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ORIGINAL PAPER

Suberoylanilide hydroxamic acid (SAHA) induces growth arrest and apoptosis in pituitary adenoma cells

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Abstract Pituitary adenomas, which account for 15–20% of intracranial tumors, are associated with significant morbidity and mortality, due in part, to hormone hypersecretion and mass effects following increased proliferation. Radiotherapy and surgery remain frontline treatment options; however, adverse side effects and surgical limitations to treat invasive tumors necessitate the need for novel therapeutic targets. This study tested the efficacy of the histone deacetylase inhibitor (HDACi), suberoylanilide hydroxamic acid (SAHA) in GH3, and MMQ pituitary adenoma cells. Clinically achievable concentrations of SAHA (500 nM–4 μM) induced growth arrest and increased cell death in GH3 pituitary adenoma cells. Further investigation into the mechanism of cell death revealed an increase in PARP cleavage and procaspase-3 activation, consistent with apoptotic cell death. SAHA also attenuated the expression of anti-apoptotic IAP (XIAP, survivin) and Bcl-2 family proteins (Bcl-2, Bcl-xL), but did not alter Bax expression. Together, these findings support a possible utility for SAHA alone or in combination with radiation for the treatment of pituitary adenoma.

Keywords Vorinostat · Histone · Pituitary adenoma · Cell cycle · HDAC · Prolactinoma

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Introduction

Pituitary adenomas, which account for 10–15% of intracranial tumors, are discrete, well-demarcated lesions that are associated with significant morbidity and mortality, due in part, to hormone hypersecretion and mass effects [1]. Although histological malignancy of the pituitary is rare, adenomas exhibit infiltrative and invasive growth patterns that are characteristic of malignant tumors and that make full surgical resection difficult [2, 3]. Radiotherapy and surgery persist as frontline treatment options; however, radiation may induce collateral damage in normal pituitary cells and in the optic nerve, and the usefulness of surgical approaches to treat invasive tumors remains limited. Furthermore, lifelong palliative treatment options to control tumor growth and hormone overproduction are associated with adverse side effects and poor patient compliance [4]. Together, the lack of clinically effective treatment options emphasize the need for improved therapeutic strategies. This study, for the first time, tests the efficacy of chromatin remodeling by histone deacetylase inhibitor in the treatment of pituitary adenoma.

Histones are highly conserved, basic, DNA-binding proteins that play a crucial role in packaging DNA into chromatin. Post-translational histone modifications, such as acetylation, control gene expression and in turn, regulate diverse biological processes, including cellular proliferation, cell cycle regulation, and apoptosis [5–8]. Histone acetyltransferase (HAT), which promotes histone acetylation, and histone deacetylase (HDAC), which catalyze the removal of acetyl groups from histones, maintain the degree of histone acetylation. Tumor progression is frequently associated with increased histone deacetylases (HDAC) activity [9–11], and histone deacetylase inhibitors (HDACi) are a novel class of anti-neoplastic agents.

Inhibition of HDAC activity increases the accumulation of acetylated core histones, which induces an open chromatin conformation and promotes the transcriptional activation of a subset of target genes, facilitating cell cycle arrest or apoptosis [12–15].

Suberoylanilide hydroxamic acid (SAHA; vorinostat) is a hydoxamic acid-based prototypical histone deacetylase inhibitor that is structurally related to the natural HDAC inhibitor trichostatin A. SAHA directly binds to the catalytic site of classes I, II, and IV HDACs [16, 17]. SAHA is currently in clinical trials for the treatment of solid and hematological tumors [12, 16, 18, 19]; however, it remains unknown whether HDACi may similarly reduce the progression of benign pituitary adenomas. In order to address this important issue, the effect of the clinically useful HDACi, SAHA, on cellular proliferation, survival, and radiosensitivity was tested in secretory pituitary adenoma cells. This study demonstrates, for the first time, a possible beneficial effect of SAHA in the treatment of pituitary adenomas.

Materials and methods

Supplies

All cell culture reagents, sera, and media were purchased from Hyclone Laboratories (Logan, UT). SAHA was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and dissolved in dimethyl sulfoxide (DMSO), which was used as a vehicle in all studies at a concentration <0.01%.

Cell culture

Rat pituitary adenoma cells GH3 and MMQ (American Type Tissue Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 5% bovine growth serum, and antibiotics in a 37°C humidified incubator at 5% CO_2. GH3 cells are a well-characterized model of secretory adenomas that oversecrete both prolactin (PRL) and growth hormone (GH), and reflect the biology of human secretory tumors [20). MMQ cells are a model of prolactinoma, which grows as a suspension culture.

