA was studied in medulloblastoma xenografts and a genetically precise mouse model of medulloblastoma, to determine whether SAHA should be considered for clinical medulloblastoma trials.

Materials and methods

Histone acetylation Westerns

Histone extraction was performed as previously described [7]. D283 medulloblastoma cells were collected by trypsinization and centrifuged at 1750 rpm×10 min, washed in ice-cold PBS, and the pellet was resuspended in 1 ml histone lysis buffer (Tris 10 mM, Na bisulfite 50 mM, Triton X-100 1%, $MgCl_2$ 10 mM, sucrose 8.6%, pH 6.5). Cells or frozen tissue samples from xenografts were dounce homogenized (5 strokes, tight pestle, or 20 strokes, respectively) and washed three times in lysis buffer, washed one time in Tris–EDTA pH 7.4 and resuspended in 100 µl water. Sulfuric acid histone extraction was performed, and isolated histones were separated on 4–15% gradient SDS PAGE mini-gel (Bio-Rad Laboratories, Hercules, CA) and transferred to a nitrocellulose membrane. Ponceau S (Sigma-Aldrich, St Louis, MO) was used to stain the membrane for 2 min, the image was preserved as a loading control, and the membrane was washed free of

stain in water. The membrane was probed using rabbit polyclonal antibody against acetylated H3 (Upstate USA Inc, Charlottesville, VA) and visualized using goat anti-rabbit IgG conjugated to horse radish peroxidase (Bio-Rad Laboratories, Hercules CA) and radiographically imaged with Lumi-light (Roche Diagnostics, Indianapolis, IL) chemical luminescence. Each lane represents one individual sample.

Cell culture and viability studies

Primary human foreskin fibroblasts were kindly contributed by the lab of Dr. William Carter and cultured in RPMI (Gibco, Grand Island, NY)+10% FBS [8]. Cells were seeded at 2×10^5/ml in 2 ml of media per condition. SAHA was added within an hour of plating cells, and both media and drug were replaced every 48 h. Cells were tested at 72 h for viability by automated Trypan blue exclusion using ViCell (Beckman Coulter Corp., Hialeah, FL). Apoptosis was measured with annexin V staining (BioVision Inc, Mountain View, CA) and fluorescence microscopy. Suberoylanilide hydroxamic acid (SAHA) was generously provided by Aton Pharma, (Tarrytown, NY) and Merck (Boston, MA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO) for a stock concentration of 10 mM.

Medulloblastoma cell lines

The human medulloblastoma D283 and D341 cell lines were obtained from American Type Culture Collection (Manassas, VA). D283 cells were cultured in Minimum Essential Medium Alpha (MEM Alpha) (Gibco, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT), sodium pyruvate 1 mM (Gibco) and penicillin–streptomycin (Gibco). D341 cells were cultured in MEM Alpha with 20% FBS, sodium pyruvate, and penicillin–streptomycin. Cells were plated at 2×10^5 cells per ml and treated for 72 h with SAHA or DMSO, then tested for viability and apoptosis as described for fibroblasts above.

Human tumor studies/CD56 sorting

Primary pediatric medulloblastoma tumor samples were obtained from participating Children's Oncology Group institutions. Samples were collected from patients undergoing diagnostic and therapeutic surgical resection after informed consent was obtained. These studies had prior approval from the Institutional Review Board of each participating hospital. Samples with greater than one million viable cells and more than 60% viability were used. Upon acquisition, samples were washed in Dulbecco's phosphate buffered saline (PBS) (Gibco) and transferred to DMEM-F12 (Gibco) with 10% FBS and antibiotic/antimycotic supplements (Gibco). Samples were minced with scalpels and further triturated with a 10 ml syringe and needles of decreasing caliber. Samples were filtered through a 70 μm nylon mesh and pipetted into 96 well assay plates at $1–2\times10^4$ cells per well. Each well received either 5 μM SAHA in DMSO or 1:1000 DMSO. Each plate was incubated in the dark (37°C at 5% CO_2) for 48 h and then assayed for viability using ViaLight HS Proliferation/Cytotoxicity Kit (Cambrex Bio Science Rockland, Inc., Rockland, ME). This assay measures bioluminescent ATP release as an indicator of metabolically active cells. A linear relationship between this assay and trypan blue exclusion has been established in D283 cells (A. R. Hallahan, unpublished results, this laboratory). For CD56 sorted samples, the cells were resuspended in 80 μL of sort buffer consisting of PBS+0.5% BSA (Sigma-Aldrich) +2 mM EDTA. CD56 microbeads (Miltenyi Biotec GmbH, Auburn, CA) were added for cell sorting. After 15 min at 4°C the cells and beads were washed with sort buffer and loaded onto an MS column (Miltenyi Biotec). Cells were washed three times with sort buffer over the column, and removed from the magnetic field. Without removing the magnetic beads, cells were pipetted into 96 well assay plates at $1–2\times10^4$ cells per well. Each well received either 5 μM SAHA in DMSO, 1:1000 DMSO as a negative control, or 10 μM etoposide (Sigma-Aldrich), 10 μM cyclosporin A (Sigma-Aldrich), and 25 μM cisplatin (Sigma-Aldrich) as a positive control. Each plate was incubated in the dark (37°C at 5% CO_2) for 48 h and then assayed for viability using the ViaLight system as described above.

Xenografts

About 10^6 D283 cells were suspended in media mixed 1:1 with Matrigel basement membrane matrix (BD Biosciences, Franklin Lake, NJ) for a total volume of 200 μl, and were injected subcutaneously into the flanks of 6–8-week-old female NU/NU athymic mice (Charles River Laboratories,Wilmington, MA). Tumors were measured with digital calipers (Stoelting Co, Wood Dale, IL) and tumor volumes were calculated as $A^2\times B/2$, where A is the short diameter of the tumor, and B is the long diameter. Mice in all studies were weighed and assessed for general health twice a week, and tumors were measured once a week. Animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Experimental Animals and approved by our Institutional Animal Care and Use Committee (IR#1573).

Minimal disease study

Mice were divided into two arms, with five mice per arm, and one tumor injection per mouse. They were fed either powder food or powder food with SAHA mixed into it at 200 mg/kg/day. SAHA was given daily beginning the day after tumors were injected. Food±drug was renewed every 3–4 days.

