Anticancer activity of SAHA, a potent histone deacetylase inhibitor, in NCI-H460 human large-cell lung carcinoma cells *in vitro* and *in vivo*

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Abstract. Suberoylanilide hydroxamic acid (SAHA), a potent pan-histone deacetylase (HDAC) inhibitor, has been clinically approved for the treatment of cutaneous T-cell lymphoma (CTCL). SAHA has also been shown to exert a variety of anticancer activities in many other types of tumors, however, few studies have been reported in large-cell lung carcinoma (LCC). Our study aimed to investigate the potential antitumor effects of SAHA on LCC cells. Here, we report that SAHA was able to inhibit the proliferation of the LCC cell line NCI-H460 in a dose- and time-dependent manner, induced cell apoptosis and G_2/M cell cycle arrest, decreased AKT and ERK phosphorylation, inhibited the expression of pro-angiogenic factors (VEGF, HIF-1α) *in vitro*, and suppressed tumor progression in an NCI-H460 cell nude mouse xenograft model *in vivo*. These results indicate that SAHA can exert its strong antitumor effects in LCC patient.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1) and can be classified into two major groups, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC mainly consists of squamous cell carcinoma (SC), adenocarcinoma (AC) and large-cell carcinoma (LCC). The prognosis of lung cancer depends on pathological stages and histological types, and the prognosis of LCC is the worst in NSCLC (2). Among subtypes of LCC, the prognosis of large-cell neuroendocrine carcinoma (LCNEC) was poorer than others even if at early stages (3-5) such as SCLC. A better therapeutic drug for this kind of lung cancer is thus urgently required to drastically reduce the number of deaths.

Histone deacetylase (HDAC) inhibitors have garnered significant attention as anticancer drugs. These therapeutic agents have been clinically validated with the market approval of vorinostat (SAHA, Zolinza) for treatment of cutaneous T-cell lymphoma (6,7). Suberoylanilide hydroxamic acid (SAHA) is a potent, reversible pan-histone deacetylase (HDAC) inhibitor. It inhibits both class I and class II HDACs, altering gene transcription and inducing cell cycle arrest and/or apoptosis in a wide variety of transformed cells (8). It has been reported that SAHA inhibited cell proliferation, induced apoptosis and had antitumor activities in various human cancer cells (9-11), including NSCLC cells (12,13). SAHA also exhibited anticancer activities by regulating a variety of signaling pathways (14). The evidence suggested that SAHA might act as an oral anticancer agent in LCNEC although few studies have yet been reported.

NCI-H460 is an LCC cell line with neuroendocrine features (15,16). The present study focused on the anticancer effects of SAHA on NCI-H460 cells *in vitro* and *in vivo*. We aimed to investigate whether SAHA exhibits anticancer effects by suppressing cell proliferation, cell apoptosis, influencing cell cycle distribution, regulating cell signaling in NCI-H460 cells, and next to confirm the antitumor activity of SAHA in a nude mouse xenograft model of NCI-H460 cells.

Materials and methods

Antibodies and regents. Suberoylanilide hydroxamic acid (SAHA, Vorinostat) with a purity of >98% was purchased from Sigma-Aldrich (St. Louis, MO, USA). It was dissolved in dimethyl sulfoxide (DMSO) and stored at -20˚C, then thawed before used. DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) were purchased from Sigma-Aldrich. Human lymphocyte separation liquid Ficoll-Hypaque was purchased from TBD Science (Tianjin, China). Hoechst 33342 was purchased from

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Beyotime (Shanghai, China). Antibodies of AKT, ERK1/2, phosphor-AKT, phosphor-ERK1/2, HIF-1α, VEGF were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Isolation of peripheral blood mononuclear cells and cell culture. The peripheral blood mononuclear cells (PBMCs) were fractionated from peripheral blood (PB) of healthy volunteers by Ficoll-Hypaque density gradient centrifugation. The isolated PBMCs were washed for 3 times and suspended with RPMI-1640 medium (Hyclone, UT, USA) containing 10% fetal bovine serum human (FBS) (Gibco, USA). Human large-cell lung carcinoma cell line NCI-H460 was obtained from the China Center for Typical Culture Collection (Wuhan, China) and maintained in RPMI-1640 medium with 10% FBS. All cells were seeded onto 50 cm^2 culture bottle at 37˚C, 5% CO_2, in an incubator.