Assessment of cellular viability

Cell viability was estimated by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) reduction assay, as detailed by our laboratory [21]. Apoptosis was quantified by flow cytometry, as described previously by our group [22]. Briefly, adherent and non-adherent cells were collected and washed following treatments. Cell suspensions were stained for 15 min at room temperature with annexin V-PE (BD Pharmigen, San Diego, CA), an early apoptotic marker, and with 7-aminoactinomycin D (7-AAD), a fluorescent marker that labels dead cells. The percentage of apoptotic cells was quantified using a FACScan flow cytometry. Cellular morphology was documented using a Zeiss Axiovert 40 light microscope (20× objective) equipped with a digital imaging system.

Cell cycle analysis

Into a 60-mm dish, 5×10^4 cells were plated for 2 days, synchronized overnight in serum free media, and treated for up to 48 h in complete culture media, as indicated in the figure legends. Cells were washed twice and permeabilized with 70% methanol overnight at −20°C. Cells were incubated with 0.5 ml of a 50 μg/ml propidium iodide (PI) solution containing 200 μg/ml RNaseA for 30 min and immediately analyzed by flow cytometry using BD Biosciences FACS Calibur analyzer.

Western blotting

Western blotting was performed as described by our laboratory [21]. Briefly, cells were washed with phosphate-buffered saline (PBS) and whole cell lysates were collected in radioimmunoprecipitation (RIPA) buffer containing protease inhibitor cocktail, phosphatase inhibitor cocktail, and phenyl methane sulfonyl fluoride (PMSF). Cell lysates were sonicated, centrifuged for 5 min at 14,000 rpm at 4°C, and protein concentrations were quantified by BCA protein assay kit (Pierce, Rockford, IL). Fifty micrograms of protein was resolved on a 4–20% sodium dodecyl sulfate–polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. Blots were incubated overnight at 4°C in primary antibody (acetyl-histone H3 and H4, Survivin, XIAP, Bcl2, Bcl-xL, and Bax (Cell Signaling Technology, Beverly, MA) and β-actin (Abcam, Cambridge, MA), caspase-3, (Santa Cruz Biotechnology, Santa Cruz, CA), or PARP (Trevigen, Gaithersburg, MD) followed by a 2-h incubation with a corresponding Alexa Fluor 750 secondary antibody. Blots were visualized using the Li-Cor Odyssey near-infrared imaging system and quantified using Quantity One software (Bio-Rad, Foster City, CA).

Statistical analysis

For cellular viability studies, $n = 6$ wells/group were used within each experiment for analysis. For western blotting, all experiments were performed at least in triplicate using independent cell cultures. All experiments were repeated at least three times for the validation of results. Data within

each time point were compared using a one-way analysis of variance (ANOVA), followed by Student–Newman–Keul's or Dunnett's post-hoc test. A $P < 0.05$ was considered to be statistically significant.

Results

SAHA inhibits the proliferation of pituitary adenoma cells

Clinically achievable concentrations of SAHA (500 nM–4 μM) significantly reduced the viability of GH3 and MMQ pituitary adenoma cells in a concentration and time-dependent manner following a 1–4 day treatment, as assessed by the MTT assay (Fig. 1a, b). The lowest tested concentrations of SAHA (500 nM, 1 μM) reduced GH3 cellular viability to 66.4 ± 3.2% ($P < 0.001$ vs. vehicle) and 44.8 ± 2.5%, respectively, ($P < 0.001$ vs. vehicle) following a 72-h treatment, with an IC50 of 1.1 μM (Fig. 1a). Similarly, SAHA (500 nM, 1 μM) reduced MMQ cellular viability to 82.4 ± 5.7% ($P < 0.05$ vs. vehicle) and 73.6 ± 5.9% ($P < 0.01$ vs. vehicle), respectively, with an IC50 of 1.5 μM following a 72-h treatment (Fig. 1b). A maximal effect in both cell lines was observed following a 96-h treatment with 4 μM SAHA, which remarkably dropped cellular viability to 3.9 ± 0.3% ($P < 0.001$ vs. vehicle) in GH3 cells and 8.9 ± 2.9% ($P < 0.001$ vs. vehicle) in MMQ cells. SAHA treatment was also associated with prominent morphological changes in GH3 cells, including cellular flattening and cytoplasmic enlargement following a 48-h treatment (Fig. 1c).