Bulk disease/oral SAHA treatment study

Mice were injected with 10^6 D283 cells as described above, and when the average tumor volume reached 200 mm^3 (approximately 2–4 weeks after tumor cells were introduced into the flank) the mice were divided into two arms, treatment (n=18) versus control (n=17). Mice in the treatment arm received SAHA 200 mg/kg/day daily in powder food, and controls got powder food alone. Food±drug was renewed every 3–4 days.

Bulk disease/intraperitoneal SAHA treatment study

Mice were injected with two flank tumors per mouse as described. When tumors reached an average of 90 mm^3 in volume, mice were divided into two arms. The treatment arm (n=9) received daily intraperitoneal (ip) injections of 4 mg SAHA (approximately 200 mg/kg/d) in 50 µl of DMSO and controls (n=12) got daily 50 µl ip DMSO alone.

TUNEL staining in flank xenografts

Animals were injected subcutaneously with one million D283 cells as described above. When tumors were at least 150 mm^3 in volume, three animals were fed powder food with 200 mg/kg/d of SAHA. After 4 days of treatment the animals were sacrificed and tumors were cryopreserved and sectioned. Control tumors were collected from three animals fed powder food without drug. Apoptosis was quantified in xenograft tumors through terminal deoxynucleotide transferase mediated dUTP nick end labeling (TUNEL) using In Situ Cell Death Detection Kit as per manufacturer instructions (Roche Diagnostics, Indianapolis, IL) and fluorescence microscopy. Ten non-overlapping high power fields per tumor were counted by an observer blinded to treatment conditions, and TUNEL-positive cells were reported as a percentage of all cells in the field as determined by DAPI staining.

Intracranial tumor apoptosis and proliferation studies

ND2:Smo transgenic mouse lines were developed in this laboratory as described previously [9]. Briefly, the neuroD2 promoter is used to drive overexpression of a mutated copy of Smoothened, a Sonic hedgehog (shh) pathway activator, in cerebellar granule neuron precursors. Two different Smoothened mutations, each a different point mutation (SmoA1 and SmoA2), were used to generate these mice. The point mutations result in constitutive activation of the shh pathway. This mechanism of tumor development models a subset of medulloblastomas known to have aberrant shh activity. Cerebellar tumors consistent with medulloblastoma occur in these genetically engineered mice at an incidence of 48% at a median age of 6 months. Once symptomatic, affected mice live for generally 4–10 days before succumbing to their tumors. Eight mice were identified as having intracranial tumors based on symptoms of head tilt, ataxia, decrease in activity, weight loss, and bulging posterior skull. Mice were then randomly assigned to the treatment (n=4) or control arm (n=4). Mice were given 5 mg of SAHA ip (dose ~200 mg/kg/mouse) every 24 h for three doses. The mice were sacrificed 2 h after the third dose of SAHA. SAHA was prepared for injection by dissolving 5 mg in 50 µl of DMSO; controls received 50 µl ip DMSO daily for three doses. All mice received 100 µg 5-bromo-2-deoxyuridine (BrdU)/gm mouse body weight by ip injection 1 h prior to sacrificing the animals. BrdU (Sigma) was prepared as 10 mg/ml in PBS.

Whole brains were fixed in paraformaldehyde 4% solution and preserved in 70% ethanol. Tissue blocks were paraffin-embedded and cut into 4 mm sections. Slides were stained with hematoxylin and eosin using standard methods and tumor morphology was confirmed. Sections were then stained for BrdU to quantify proliferation and cleaved caspase-3 (Biocare Medical, Concord, CA) to quantify apoptosis. Hematoxylin was used to counterstain both of these stains. An observer blinded to treatment information quantified the number of cells BrdU or cleaved caspase-3 positive compared to total cells in 10 non-overlapping high-power fields per mouse using the cell counter feature of the Image J program.

Statistics

Statistical analyses were performed on the studies indicated using a two-tailed t-test of two samples assuming equal variance. Calculations were done in Microsoft Excel. Dr. Michael Leblanc, senior statistician for the Southwestern Oncology Group and faculty member at the Fred Hutchinson Cancer Research Center, generously provided advice and review of statistical methods.

Results

Increased histone acetylation in D283 medulloblastoma cells treated with SAHA in vitro and in vivo

In vitro

As a means of identifying biological activity of SAHA in medulloblastoma cell lines, cell cultures and tumors were

analyzed for histone acetylation. D283 cells were cultured for 3 h with concentrations of SAHA ranging from 10 nM to 10 μM. Western analysis was performed with an antibody that specifically recognizes acetylated H3 (AcH3). Accumulation of AcH3 was seen at every concentration, and band intensity increased with increasing SAHA concentrations, displaying a dose–response relationship (Fig. 1a).

In vivo

To demonstrate histone deacetylase inhibition in a mouse system, and to determine the duration of SAHA's effect on accumulation of acetylated histones, tumors were removed from medulloblastoma xenograft animals at 1, 6, 16 and 24 h post-treatment with intraperitoneal (ip) SAHA. Two negative controls are included to represent the natural variation in acetylation status in untreated mice. Increased acetylation is seen in the tumors of mice treated 1–6 h before tumor removal but not in mice treated at earlier time points prior to sacrifice (Fig. 1b). This finding is consistent with the known drug half-life of SAHA in mice, approximately 1 h. Histone hyperacetylation as shown by these Westerns confirms SAHA activity in D283 cells in semi-adherent culture and in bulky tumors.

No significant cell death in non-transformed fibroblasts treated with SAHA in vitro

The goal of new drug development for cancer therapy is to find agents that specifically act on cancer cells, sparing normal tissue. To assess non-specific cell damage, normal fibroblasts were exposed to high dose SAHA. Human foreskin fibroblasts were grown in culture and treated with SAHA at concentrations from 0–10 μM. At 72 h, viability decreased from 93% in controls to 85% in cells treated with the highest concentration of SAHA. Apoptosis was seen in 9% of cells treated with 10 μM SAHA, while 3% of untreated controls underwent apoptosis (Fig. 2a, b). This result, showing little damage to normal fibroblasts, is consistent with previous studies showing that SAHA is minimally toxic to non-neoplastic cells [10].