Cell proliferation assay. NCI-H460 cells ($2x10^3$) and PBMCs ($2x10^3$) were cultured in RPMI-1640 medium with 10% FBS in 96-well plates. 1, 2.5, 5 and 10 μM of SAHA were added respectively and cells were incubated for 24, 48 and 72 h. The anti-proliferative effect of SAHA was determined by using the MTT dye uptake method. MTT solution (20 μl) (5 mg/ml) was added to each well. After incubation for 4 h at 37˚C, the supernatants were removed and 150 μl DMSO was added to each well. Optical density (OD) was detected with a microplate reader (Biotech, NY, USA). IC_{50} was taken as the concentration that caused 50% inhibition of cell proliferation. Cell proliferation inhibited (%) = [1-(OD of the experimental sample/OD of the control)] x 100% (n=5).

Cell apoptosis assay. NCI-H460 cells were exposed to increasing concentration of SAHA and the proportion of apoptotic cells was quantified by Annexin V-FITC/PI dual staining (Beyotime). SAHA-treated and untreated cells cultured for 12 h and resuspended in 100 μl of binding buffer. Then cells were stained with 5 μl Annexin V-FITC and 10 μl PI for 15 min in the dark at room temperature, then analyzed by flow cytometry (BD Biosciences, CA, USA) within 1 h.

Hoechst 33342 staining. Cells were exposed to various concentration of SAHA (5, 10 and 20 μM) for 12 h and cells without SAHA treatment served as control. Cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min, then stained with 10 μl Hoechst 33342 for 10 min. Finally cells were viewed by a confocal microscope (Olympus, Tokyo, Japan).

Cell cycle assay. After 24-h treatment with 2.5 and 5 μM SAHA in NCI-H460 cells, we performed DNA flow cytometric analysis to study cell cycle distribution. Cell cycle distribution was determined by DNA staining with PI. Briefly, cells were washed in PBS and fixed in 70% ethanol overnight after treated with SAHA, the next day cells were collected and resuspended in PBS containing 40 μg/ml PI, 0.1 mg/ml RNase (Beyotime), and 5% Triton X-100 and incubated at 37˚C for 30 min. Finally cells were analyzed by flow cytometry (BD Biosciences).

Western blot analysis. Lysates were prepared from $1x10^7$ cells by dissolving cell pellets in 100 μl of lysis buffer. Lysates were centrifuged 12,000 g for 15 min at 4˚C and the supernatants were collected. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added and lysates were heated at 100˚C for 5 min and 50 μg of protein was loaded into each well of 10% SDS-PAGE gels. Proteins were electrophoretically transferred to nitrocellulose membranes blocked with 5% non-fat milk or 5% BSA, and incubated overnight at 4˚C with the primary antibodies (AKT 1:500, p-AKT 1:500, p-ERK1/2 1:500, ERK1/2 1:1,000, VEGF 1:400; HIF-1α 1:500 and β-actin 1:1,000). The blots were washed, exposed for 1 h to corresponding HRP-conjugated secondary antibodies, and detected using ECL (Pierce Biotechnology, Rockford, IL, USA).

Nude mouse xenograft model. Four-week-old female BALB/c nude mice were purchased from the Jackson Laboratory (Vital River, Beijing, China). Experimental animals were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Tongji Medical College. Mice were injected subcutaneously with $1x10^7$ NCI-H460 cells per mouse into the right armpit. When the maximum diameter of tumor reached 5 mm, mice were randomly grouped into a blank group, a vehicle control group (DMSO) and SAHA group. The tumors were observed and measured every day. The SAHA group and DMSO group, respectively, received intraperitoneal injection of SAHA (50 mg/kg/day, SAHA was first dissolved in DMSO, then normal saline diluted to 0.2 ml per mouse, was added, the final concentration of DMSO is 40%) or normal saline (0.2 ml/day, 40% DMSO) for 10 days.

Statistical analysis. All data are expressed as the mean ± SD of at least 3 independent experiments. Statistical differences between experimental groups were analyzed by Student's t-test, and ANOVA (GraphPad Prism 5.0), $P<0.05$ was considered a statistically significant difference.

Results

Effects of SAHA on the proliferation of NCI-H460 cells and PBMCs. To explore the effect of SAHA on the growth of NCI-H460 cells, MTT assay was used to assess the cell viability. NCI-H460 cells were treated with increasing concentration of 1, 2.5, 5 and 10 μM SAHA for 24, 48 and 72 h, respectively. SAHA caused inhibition of cell viability in a time- and dose-dependent manner as shown in Fig. 1A. The 24, 48 and 72 h IC_{50} of SAHA in NCI-460 cells were 43.23, 4.07 and 1.21 μM, respectively. The IC_{50} values decreased gradually with time passing. Fig. 1B shows the curve of growth inhibition of SAHA on NCI-H460 cells and PBMCs. When treated with various concentrations of SAHA for 24 h, SAHA inhibited the proliferation of NCI-H460 cells in a dose-dependent manner, but had no inhibitory effect on PBMCs.