SAHA induces growth arrest and apoptosis

MTT measures cellular viability as a function of mitochondrial activity; however, a reduction in cellular proliferation and/or an increase in cell death cannot be differentiated using this assay alone. Therefore, to delineate the mechanism underlying this effect, cell cycle analysis was performed using flow cytometry to assess the effect of SAHA on cellular proliferation. Consistent with an anti-proliferative effect, SAHA (500 nM) increased the percentage of GH3 cells in the sub-G1 phase (1.2 ± 0.1% vs. 0.4 ± 0.02% in control; $P < 0.01$ vs. vehicle) and G1 phase (89.4 ± 0.3% vs. 72.0 ± 0.4% in control; $P < 0.001$ vs. vehicle), with a corresponding reduction in the percentage of cells within the G2/M phase (3.6 ± 0.2% vs.

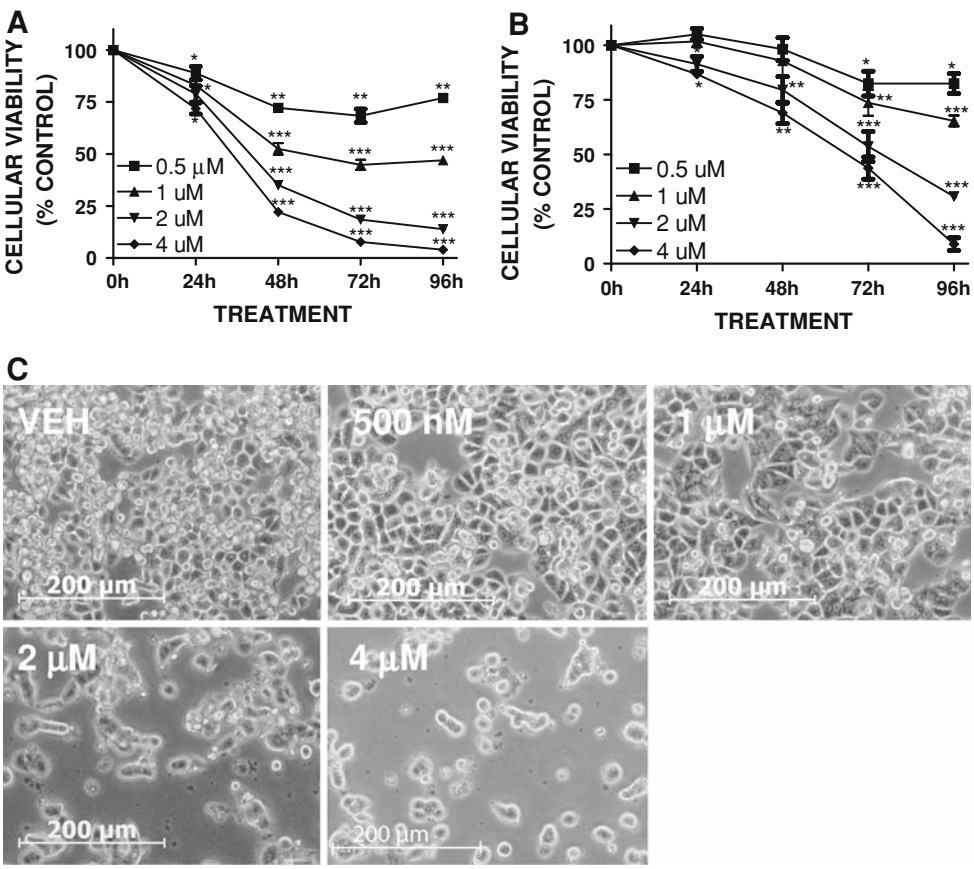

Fig. 1 Effect of SAHA on pituitary adenoma cellular viability. SAHA (500 nM–4 μM) concentration-dependently reduced the viability of **a** GH3 or **b** MMQ pituitary adenoma cells over the first 4 days following treatment, as assessed by MTT reduction assay. Data are representative of at least three independent experiments ($n = 6$/experiment) and are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle-treated cultures. **c** Cellular morphology of GH3 cells following a 48-h treatment with increasing concentrations of SAHA. Scale bar = 200 μm