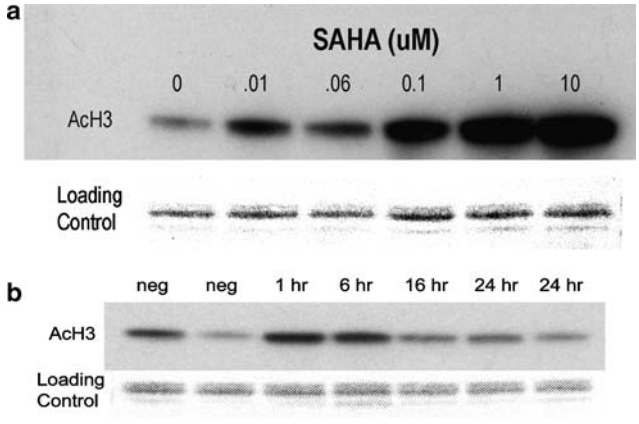

Fig. 1 Western analysis with antibody to acetylated histone H3 shows accumulation of hyperacetylated histones extracted from (**a**) cultured medulloblastoma D283 cells treated for 3 h with SAHA (0–10 μM), and (**b**) D283-derived medulloblastoma xenograft tumors removed 1–24 h after treating the animals with 4 mg (200 mg/kg) ip SAHA. Each band represents one tumor. 'neg' indicates tumors from two animals never treated with SAHA

Increased apoptosis in vitro in medulloblastoma cell lines treated with SAHA

To assess the effects of SAHA on medulloblastoma cell lines, we treated D283 and D341 cells in culture for 72 h with varying concentrations of SAHA. The fraction of D341 cells that was viable under control conditions was lower than D283, and background annexin V staining was greater. At the density used in these experiments, this represents the baseline ratio of viable cells to those undergoing apoptosis for this cell line. We saw that SAHA caused cell death at concentrations as low as 500 nM (Fig. 2c). More than 90% of cells were dead by Trypan blue exclusion with continuous exposure to 10 μM SAHA for 72 h compared to 10–15% cell death in controls treated with vehicle (DMSO) alone. Parallel cultures were stained for annexin V expression as a measure of apoptosis (Fig. 2d). The concentration-dependent loss of viability and induction of apoptosis was statistically significant at a concentration of 1 μM and greater in D283 cells, and 5 μM and greater in D341 cells. In all experiments, decreased viability was correlated with increased annexin V positivity, confirming that this high level of cell death is due to an apoptotic mechanism. The dose used in these in vitro experiments, 10 μM, is approximately the maximum plasma concentration reported in patients given a SAHA dose of 300 mg/m^2 [11].

Decreased viability of primary tumor samples treated with SAHA

Primary tissue samples from pediatric patients with medulloblastoma were used to validate the data generated in established pediatric medulloblastoma cell lines. These studies were conducted over 48 rather than 72 h because we previously determined this to be the optimal window for assessing drug efficacy in primary medulloblastoma samples [9, 12, 13]. Originally, tumor samples were treated without selecting any specific cell population. The results after six samples were tested with 5 μM SAHA showed sensitivity to SAHA in all cases. All of these had a final

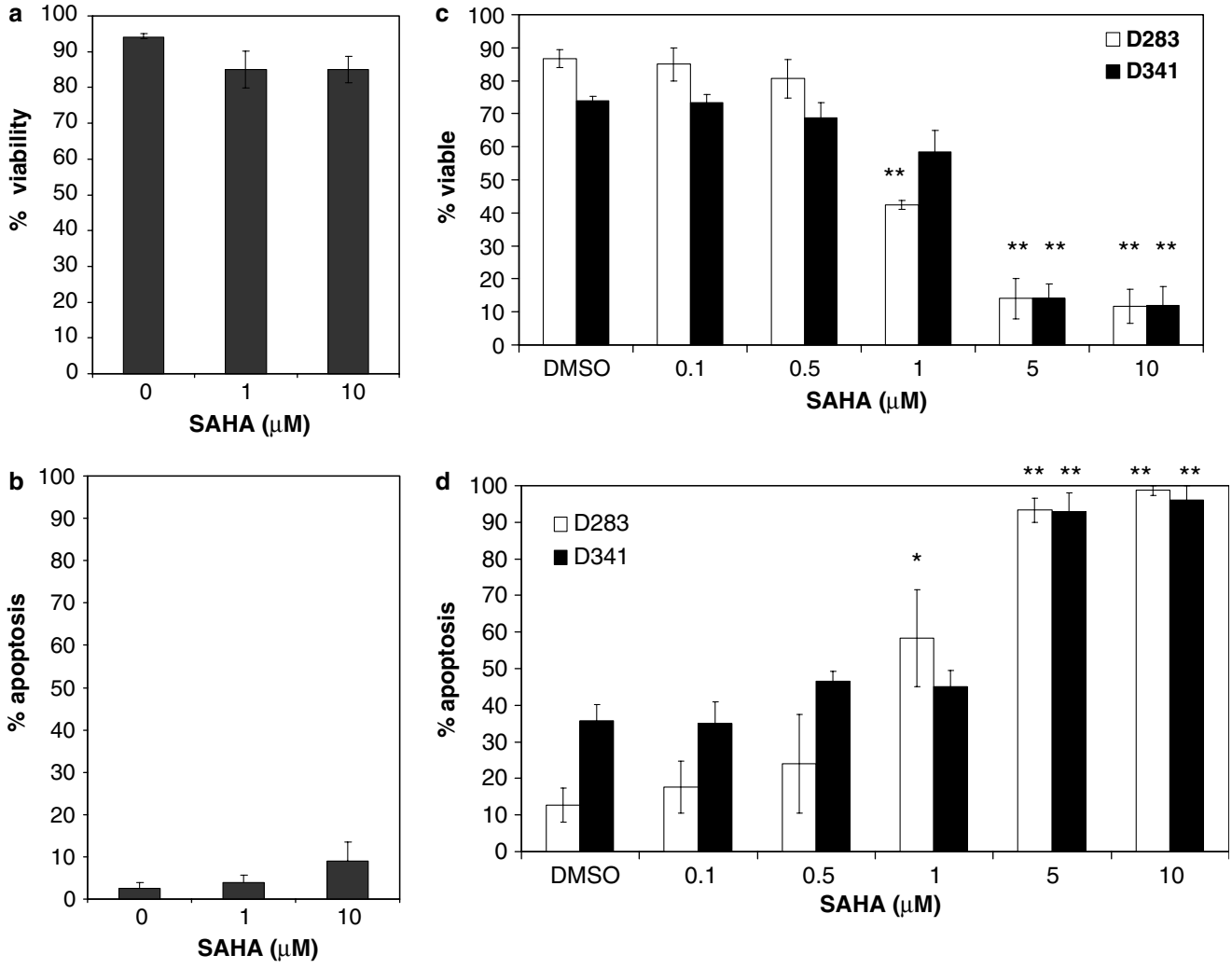

Fig. 2 In vitro assays in cells treated with SAHA. (**a**) Viability of fibroblasts measured by Trypan blue exclusion and (**b**) apoptosis of fibroblasts measured by annexin V staining. Error bars represent SE from three independent experiments. The differences are not statistically significant. (**c**) D283 and D341 cells treated with SAHA (0–10 μM) for 72 h. Cells were evaluated by Trypan blue exclusion for cell viability (n = 3 independent experiments). Error bars represent SE. $^{**}P < 0.001$. (**d**) Cells under the same conditions as in (**c**), evaluated for apoptosis by annexin V staining (n = 3 independent experiments). Error bars represent SE. $^{*}P$=0.03 and $^{**}P < 0.001$