Effects of SAHA on cell apoptosis of NCI-H460 cells. To determine whether the impaired cell viability of NCI-H460 cells induced by SAHA involved apoptosis, NCI-H460 cells were exposed to increasing concentration of SAHA. The proportion of apoptotic cells was quantified by Annexin V

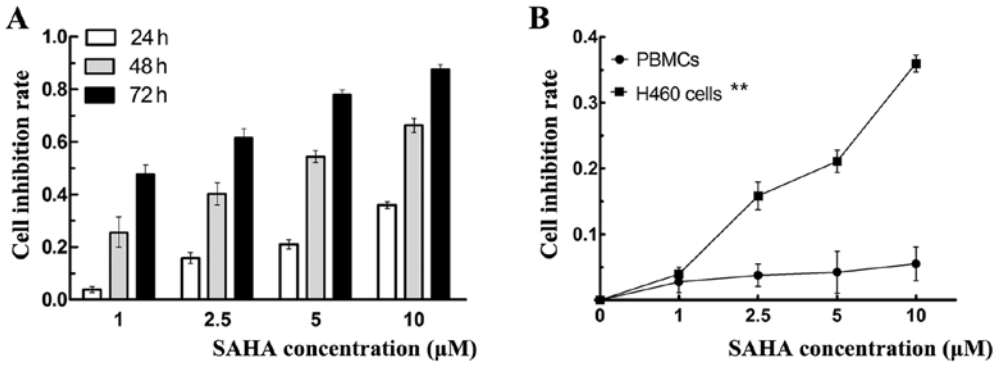


Figure 1. Effects of SAHA on NCI-H460 cells and PBMCs. (A) NCI-H460 cells treated with increasing concentration SAHA for 24, 48 and 72 h, respectively. Data are the mean ± SD of at least three independent experiments. (B) Comparison of inhibition effects of different concentration SAHA on NCI-H460 cells and peripheral blood monocular cells (PBMCs). Data are the mean ± SD of at least three independent experiments (**P<0.01).

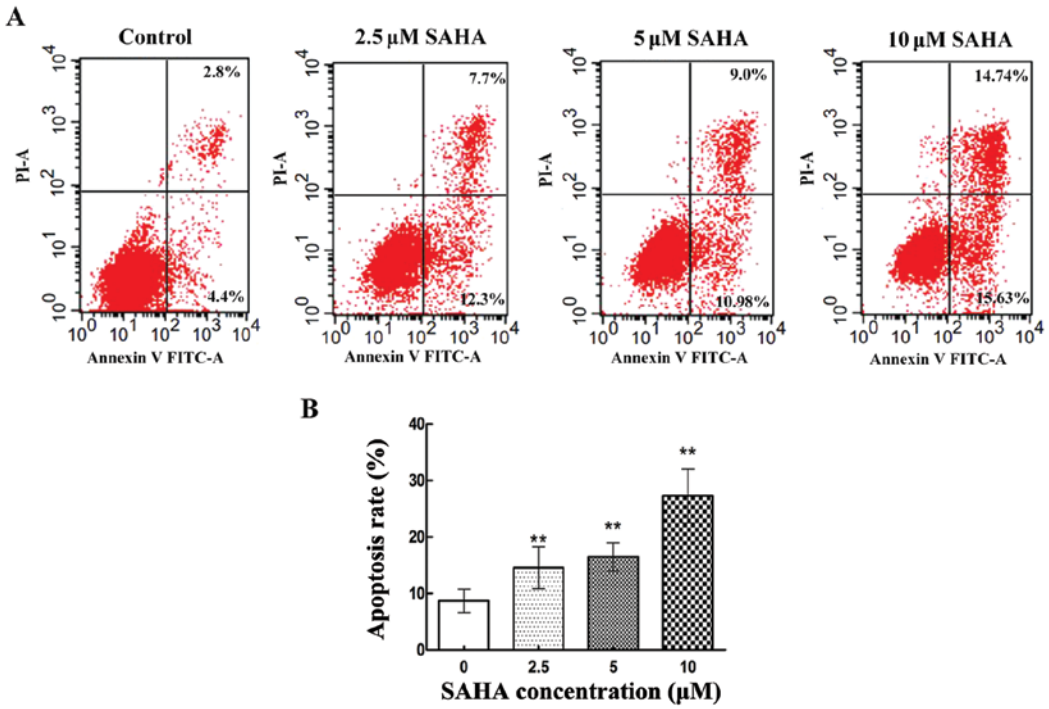


Figure 2. SAHA causes cell apoptosis of NCI-H460 cells. (A) Cells were treated with SAHA (2.5, 5 and 10 μM) for 24 h and cells without SAHA treatment served as control. Cell apoptosis was quantified by Annexin V-FITC/PI dual staining assay. Representative image of three independent experiments. (B) The rate of apoptotic cells was quantified. Data are the mean ± SD of at least three independent experiments (**P<0.01).