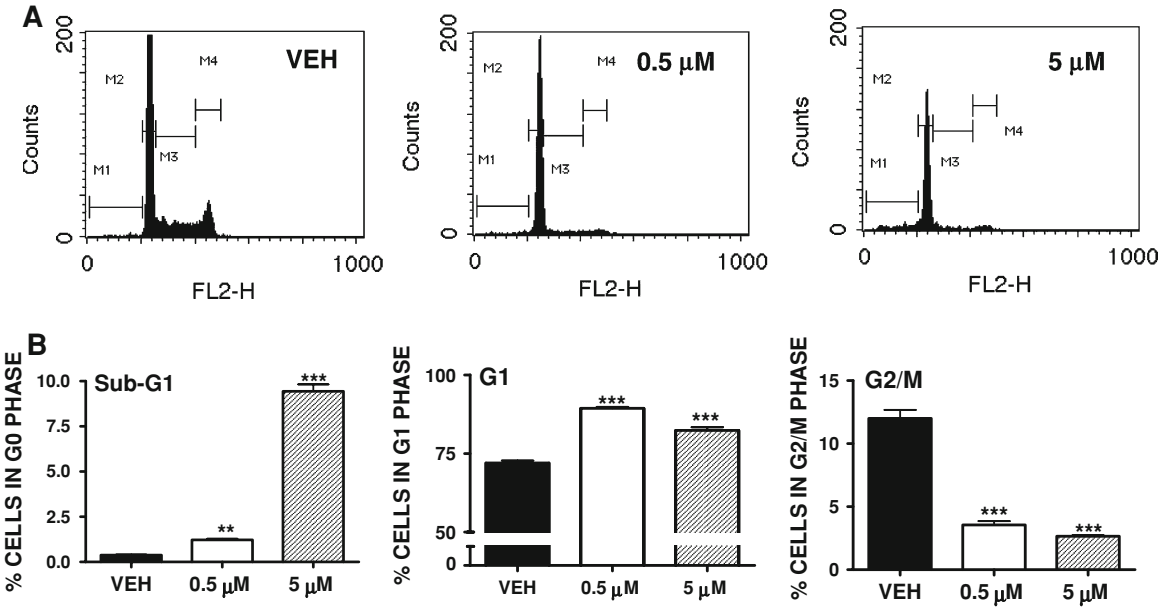

Fig. 2 Cell cycle analysis of GH3 cells following SAHA treatment. **a** Cells were treated for 48 h with SAHA (0, 0.5, or 5 μM) for 48 h, and then analyzed by flow cytometry. M1, M2, M3, and M4 represent the sub-G1, G1, S, and G2/M phase, respectively. Data are representative of the three independent experiments. **b** Quantification of cell cycle distribution following SAHA treatment. SAHA increased the percentage of cells in the sub-G1 and G1 phases and decreased the percentage of cells in G2/M phase of the cell cycle. Data are expressed as the mean ± SEM from the three independent experiments. Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test (**$P < 0.01$, ***$P < 0.001$ vs. vehicle-treated cultures)

12.0 ± 0.4% in control; $P < 0.001$ vs. vehicle) following a 48-h treatment (Fig. 2a). Similarly, 5 μM SAHA increased the percentage of cells in the sub-G1 phase (9.4 ± 0.4%; $P < 0.001$ vs. vehicle, $P < 0.01$ vs. 500 nM SAHA) and G1 phase (82.3 ± 0.9%; $P < 0.001$ vs. vehicle, not significantly different from 500 nM SAHA). Apoptosis is associated with the accumulation of cells within the sub-G1 fraction; thus. studies were performed to determine whether SAHA increased annexin V labeling, a measure of apoptotic cell death. The percentage of early apoptotic cells (annexin V^+/7-AAD^-) increased from 0.4% in vehicle-treated cultures to 4.3 ± 0.1% ($P < 0.05$ vs. vehicle), 14.1 ± 0.2% ($P < 0.001$), and 22.4 ± 2.5% ($P < 0.001$) following a 48-h treatment with 1, 2, or 4 μM SAHA, respectively, (Fig. 3a, b).

SAHA exhibits HDACi activity in pituitary adenoma cells

SAHA increased the acetylation of histone H3 and H4, the principal targets of HDACs, at concentrations which correlated with the induction of cell death. Concentrations of SAHA as low as 1 μM increased histone acetylation following a 48-h exposure, with a pronounced effect noted after treatment with 2 μM (Fig. 4a). Consistent with the maximal loss of cellular viability, 4 μM SAHA dramatically increased histone H3 and H4 acetylation at this concentration, suggesting an HDACi mechanism of action. In further support of this possibility, trichostatin A, a first-generation HDACi that is structurally related to SAHA, significantly reduced GH3 cellular viability following a 48-h treatment with a concentration range of 10–160 nM (data not shown).