pathologic diagnosis of classic medulloblastoma except for R346, which had nodular features consistent with the desmoplastic variant of medulloblastoma. The fraction of viable cells ranged from 42.5–78% ($P < 0.001$ for five of six samples) (Fig. 3a). This set of results did not reflect the very high cell death ratio seen in established cell lines. We reasoned that the difference was due to contamination of surgical specimens with non-neoplastic cells, including normal brain, blood, stroma and endothelial elements, since we have established that non-neoplastic tissue is relatively resistant to SAHA. To overcome this sample heterogeneity subsequent samples were sorted to enrich for cells that express neural cell adhesion molecule (NCAM, or CD56), a known cell surface antigen on medulloblastomas. The cell suspension was sorted using magnetic beads coated with antibodies that recognize CD56. Flow cytometry studies show that this method isolates a >95% pure population of cells with uniform size and side scatter (data not shown). Three cases were enriched for CD56 cells, and 5 μM SAHA reduced viability to 11±2% ($P < 0.001$), 6±7% ($P < 0.001$), and 51±4% compared to vehicle controls (Fig. 3b). The final pathologic diagnosis on the first two cases was classic medulloblastoma, whereas the third specimen was reported as medulloblastoma with possible anaplasia. Anaplastic medulloblastoma is historically more resistant to therapy [14], and this sample may reflect that observation. The vehicle control for this sample also showed low metabolic activity, consistent with studies our lab has performed on past anaplastic medulloblastoma samples, in which viability at 48 h was very low. It is

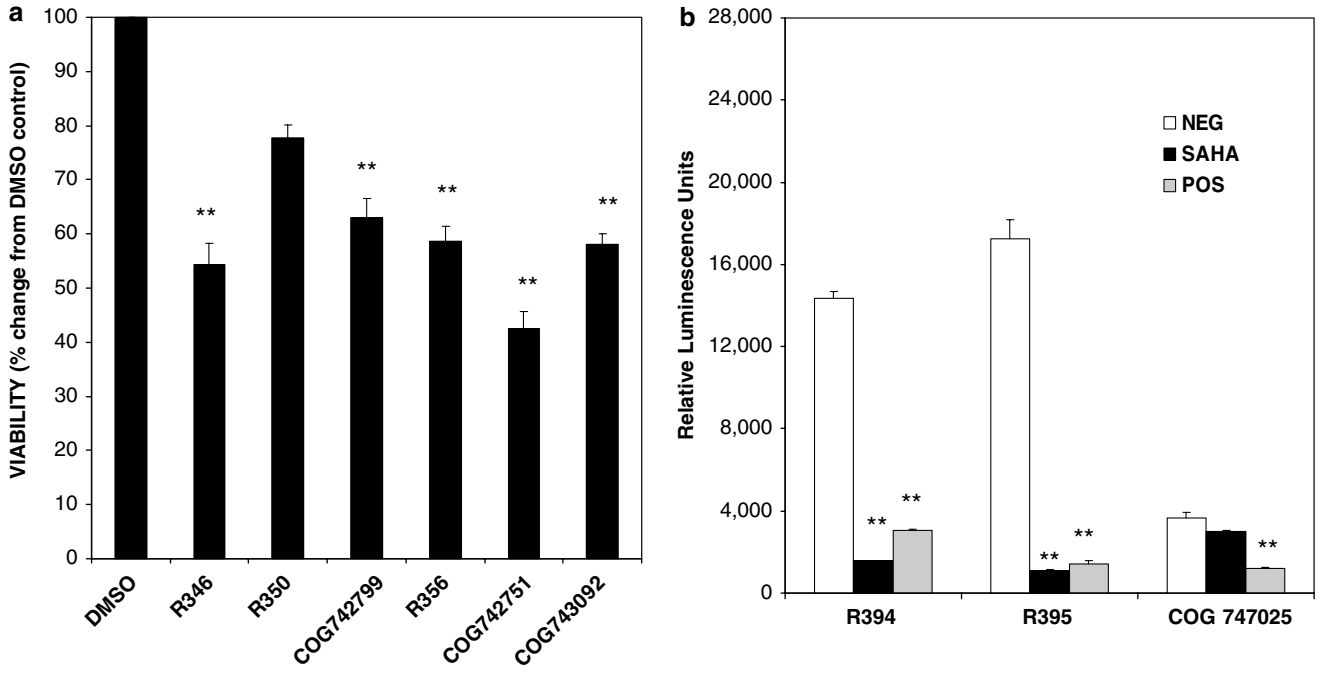

Fig. 3 Primary medulloblastoma samples treated for 48 h in near-single cell suspension and evaluated by ATP/luciferase assay for viability. (**a**) Six primary tumors treated with 5 μM SAHA ($n = 24$–48 wells). Viability represents quantity of ATP in treated cell samples divided by ATP in samples with vehicle alone (DMSO). Error bars represent SE. $^{**}P < 0.001$ for each individual tumor (P value range: 3 E-17 to7 E-09) except R350 ($P = 0.058$). Also, $P < 0.001$ between six sets of treated versus untreated samples. (**b**) CD56-sorted samples treated in three conditions: 'NEG' (DMSO at 1:1000), 5 μM SAHA and POS (25 μM cisplatin+10 μM cyclosporine A+10 μM etoposide) ($n = 12$ wells). Error bars represent SE. $^{**}P < 0.001$ in R394 and R395 for SAHA or positive control versus DMSO. $^{*}P < 0.001$ in COG747025 for positive control versus DMSO

difficult to interpret drug responsiveness when background cell death is so high. Further studies need to be completed to determine what utility SAHA may have in this subset of medulloblastoma. The robust response of classic medulloblastomas supports advancement of SAHA to human clinical trials.