-FITC/PI dual staining cytometry. As shown in Fig. 2A, untreated NCI-H460 cells exhibited little Annexin V staining. In contrast, after treated with SAHA at 2.5, 5 and 10 μM for 12 h, NCI-H460 cells showed an increasing degree of Annexin V staining. The apoptosis rate was 14.6±3.72, 16.5±2.49 and 27.3±4.74% respectively, which was statistically different from the untreated cells (Fig. 2B). In addition, we also assessed the effect of SAHA on apoptosis of NCI-H460 cells by fluorescent Hoechst 33342 staining of cell nucleus. Nucleus of cells in control was regular in shape, but a part of the nucleus SAHA-treated cells were fragmented and condensed, being typical apoptotic morphology (Fig. 3). The number of cells with apoptotic morphology was increased progressively with SAHA concentration.

Effects of SAHA on cell cycle of NCI-H460 cells. To assess whether SAHA affects the cell cycle, we performed DNA flow cytometric analysis to study cell cycle distribution. After 24-h treatment with 2.5 and 5 μM SAHA in NCI-H460 cells, the cell proportion in the G_2/M phase increased gradually in a dose-dependent manner. The rate of G_0/G_1 phase cells decreased accordingly and the cells in S phase had no significant change (Fig. 4). This result indicated SAHA could induce G_2/M cell accumulation and cause G_2/M phase cell cycle arrest.

Effects of SAHA on the expression of AKT and ERK signaling. Extensive studies showed that the activation of AKT (phospho-AKT) and ERK1/2 (phospho-ERK1/2) play a crucial role in tumor cell growth and survival, which regulated many related factors for anti-apoptotic functions. To investi-

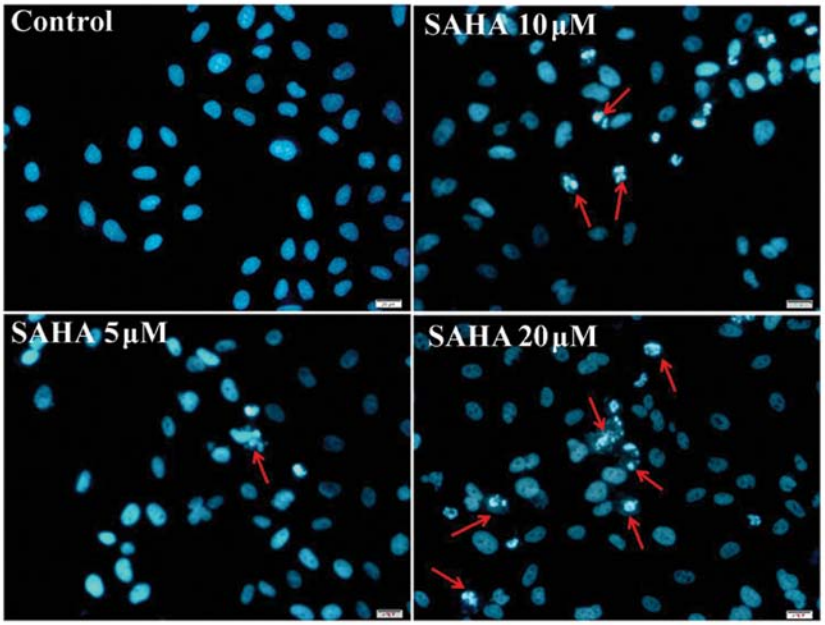
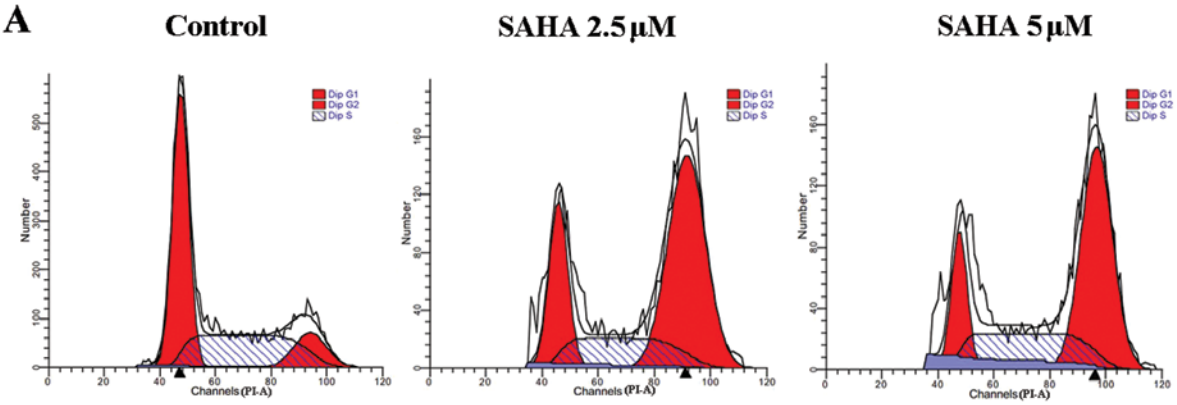