SAHA attenuates anti-apoptotic protein expression in GH3 cells

Coincident with the increase in apoptotic cell death, SAHA downregulated the expression of the anti-apoptotic Bcl-2 family proteins Bcl–xL and Bcl-2 (Fig. 5). A maximal reduction was observed following a 48-h treatment with 4 μM SAHA, which reduced Bcl-2 and Bcl-xL expression by 72 ± 6% and 63 ± 11%, respectively ($P \leq 0.01$ vs. vehicle), whereas the pro-apoptotic protein, Bax, remained unchanged at all tested concentrations. SAHA also attenuated the expression of the IAP (inhibitor of apoptosis) family members, XIAP (Fig. 5) and survivin (Fig. 6). Of particular interest, XIAP expression was reduced by 59.5 ± 0.5% and 71.5 ± 3.5% following a 48-h treatment with 2 or 4 μM SAHA, respectively ($P \leq 0.01$ vs. vehicle). Similarly, survivin expression was reduced by 92.7 ± 1.4% and 98.3% ± 0.8% ($P \leq 0.001$ vs. vehicle), as compared to vehicle-treated cultures (Fig. 6).

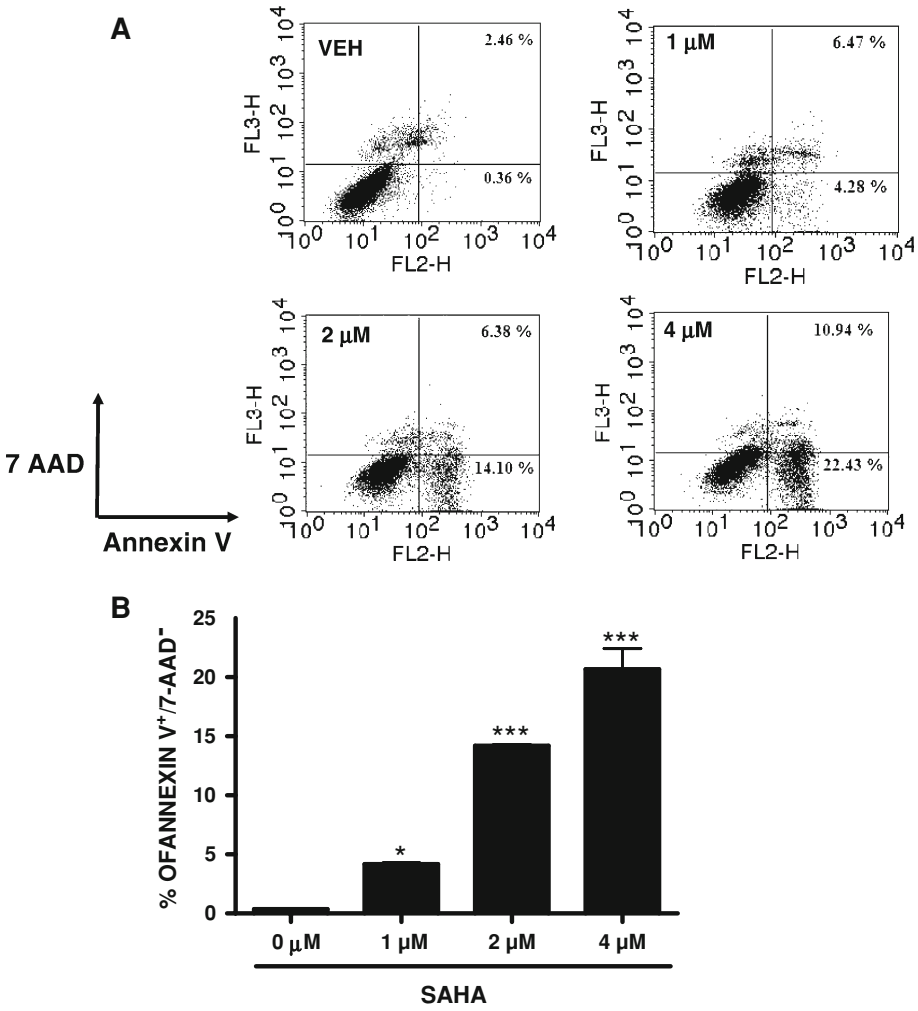

Fig. 3 SAHA increases apoptotic cell death in GH3 cells. **a** Determination of apoptotic cell death using flow cytometry. Following a 48-h treatment with SAHA (0.5–4 μM), cells were stained with 7-AAD (*y*-axis), a marker of dead cells and Annexin V (*x*-axis), a marker of early apoptotic cell death. **b** The percentages of early apoptotic cells, which represent Annexin $V^+/7\text{-}AAD^-$ (lower right quadrant in panel A), are shown. SAHA concentration dependently increased early apoptotic cell death in GH3 cells. Data are expressed as the mean ± SEM from the three independent experiments. Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test ($^*P < 0.05$, $^{***}P < 0.001$ vs. vehicle-treated cultures)