SAHA induces tumor regression or slows tumor growth in vivo without evidence of toxicity

To assess in vivo activity of SAHA against medulloblastoma, studies were performed using D283 cells to create flank xenograft medulloblastoma tumors in athymic mice. Each of the described xenograft studies showed efficacy of SAHA, and no mouse experienced significant weight loss or change in activity. In all, 38 athymic mice were treated daily for up to 8 weeks with 200 mg/kg/d of SAHA. The dose of 200 mg/kg/d is based on the previously determined maximum tolerated dose (MTD) in rats [15] and our own confirmatory MTD study in athymic mice (data not shown). By observation and basic blood studies, no weight loss, change in activity, renal dysfunction or hepatic insults were identified. Mild pancytopenia was the only toxicity observed, and this is a side effect previously reported in human clinical trial data.

Trial 1

Most patients diagnosed with medulloblastoma undergo a gross total resection of the tumor, leaving behind only microscopic residual disease. Therefore, we devised a pilot study to investigate the role of SAHA in flank xenografts that were very small and just beginning to grow, modeling a minimal disease state.

Five mice per arm began treatment with oral SAHA the day after injection of tumor cells into their flanks. Food consumption was measured on a five mouse per cage basis, and is estimated to range from 40–73% of food presented. Therefore it can be assumed that mice received on average about 55% of the prescribed SAHA dose (112 mg/kg/day). Dosing for each individual mouse may have varied.

The average tumor volume in the control group reached 100 mm^3 by 3 weeks after injections, and in the SAHA treated animals the average volume reached 100 mm^3 at 5 weeks. One mouse in the control arm did not develop a flank tumor, and two treated mice did not have tumors. The study was terminated at 8 weeks, at which time the average tumor volume in control animals was 1072 mm^3, while the average volume in treated mice was 362 mm^3 (Fig. 4a), demonstrating slowed rate of tumor growth with treatment.

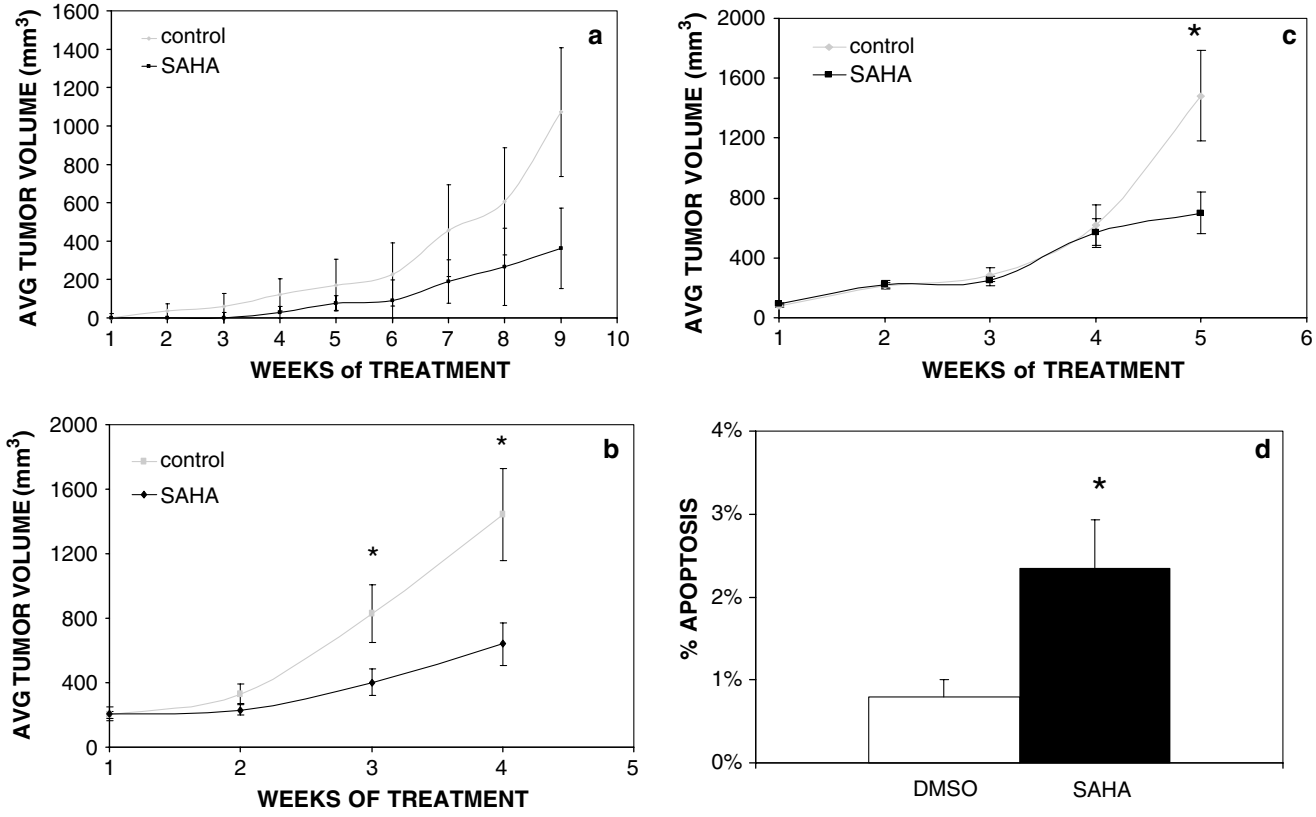

Fig. 4 In vivo studies of athymic mice, treated with SAHA, bearing flank tumors of D283 medulloblastoma cells. (**a**) Average growth of tumors in animals injected with D283 cells that were treated with 200 mg/kg/day SAHA in powder food starting the day following tumor injection. (n=5 mice per arm) versus powder food alone. (**b**) Average tumor growth in 17 control mice versus 18 mice treated with 200 mg/kg/day SAHA mixed into powder food daily × 4 weeks. Each mouse had one flank tumor. *P=0.036 at week 3, 0.014 at week 4. (**c**) Average tumor growth in 9 control animals (total of 16 tumors) injected daily with vehicle alone (50 µl ip DMSO) compared to 12 mice (23 tumors) injected with 200 mg/kg ip SAHA daily for 28 days. *P=0.014 (**d**) Apoptosis in tumor tissue quantified by TUNEL stain. Graph represents the percent of TUNEL + cells per tumor for three independent tumors in each condition based on 10 fields of 600–1200 cells per high power field. The control bar shows tumors in untreated animals compared to animals treated with 200 mg/kg/day of SAHA orally for 4 days. Error bars represent SE. *P=0.018