Figure 3. SAHA causes cell nucleus morphological changes of NCI-H460 cells. Cells were exposed to various concentrations of SAHA (5, 10 and 20 μM) for 12 h and cells without SAHA treatment served as control, then stained with 1 μg/ml Hoechst 33342. As the red arrows indicate, fragmented and condensed cell nucleus morphology could be observed in SAHA-treated cells, while the nucleus of untreated cells was regular in shape (x400). The apoptotic cells were increased with SAHA concentration. Scale bar, 20 μm.

A



B

SAHA (μM)	G0/G1 (%)	S (%)	G2/M (%)
Control	64.1±3.2	24.8±2.0	11.0±1.5
2.5 μM	39.6±16.5*	21.3±0.2	39.0±16.3*
5 μM	34.8±17.1*	24.4±5.0	40.8±14.4*

Figure 4. Effects of SAHA on cell cycle distribution in NCI-H460 cells. (A) Cells were incubated with 2.5 and 5 μM SAHA for 24 h and cells without SAHA treatment served as control, the distribution of cell cycle was detected by PI staining. Representative images of three independent experiments. (B) The cell cycle distribution was quantified in SAHA-treated and untreated cells. Data are the mean ± SD of at least three independent experiments (*P<0.05).

gate whether SAHA could affect the activation of AKT and ERK1/2, NCI-H460 cells were treated with SAHA of 2.5, 5 and 10 μM for 24 h to detect the protein expression. As a result, constitutively activated AKT (phospho-AKT) and ERK (phospho-ERK1/2) were seen in untreated cells. SAHA treatment induced a dose-dependent decline of phospho-AKT and phospho-ERK1/2, while total AKT and total ERK1/2 proteins had no significant change (Fig. 5A).

Effects of SAHA on the expression of HIF-1α and VEGF. We next determined the effects of SAHA on the expression of pro-angiogenic factors. HIF-1α and VEGF are the two important