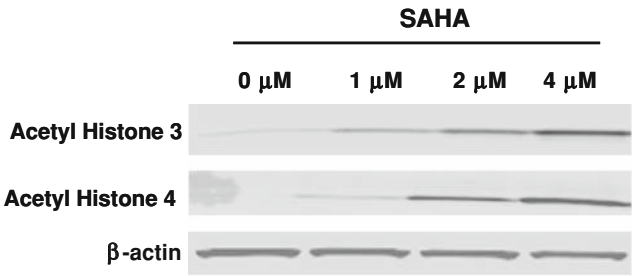

Fig. 4 SAHA functions as an HDACi in GH3 adenoma cells. Cells were treated with increasing concentrations of SAHA (1–4 μM) for 48 h, followed by western blotting for acetyl histone 3 and acetyl histone 4. *β*-actin was used as a loading control. Data are representative of the three independent experiments. Data are expressed as mean ± SEM ($n = 6$/experiment). Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test ($^*P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated cultures)

SAHA induces caspase activation and PARP cleavage in GH3 cells

SAHA augmented pro-caspase-3 activation and increased the proteolysis of PARP a hallmark of apoptotic cell death (Fig. 7a) following a 48-h exposure. Densitometry analysis revealed a 46.7 ± 3.7% decrease in pro-caspase-3 ($P < 0.05$ vs. vehicle) following treatment with 4 μM SAHA. Similarly, 4 μM SAHA dramatically induced PARP cleavage, consistent with caspase-3 activation and apoptotic cell death (Fig. 7b).

Discussion

This study documents, for the first time, an anti-proliferative and pro-apoptotic effect of the FDA-approved HDACi, SAHA, in pituitary adenoma cells. The effect of SAHA was associated with elevated sub-G1 and G1 cell cycle arrest, and increased apoptotic cell death. Together, these findings support a novel role for HDACi in the treatment of pituitary adenoma and indicate that SAHA may limit pituitary tumor proliferation and survival.

Benign pituitary adenomas exhibit progressive tumor growth, locally invasive growth, hormone hypersecretion, and compression of surrounding structures, which may

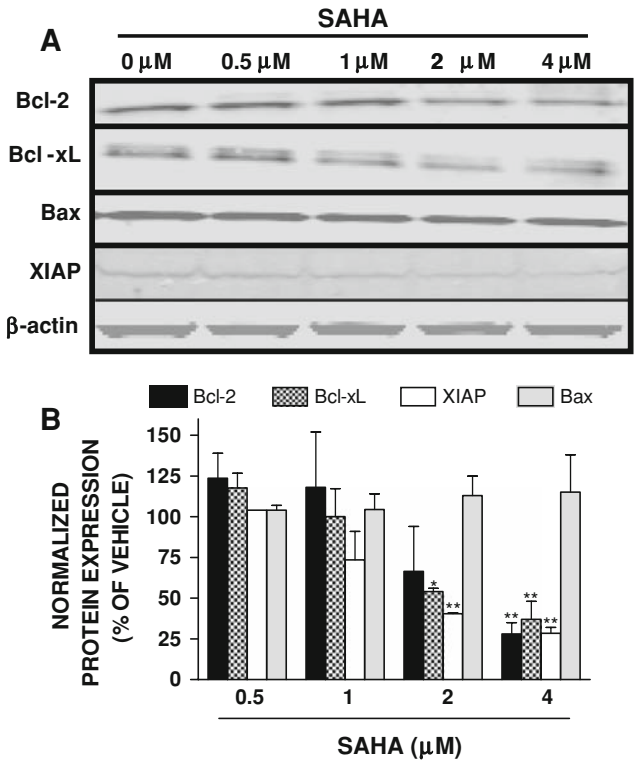

Fig. 5 SAHA attenuates anti-apoptotic protein expression in GH3 cells. **a** Concentration-dependent effect of SAHA (0.5–4 μM) on the expression of Bcl-2, Bcl-xL, XIAP, and Bax following a 48-h treatment. Blots are representative of the three independent experiments. **b** Densitometric analysis of western blotting data. Data are the mean ± SEM from the three independent experiments and were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs vehicle