Trial 2

The pilot study had a small number of mice, but demonstrated that mice could tolerate orally administered SAHA for several weeks, though the dose received was only about half of the dose intended. The next study was designed to evaluate tumor response to therapy. We tried to more closely calculate food intake to improve dose delivered, and used only mice in which bulky tumors were clearly established. Mice with bulk tumors averaging 200 mm^3 were given SAHA at 200 mg/kg/d as powder added to powder food (n=18), or powder food alone (control n=17). In the SAHA arm, one mouse showed tumor regression, and nearly half grew to less than double the original volume in 4 weeks (range: 0.1–6 × original volume). Tumors in the control group had a 7-fold increase in volume on average (range: 0.2–30 × original) (Fig. 4b). Overall, control tumors increased to an average volume of 1,445 mm^3 while tumors in treated animals averaged 642 mm^3, which is a 56% difference (P=0.014). When all tumor volumes are summed together, the control group had 24,572 mm^3 of tumor, while SAHA treated animals had 11,557 mm^3 of tumor, a 53% difference. The sum for the control group does not include the single mouse whose tumor grew to >2000 mm^3 at 3 weeks and was removed from the study early.

Food consumption was measured on a per cage basis, and is estimated to range from 75 to 100% of food presented. Therefore it can be assumed that mice received at least 75% (150 mg/kg/day) of the prescribed SAHA dose in general, while dosing for each individual mouse may have varied.

Trial 3

Positive tumor effect in the oral studies prompted a trial of intraperitoneal (ip) SAHA, a more dependable, controlled and efficient delivery system. This trial was performed

after injection of two flank tumors per mouse, which resulted in bilateral tumors in 85% of mice. The control arm included 9 mice with a total of 16 tumors; in the SAHA arm there were 12 mice and 23 tumors total. Due to rapid tumor growth, eight mice originally injected with tumors were taken off study early and not included in the data reported, since the animals had to be euthanized before 4 weeks. Five of those mice were in the control arm and three were in the SAHA arm. The mice received daily ip injections of 200 mg/kg/day SAHA, the same dose as the oral trial. Mice with bulk tumors averaging 90 mm^3 were treated with SAHA given by ip injection daily (Fig. 4c). After 4 weeks of treatment, the average tumor volume in the control group was 1483 mm^3, and the average SAHA treated tumor was 699 mm^3 (P=0.014). While few tumors resolved completely, nearly all of them responded to some degree, with a statistically significant difference in average tumor bulk in the treated mice after 28 days of daily therapy.

SAHA causes apoptosis in xenograft tumors

D283 xenograft tumor tissue was examined for apoptosis using TUNEL staining and fluorescence microscopy. Three control tumors showed 0.79% positive cells and three tumors from animals treated with SAHA for 4 days showed 2.35% positive cells (P=0.018). These data confirm that SAHA invokes an apoptotic mechanism of cell death in medulloblastoma, as was seen in the in vitro system (Fig. 4d). While baseline apoptosis in these tumors seems low, it is not outside of the range reported in human tissue samples tested with cleaved caspase-3 (0.1–25.9%) [16].

Intracranial tumors affected by intraperitoneal SAHA

A genetically faithful model of medulloblastoma was developed using the neuroD2 promoter to drive overexpression of two different mutated copies of Smoothened, a Sonic hedgehog (shh) pathway activator, in cerebellar granule neuron precursors. The two different mutations in these mice, SmoA1 and SmoA2, linked to the neuroD2 promoter are collectively referred to as ND2:Smo. Hematoxylin and eosin staining for histologic evaluation of intracranial tumors in ND2:Smo mice revealed various stages of tumor formation from grossly normal cerebellum with islands of advanced hyperplasia to complete obliteration of all recognizable patterns of normal cerebellar architecture, as described [9]. Four mice were treated with ip SAHA and four received ip DMSO daily for 3 days before collecting brains. 5-bromo-2-deoxyuridine staining was used to assess proliferation. Patterns of staining in these tumors were not consistent within any tumor, or among tumors in either arm. We therefore conclude that response to SAHA is not due to decreased proliferation.

Apoptosis was measured by staining for cleaved caspase-3. Figure 5a demonstrates representative slides of a control (DMSO) mouse compared to a SAHA treated mouse after staining with cleaved caspase-3. Within areas of normal cerebellum and the remainder of brain, no excess cleaved caspase-3 stain was seen in SAHA treated animals or controls. Tumors of treated animals showed nearly four-fold more cleaved caspase-3 staining than controls (Fig. 5b). This confirmed that SAHA entered these intracranial tumors, and induced apoptosis.

Discussion

Findings presented here indicate that SAHA has potential as an effective treatment for medulloblastoma. Importantly, SAHA was administered systemically and crossed the blood–brain barrier to demonstrate anti-tumor activity at a dose that did not cause prohibitive toxicity in the mouse model.

One of the more potent traditional chemotherapeutic agents currently used to treat medulloblastoma, cisplatin, achieved 60–70% cell death in established cell culture as a single agent. This agent often has to be given at reduced doses, due to renal and ototoxicity [1]. Recently, retinoic acid has been introduced as a pro-apoptotic agent in medulloblastoma, and showed 70–80% cell death in D283 and D341 cells [13]. SAHA, in comparison, caused >90% cell death in tissue culture. If this in vitro efficacy in comparison to accepted agents translates into improved clinical results, a better, less toxic plan for children with medulloblastoma may soon include SAHA.