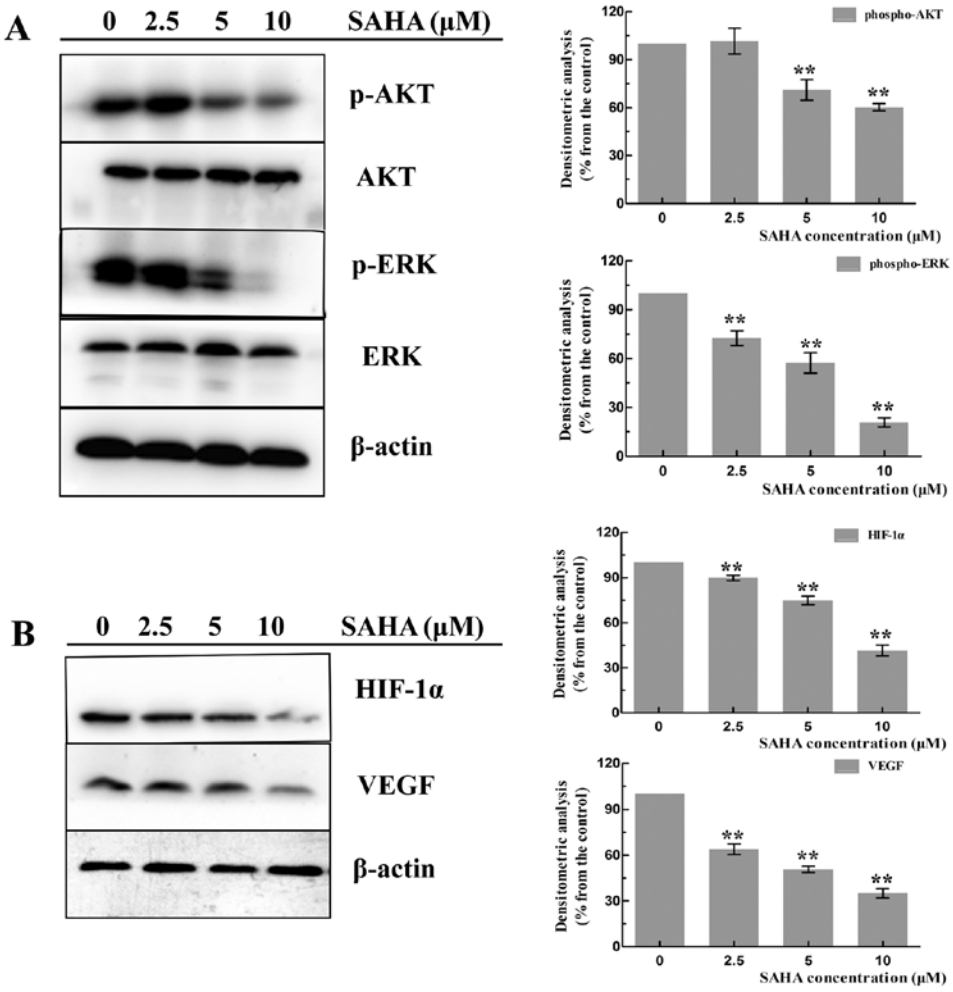


Figure 5. Effects of SAHA on the phosphorylation of AKT, ERK1/2 and VEGF, HIF-1α. NCI-H460 cells were treated with increasing concentrations (0, 2.5, 5 and 10 μM) of SAHA for 18 h, then cell proteins were subjected to western blotting with anti-phospho-AKT, anti-phospho-ERK1/2, anti-total-AKT, anti-total-ERK1/2, anti-VEGF, anti-HIF-1α, β-actin antibodies. (A) A representative western blotting of phospho-AKT, phospho-ERK1/2, total-AKT, total-ERK1/2. The expression of phospho-AKT and phospho-ERK1/2 proteins in SAHA-treated and untreated cells was quantified by densitometry. Data represent the mean ± SD of at least three separated experiments (**P<0.01). (B) A representative western blotting of VEGF, and HIF-1α. The expression of two proteins in SAHA-treated and untreated cells were quantified by densitometry. Data represent the mean ± SD of at least three separated experiments (**P<0.01).

components in tumor angiogenesis. Many investigations have found that SAHA could inhibit angiogenesis to play an anti-tumor effect. To test this hypothesis in NCI-H460 cells, we detected the expression of HIF-1α and VEGF in SAHA-treated and untreated cells. As shown in Fig. 5B, SAHA-treated cells exhibited significant decreased levels of HIF-1α and VEGF in a dose-dependent manner compare with the untreated cells.

Effects of SAHA on the growth of NCI-H460 cell xenograft in vivo. To assess the therapeutic efficiency of SAHA *in vivo*, a xenograft murine model of NCI-H460 cells was established. After cell injections for 7 days, NCI-H460 cells quickly developed a tumor at the site of subcutaneous injection. As shown in Fig. 6A and B, compared with vehicle control group (DMSO) and blank group, SAHA group significantly reduced the tumor size (P<0.01). DMSO group had almost no effect on the tumor and the tumor growth between control and blank groups had no significant difference (P=0.99). The mice were weighed every day, and no significant difference occurred in their weights (data not shown). The mice were euthanized and tumors were carefully dissected and weighed (Fig. 6C). Tumor weight of SAHA treatment group was significantly less than control group and blank group (P<0.01) (Fig. 6D).

Discussion

Chromatin protein acetylation is part of a complex signaling system that is largely involved in the control of gene expression (17). Histone acetyltransferases and HDACs act in opposing manner to control the acetylation state of nucleosomal histones. Epigenetic modification by small-molecule HDAC inhibitors is a promising new anti-neoplastic approach for various solid and hematological malignancies (18). SAHA, a second generation hybrid polar compound, is a potent pan-HDAC inhibitor and has been clinically approved for treatment of cutaneous T-cell lymphoma (CTCL) (18,19). HDACs act not only on histones, but rather have many different cellular substrates and target proteins involving physiological and pathological conditions, therefore SAHA is able to exert a variety of anticancer activities in many tumor types as a pan-HDAC inhibitor (20). It has been reported that SAHA exhibited antitumor effects by prompting tumor cells to enter apoptosis, interfering with the cell cycle,