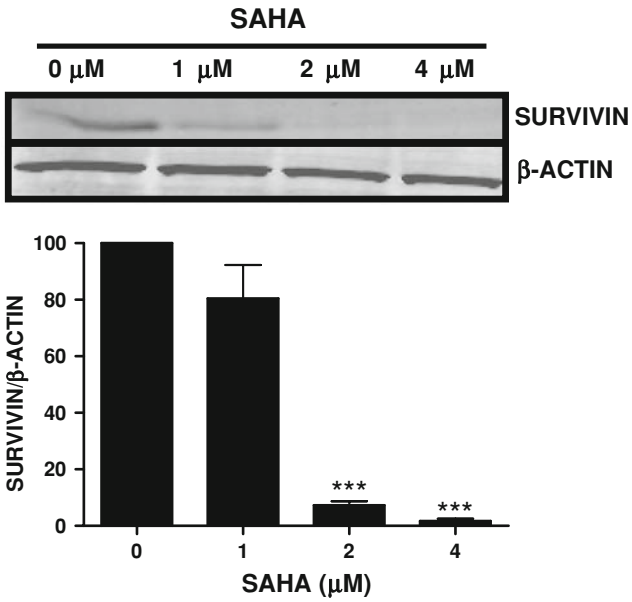

Fig. 6 Survivin expression is reduced by SAHA treatment. GH3 cells were treated for 48 h with SAHA (1–4 μM), followed by western blot analysis for survivin, an anti-apoptotic protein. Densitometry is expressed as the mean ± SEM from the three independent experiments, and data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test ($^{*}P < 0.05$ vs. vehicle-treated cultures)

cause headaches, vision loss, and amenorrhea [1, 23–28]. Approximately, one-third of pituitary adenomas are endocrinologically active, hyper-secreting hormones such as prolactin (PRL), growth factor (GH), adenocorticotrophin releasing hormone (ACTH), or gonadotropins. The clinical management of prolactomas, the most common type of secretory pituitary adenoma, frequently involves the normalization of hormone levels, using bromocriptine or cabergoline [29] and radiotherapy to limit the growth of drug resistant tumors. While these treatment regimens generally control patient morbidity, dopamine agonists require lifelong administration and are frequently associated with significant side effects and poor rates of compliance. Similarly, the optic apparatus exhibits exquisite sensitivity to radiation-induced injury, an important cause of visual deficits and blindness. Thus, the clinical risks and associated toxicity of current treatment modalities emphasize the need for novel therapeutic approaches.

Histone acetylation modulates diverse biological processes including cellular proliferation, cell cycle regulation, and apoptosis, suggesting a potential role in the

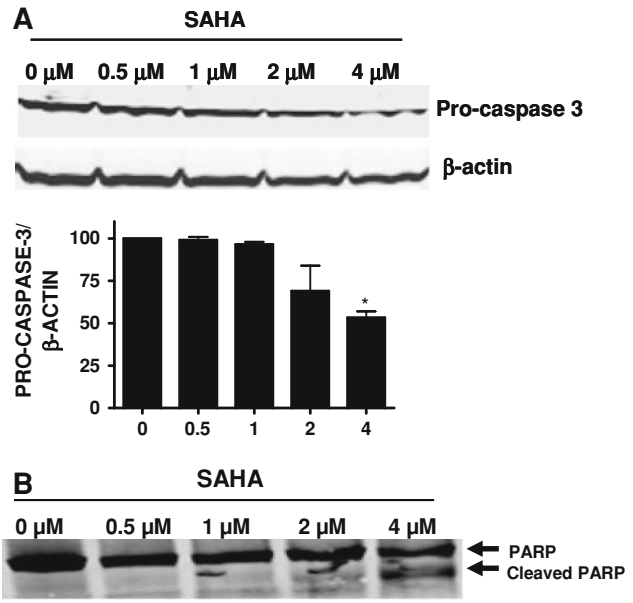

Fig. 7 SAHA modulates hallmarks of apoptotic death. GH3 Cells were treated for 48 h with SAHA (0.5–4 μM), followed by western blotting. **a** SAHA decreased the expression of pro-caspase-3, consistent with caspase-3 activation. β-actin was used as a loading control. Densitometry is expressed as the mean ± SEM and data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test ($^{*}P < 0.05$ vs. vehicle-treated cultures). **b** SAHA increased PARP cleavage, consistent with caspase activation. Blots are representative of at least three independent experiments