Primary tumor samples give us the opportunity to overcome some of the limitations of cell lines. Resected tumor specimens, while a less pure population than cell lines, more accurately represent human disease in terms of cell heterogeneity, gene expression levels, and epigenetic modifications. The data showing high SAHA sensitivity of CD56-positive patient samples indicate that the cell line work is valid and may be translatable to primary disease efficacy. It also reinforces the assertion that the non-neoplastic elements of a surgical specimen do not experience cytotoxicity from SAHA the way that medulloblastoma cells from affected patients do. This is an encouraging finding, considering the goal of improving cures, but also sparing normal tissue. The studies on human fibroblasts and the absence of toxicity in mice treated with SAHA are consistent with previous reports that this drug has a wide therapeutic window. The report of a Phase I trial of SAHA [11] described minimal toxicity among 37 adults with

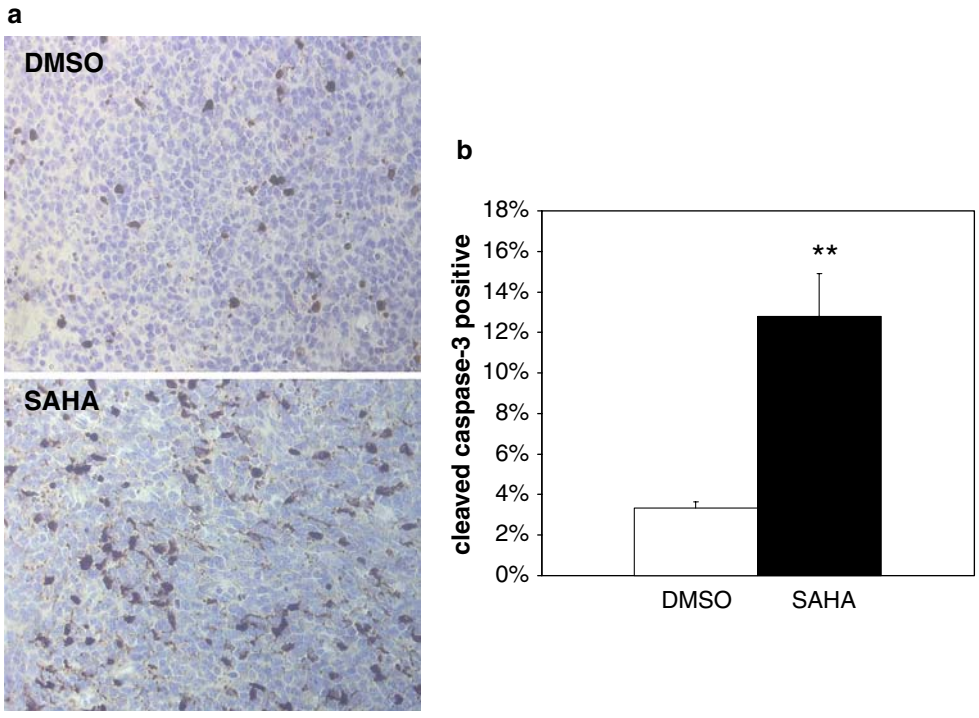

Fig. 5 Intracranial medulloblastoma model ND2:Smo. (**a**) Photomicrographs of tumors from ND2:Smo mice treated for 3 days with intraperitoneal DMSO versus 200 mg/kg/day of intraperitoneal SAHA after paraformaldehyde fixation, paraffin embedding, and cleaved caspase-3 staining. Representative images are shown. (b) Apoptosis in tumor tissue quantified by cleaved caspase-3 stain. Graph represents the percent of cleaved caspase-3 + cells per tumor for four independent tumors in each condition base on cells 10 fields of 400–1500 cells per high power field. Treated animals received 200 mg/kg/day of intraperitoneal SAHA for 3 days. Error bars represent SE. $^{**}P < 0.001$

hematologic malignancies and solid tumors treated with intravenous SAHA at doses from 75–900 mg/m^2/d×5 days for up to 3 weeks. Toxicities included Grade 3 hyperglycemia in 16% of patients, reversible thrombocytopenia, fatigue, low grade renal insufficiency in less than one quarter of patients, and adult respiratory distress syndrome in one patient. This pulmonary complication was the only serious adverse event reported that was believed to be drug related. The safety and efficacy of SAHA continues to be evaluated in adult trials. No data are currently available about safety of SAHA in children, and none of the adult patients in the above report or other reported adult SAHA trials had brain tumors. Children's Oncology Group currently has a Phase I study of SAHA underway for children with solid tumors, based in part on data presented here.

We conclude from the mouse studies that oral and parenteral routes of administering SAHA are effective at reducing the rate of tumor growth and, in a few cases, eliminating the tumor cells altogether. The dose of SAHA used in xenograft studies is comparable to the dose used in human trials, based on formulas found in Guidance for Industry and Reviewers (http://www.fda.gov/cber/gdlns/dose.pdf). Since pharmacokinetics of SAHA in the mouse are not published, actual drug exposure in mice may be different than in man despite comparable dosing, making it difficult to confidently predict the human response to a similar dose of SAHA.

The ND2:Smo mouse medulloblastoma model provides a histological and gross anatomical replica of a human posterior fossa small round blue cell tumor. This tumor arises spontaneously in granule cells, without the insults of surgical or needle instrumentation that would disrupt vascular integrity or fidelity of cell–cell communication. Demonstration of apoptosis by cleaved caspase-3 staining not only confirms that SAHA is able to cross the blood–brain barrier and enter the tumors in the ND2:Smo model, but that the local concentration of SAHA is high enough to be anti-neoplastic. A limitation of the ND2:Smo mouse model is that mice tolerate smaller tumors without observable impairment, and only develop neurological indicators of their tumors at end stage. This time point is too late to initiate preclinical trials because some ND2:Smo mice die within days of symptom onset. Studies are underway to assess the feasibility of radiographic early tumor detection and size monitoring. At that time studies could be performed to look at clinical markers such as time from radiographic identification of tumor to symptom development, drug response based on reduction in tumor size, or even cure.

A number of studies suggest that decreased histone acetylation or enhanced deacetylation may, at least in part, contribute to tumorigenesis in a variety of cancers. The importance of normal histone acetylation in tumor suppression is highlighted by the finding that BRCA2, a tumor suppressor gene mutated in breast cancer, functions as a histone acetyl transferase [17]. The histone deacetylase HDAC1 is overexpressed [18] and HDAC4 recruitment to the nucleus [19] has been demonstrated in hormone

refractory prostate cancer. Early studies of other solid tumors have demonstrated increased expression of histone deacetylase HDAC2 by gene array including MYCN amplified neuroblastoma cell lines [20], APC mutant colon cancer [21], and the medulloblastoma mouse model ND2:SmoA1 (A. R. Hallahan, unpublished results, this laboratory). These studies suggest that aberrant control of histone acetylation status may play a role in solid tumor genesis or progression.