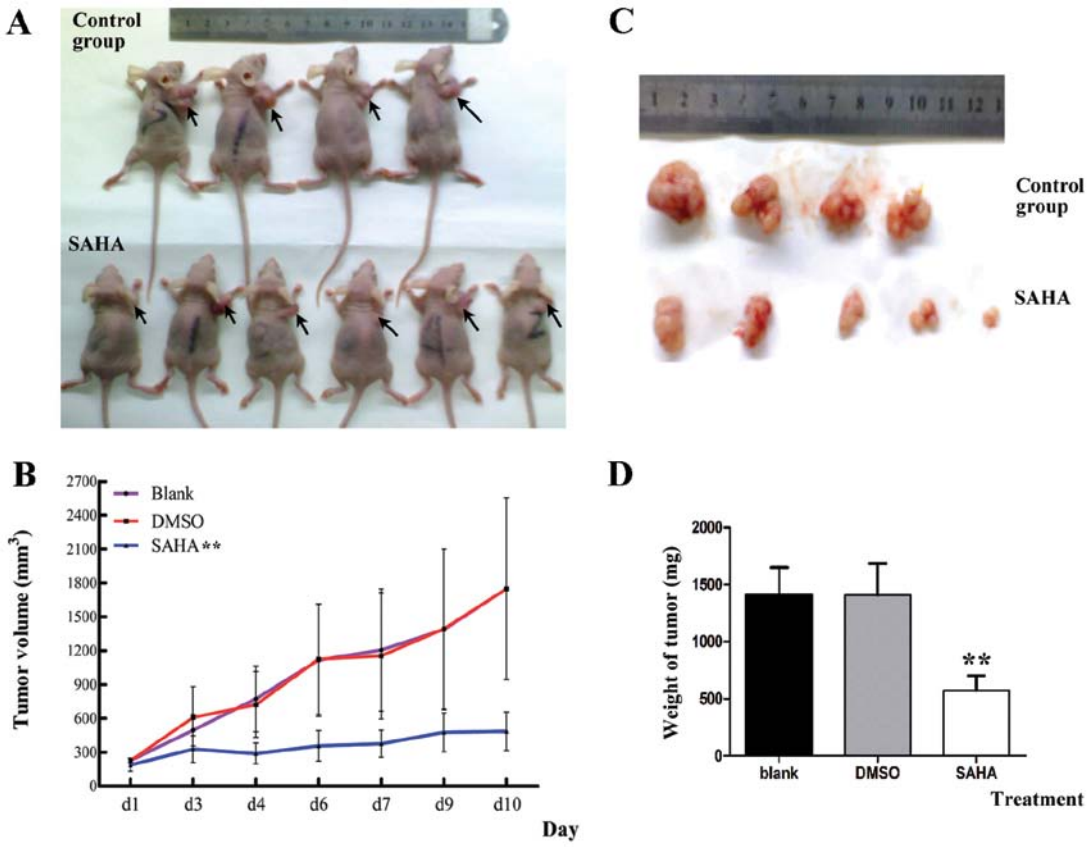


Figure 6. SAHA suppresses NCI-H460 cell tumor progression in the mouse xenograft model. NCI-H460 cells ($1x10^7$ per mouse) were injected into 4-week-old nude mice. When solid tumors grew to ~200 mm^3, the mice were treated intraperitoneally with either a vehicle control (DMSO) or SAHA at 50 mg/kg daily for 10 days. (A) After establishment of subcutaneous tumors, SAHA was injected during treatment. Blank arrows indicate locations of tumor formation of treated and untreated groups. (B) Effect of SAHA on xenograft tumor volume. The SAHA treatment groups had significantly smaller tumor volume than the control group whereas there is no significant difference among the control and blank groups, $^{**}P<0.01$. (C) Image of xenograft tumor with SAHA-treated and untreated. (D) Effect of SAHA on xenograft tumor weight. $^{**}P<0.01$ (compared to control).

inducing DNA damage, disturbing cell signaling, inhibiting tumor angiogenesis in many solid and hematological tumors (20,21), but few studies have been reported in large-cell lung carcinoma (LCC).

LCC has the worst prognosis in NSCLC (2). Among subtypes of LCC, large-cell neuroendocrine carcinoma (LCNEC) is the common type and the prognosis of it was poorer than others even if at early stages (22,23). The 5-year overall survival is ~15-25% (23). The optimal treatment of LCNEC has not been established. Because it is an uncommon malignancy, prospective, randomized trials have not been performed (23). Thus, there is an urgent need for new drug that can target this kind of tumor cells and benefit LCNEC patients. Since SAHA is able to inhibit tumor progress and has been approved for treatment of CTCL, we proposed that SAHA can also exert antitumor effects on LCNEC. For confirmation, we chose the large-cell lung carcinoma neuroendocrine cell line NCI-H460 (15,16) as our experimental cells and the NCI-H460 cell nude mouse xenograft model to further verify the effects of SAHA *in vitro* and *in vivo*.