regulation of tumor progression [5–11]. In this study, clinically achievable concentrations of the HDACi and SAHA reduced the viability of the secretory pituitary adenoma cell lines, GH3 and MMQ. The concentrations of SAHA required to inhibit cellular growth directly correlated with the degree of histone H3 and H4 acetylation, consistent with an HDACi mechanism of action, and notably, were less than the concentrations needed to restrict the growth of glioblastoma cell lines [30]. That SAHA may act via a HDACi-mediated mechanism that was further supported by the observation that trichostatin A, a first-generation HDACi that is structurally similar to SAHA, also potently reduced cellular viability (data not shown); however, in contrast to trichostatin A, SAHA is non-toxic in non-transformed cells [16, 31, 32].

SAHA increased the proteolysis of poly(ADP)-ribose polymerase (PARP), a hallmark of apoptotic cell death that is catalyzed by the activation of caspase-3 [33], and decreased the expression of anti-apoptotic proteins, including Bcl-2, Bcl-xL, Survivin, and XIAP prior to induction of apoptotic cell death; however, the irreversible, pan-caspase inhibitor, Z-VAD.fmk (100 μM) only slightly (but significantly) reduced cytotoxicity following co-treatment with higher (2–4 μM), but not lower (500 nM, 1 μM) concentrations of SAHA (SRS, JV, and KMD, unpublished observations). These findings suggest that SAHA may utilize both caspase-dependent and -independent pathways of cell death. Although the mechanisms underlying this effect remain unclear in pituitary cells, SAHA induced G1 growth arrest and caspase-independent cell death via the activation of autophagy in endometrial stromal sarcoma cells and chondrosarcoma cells [34, 35]. Of particular interest to the present report, down regulation of the Akt-mTOR signaling pathway increased autophagic cell death in prostate cancer cells, an effect that was dependent upon the reduced survivin expression [36]. Similar to this study, our laboratory recently demonstrated constitutive Akt activation in GH3 cells and that inhibition of this signaling pathway dramatically increased cell death [4]. Coupled with the basal activation of Akt in human pituitary tumors [37], these data suggest Akt signaling may restrain autophagic cell death and promote pituitary adenoma progression.

Pituitary adenomas exhibit a relatively slow rate of growth, as compared with malignant brain tumors; however, aberrant tumor proliferation contributes to patient morbidity. Consistent with a beneficial effect in malignant cell lines [38–41], clinically relevant concentrations of SAHA induced sub-G1 and G1 growth arrest in pituitary tumor cells. Expression of survivin, which is dramatically increased in human secretory and non-secretory pituitary adenoma [42], is tightly coupled to the cell cycle and regulation of cellular proliferation, with highest expression in the G2/M phase of the cell cycle and a reduction observed in the G1 phase [43]. Thus, it is unclear whether SAHA directly inhibits survivin expression or whether this reduction may be a secondary response to an increase in G1 growth arrest following SAHA treatment. Regardless of the underlying mechanism, a reduction in the expression of survivin may have broad biological consequences. Survivin is reported to directly inhibit the activity of the pro-apoptotic proteases, caspase-3, and -7 [44]; however, a recent, dissenting report suggests that survivin may not directly bind caspases [45]. Survivin overexpression is also associated with tumor radioresistance in several malignancies, including brain, pancreatic, and rectal cancer [46–48]. Radiation remains a frontline treatment option for pituitary adenoma; however, radiotherapy may induce cognitive side effects and hypopituitarism in over one-third of patients by 24 months and nearly all patients by 10 years [49], demonstrating the need for novel strategies to reduce the amount of radiation required to reduce tumor size. Consistent with a possible benefit, 1 μM SAHA radiosensitized GH3 cells such that the combination of 2.5 Gy with SAHA more effectively reduced cell death as compared to radiation or SAHA alone (SRS, JV, and KMD, unpublished observations). Thus, survivin may influence cellular viability via both caspase-dependent and -independent mechanisms of action, and may be a useful adjunct therapy to sensitize pituitary adenoma cells to radiation.

In summary, this study suggests, for the first time, a possible anti-proliferative and pro-apoptotic effect of SAHA in benign pituitary adenoma cells. These studies provide an intriguing framework for future testing of SAHA in the medical management of pituitary tumors, both as a single agent to reduce cellular proliferation and survival and possibly as an adjunct to radiotherapy.

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