Gene expression studies of various HDIs implicate genes that control advancement through cell cycle and apoptosis initiation as effectors of their activity. Some effectors that have been linked to HDIs include but are probably not limited to $p21^{WAF1}$ [22], $p15^{Ink4b}$ [23], GADD45 [24] and ErbB2 [25]. As cyclin dependent kinase inhibitors, $p21^{WAF1}$ and $p15^{Ink4b}$ control progression through cell cycle. G1/S phase in particular is influenced by $p15^{Ink4b}$. GADD45 works at the G2-M phase of cell cycle control. ErbB2 reportedly increases invasive and metastatic potential of medulloblastomas [26]. We may discover that manipulation of chromatin by altering the histone acetylation state of malignant cells gives those cells an opportunity to correct aberrant epigenetic changes and proceed towards apoptosis, regardless of what the aberrant signals are. Perhaps normal tissue is protected from apoptosis because it has appropriate epigenetic controls in place and can correct for non-physiologic hyperacetylation without being driven towards early cell death.

Understanding the mechanism of action of drugs against tumors is important if we are to improve on the current drug treatment plans for patients, making intelligent drug choices based on knowledge of target sites and potential synergy among agents. However, introducing effective drugs into clinical trials based on strong preclinical data showing safety and efficacy in vitro and in vivo need not wait for full elucidation of molecular mechanisms. The results presented here warrant further investigation and consideration of SAHA in clinical trials in medulloblastoma.

Acknowledgements We thankfully acknowledge Victoria Richon, Paul Secrist, Andrew Hallahan and Stacey Hansen for helpful discussions. This work was supported by the Emily Dorfman Foundation Fellowship Award of the American Brain Tumor Association (S. E. S.), the Jordyn Dukelow Memorial Guild (S. E. S.), the Damon Runyon Clinical Investigator Award (J. M. O.) and NIH Grant CA112350-01 (J. M. O.)

References

1. Packer RJ, Goldwein J, Nicholson HS et al (1999) Treatment of children with medulloblastomas with reduced-dose craniospinal radiation therapy and adjuvant chemotherapy: a Children's Cancer Group study. J Clin Oncol 17:2127–2136
2. Mulhern RK, Merchant TE, Gajjar A, Redddick WE, Kun LE (2004) Late neurocognitive sequelae in survivors of brain tumours in childhood. Lancet Oncol 5: 399–408
3. Richon VM, Sandhoff TW, Rifkind RA, Marks PA (2000) Histone deacetylase inhibitor selectively induces $p21^{WAF1}$ expression and gene-associated histone acetylation. Proc Natl Acad Sci 97:10014–10019
4. O'Connor OA, Heaney ML, Schwartz L et al (2006) Clinical experience with intravenous and oral formulations of the novel histone deacetylase inhibitor suberoylanilide hydroxamic acid in patients with advanced hematologic malignancies. J Clin Oncol 24:166–173
5. Hockly E, Richon VM, Woodman B et al (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. Proc Natl Acad Sci 100: 2041–2046
6. Eyupoglu IY, Hahnen E, Buslei R et al (2005) Suberoylanilide hydroxamic acid (SAHA) has potent anti-glioma properties in vitro, ex vivo and in vivo. J Neurochem 93: 992–999
7. Cousens LS, Gallwitz D, Alberts BM (1979) Different accessibilities in chromatin to histone acetylase. J Biol Chem 254: 1716–1723
8. Sigle RO, Gil SG, Bhattacharya M et al (2004) Globular domains 4/5 of the laminin α3 chain mediate deposition of precursor laminin 5. J Cell Science 117:4481–4494
9. Hallahan AR, Pritchard JI, Hansen S et al (2004) The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of Sonic Hedgehog-induced medulloblastomas. Cancer Research 64:7794–7800
10. Marks PA, Richon VM, Rifkind RA (2000) Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J Natl Cancer Inst 92:1210–1216
11. Kelly WK, Richon VM, O'Connor O et al (2003) Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. Clin Cancer Res 9:3578–3588
12. Berman DM, Karhadkar SS, Hallahan AR et al (2002) Medulloblastoma growth inhibition by hedgehog pathway blockade. Science 297:1559–1561
13. Hallahan AR, Pritchard JI, Chandraratna RAS et al (2003) BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect. Nature Med 9:1033–1038
14. Eberhart CG, Kepner JL, Goldthwaite PT et al (2002) Histopathologic grading of medulloblastomas. Cancer 94:552–560
15. Cohen LA, Amin S, Marks PA, Rifkind RA, Desai D, Richon VM (1999) Chemoprevention of carcinogen-induced mammary tumorigenesis by the hybrid polar cytodifferentiation agent, suberanilohydroxamic acid (SAHA). Anticancer Res 19:4999–5006
16. Pizem J, Cor A, Zaletel LZ, Popovic M (2005) Prognostic significance of apoptosis in medulloblastoma. Neurosci Lett 381:69–73
17. Siddique H, Zou JP, Rao VN, Reddy ESP (1998) The BRCA2 is a histone acetyltransferase. Oncogene 16:2283–2285
18. Halkidou K, Gaughan L, Cook S, Leung HY, Neal DE, Robson CN (2004) Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate 59:177–189
19. Halkidou K, Cook S, Leung HY, Neal DE, Robson CN (2004) Nuclear accumulation of histone deacetylase 4 (HDAC4) coincides with the loss of androgen sensitivity in hormone refractory cancer of the prostate. Eur Urol 45:382–389
20. Raetz EA, Kim MKH, Moos P, et al (2003) Identification of genes that are regulated transcriptionally by myc in childhood tumors. Cancer 98:841–853
21. Zhu P, Martin E, Mengwasser J, Schlag P, Janssen KP, Gottlicher M (2004) Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell 5:455–463

22. Gui CY, Ngo L, Xu WS, Richon VM, Marks PA (2004) Histone deacetylase (HDAC) inhibitor activation of $p21^{WAF1}$ involves changes in promoter-associated proteins, including HDAC1. Proc Natl Acad Sci 101:1241–1246
23. Hitomi T, Matsuzaki Y, Yokota T, Takaoka Y, Sakai T (2003) $p15^{INK4b}$ in HDAC inhibitor-induced growth arrest. FEBS Lett 554:347–350
24. Chen Z, Clark S, Birkeland M et al (2002) Induction and superinduction of growth arrest and DNA damage gene 45 (GADD45) α and β-messenger RNAs by histone deacetylase inhibitors trichostatin A (TSA) and butyrate in SW620 human colon carcinoma cells. Cancer Lett 188:127–140
25. Scott GK, MardenC, Xu F, Kirk L, Benz CC (2002) Transcriptional repression of ErbB2 by histone deacetylase inhibitors detected by a genomically integrated ErbB2 promoter-reporting cell screen. Mol Cancer Ther 1:385–392
26. Gilbertson RJ (2004) Medulloblastoma: signaling a change in treatment. Lancet Oncol 5:209–218