We show that SAHA acted as potent chemotherapeutic agent against LCNEC. Our data indicated that SAHA inhibited the proliferation of NCI-H460 cells significantly, while had low toxicity on human peripheral blood monocular cells (PBMCs). Its biological effects on NCI-H460 cell growth varied with drug concentration and duration of exposure. Cell cycle and apoptosis detected by flow cytometry demonstrated that SAHA arrested NCI-H460 cells at G_2/M phase and induced apoptosis in a dose-dependent manner. With the increasing dose of SAHA (2.5, 5 and 10 μM for 12 h), the apoptosis rate was 14.6±3.72, 16.5±2.49 and 27.3±4.74%, respectively. NCI-H460 cells treated with SAHA also manifested typical apoptotic morphological alterations with cell nucleus presenting chromatin condensation or fragmented into smaller structures by Hoechst 33342. Along with the drug dose elevation, the cells of apoptotic morphology increased in number accordingly. Further investigation indicated that since activities of phospho-AKT and phospho-ERK1/2 both decreased in a dose-dependent manner, inhibition of AKT and ERK1/2 signaling seemed to be the potential mechanism of SAHA-induced anti-proliferation effects. We also found that SAHA could inhibit the expression of HIF-1α, VEGF of NCI-H460 cells dose-dependently. Finally, we confirmed SAHA could significantly suppress the tumor progression in the xenograft model, possibly as a result of anti-proliferation and anti-angiogenesis effects of SAHA.

Extracellular growth factor-mediated signaling, which is essential for cell proliferation, is frequently disrupted in cancers. Activation of growth factor receptors leads to the stimulation of numerous downstream pathways that modulate

cell metabolism, control gene transcription, and affect the cell cycle. Of these pathways, the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) pathways play a central role (24). PI3K/AKT pathway is a key signal transduction pathway that mediates cell growth and blocks apoptosis (25). AKT, is also called protein kinase B (PKB), plays an important role in cell survival (26). Increased activation of this survival cascade is a characteristic feature of a large variety of human malignancies and has been associated with carcinogenesis. Activation of AKT induce tumor cell proliferation by regulating transcription factors which modulate distinct sets of genes involved in cell cycle, apoptosis and DNA repair (27). MAPK cascades are also key signaling pathways involved in the regulation of normal cell proliferation, survival and differentiation. Aberrant regulation of MAPK cascades contribute to cancer and other human diseases (28). ERK1/2 (extracellular signal regulating kinase 1/2) are two isoforms that belong to the family of MAPKs, which include ERK5, the c-Jun-NH2-terminal kinases (JNK1/2/3) and the p38 MAP kinases (p38 MAPK). These enzymes are activated through a sequential phosphorylation cascade that amplifies and transduces signals (29). Our study showed that inhibition of cell proliferation, intervention in the cell cycle and induction of apoptosis by SAHA was accompanied by a downregulation of ERK1/2 and AKT signaling, suggesting that SAHA by inhibiting HDACs in LCNEC targets multiple proliferation and growth regulatory pathways. The overexpression of ERK1/2 and AKT also confirmed that cell survival signaling pathways may be the cause of LCNEC cell growth out of control.

Angiogenesis is essential for the growth, progression, and metastasis of solid tumors and efficient inhibition of angiogenesis is considered a promising strategy for the treatment of cancer (30). Hypoxia induced factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) are important pro-angiogenic factors in tumor angiogenesis. HIF-1α plays a key role in tumor angiogenesis by regulating the expression of angiogenic factors, including VEGF (31). HIF-1α overexpression has been implicated in many human cancers and is associated with increased vascularization, drug resistance and poor diagnosis (32,33). HIF-1α is acetylated and hydroxylated in normal conditions, interacting with the von-Hippel-Lindau and rapidly triggers degradation, but HIF-1α accumulated in tumor hypoxia condition (33). SAHA can promote HIF-1α degradation in tumor cells (20). It has been reported that SAHA strongly impaired the hypoxia-induced secretion of VEGF by neuroblastoma cells (34) and downregulated HIF-1α and VEGF in a breast cancer cell line xenograft model (35). Our data showed SAHA could downregulate the expression HIF-1α and VEGF in LCNEC and to suppress tumor xenograft growth by its anti-proliferation and anti-angiogenesis effects.

In conclusion, our data provide a novel mechanism-based therapeutic intervention for LCC. SAHA can block pathogenesis of aggressive LCC and may be utilized to treat LCC, particularly in LCNEC patients.

Acknowledgements

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