Histone deacetylase inhibitors suppress the growth of human osteosarcomas in vitro and in vivo

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Summary

Purpose: Although the antitumor efficacy of histone deacetylase inhibitors (HDACIs) has been referred to as a promising new treatment strategy in malignancies, how they exert their effects on human osteosarcoma in vitro and in vivo is yet not well understood. In this study, we employed HDACIs suberoylanilide hydroxamic acid (SAHA) and sodium butyrate (SB) to investigate their effects on human osteosarcoma in vitro and in vivo.

Methods: The in vitro effects of HDACIs SAHA and SB were evaluated in SaOS2 and U2OS human osteosarcoma cell lines. Cell growth, cell cycle progression and histone acetylation were investigated by MTS, flow cytometry and western blotting, respectively. In addition, SAHA or SB was administered for 4 weeks in mice xenograph models for assessing the in vivo effects.

Results: MTS assays revealed that SAHA and SB significantly suppressed the growth of SaOS2 and U2OS cells in a concentration-dependent manner. Western blotting analysis indicated that the levels of acetylated H3 were increased after HDACIs treatment. Flow cytometry showed that SAHA arrested the cell cycle in G1 and G2/M phase, while SB arrested the cell cycle in G2/M phase. The tumor growth of mice xenograph models with SaOS2 was inhibited by SAHA and SB compared with vehicle control.

Conclusion: HDACIs SAHA and SB significantly inhibit the growth of human osteosarcoma cells and induce cell cycle arrest. The tumor inhibitory effects were also validated in mice xenograft models.

Key words: cell cycle, osteosarcoma, proliferation, SAHA, SB, xenograft

Introduction

Osteosarcoma is the most common type of primary tumor of bone in humans [1,2]. In the past two decades, despite the advances in the treatment of osteosarcoma including surgery and multimodal chemotherapy, the current therapeutic strategies offer a rather poor benefit and the different chemotherapy regimens have not significantly improved the survival of patients with osteosarcomas [1-4]. The need for new therapeutic methods for osteosarcoma is urgent.

The acetylation and deacetylation of histones influence the interaction of chromatin and DNA and modulate gene expression [1,5]. Histone acetylation is associated with genes’ transcriptional activation, and histone deacetylation correlates with genes’ transcriptional silencing [6]. Reversible histone acetylation and deacetylation is regulated by histone acetyltransferase and histone deacetylase [7]. HDACIs, which enhance the acetylation of histones and are required for gene transcription, have been shown to induce cell cycle arrest, differentiation and apoptosis of many malignancies [3,8-10]. As previously reported, HDACI Trichostatin A induced apoptosis in human osteosarcoma cells [1]. HDACI PCI-24781 showed significant anticancer activity in soft tis-
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Sue sarcoma by inducing apoptosis and inhibiting growth of bone sarcoma cells [5,11].

Based on the above studies, we employed HDACIs SAHA and SB to investigate their antitumor activities in human osteosarcoma both in vitro and in vivo.

Methods

Cell culture and reagents

The human osteosarcoma cell lines SaOS2 and U2OS were purchased from the American Type Culture Collection (ATCC) and maintained in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 50 U of penicillin/ml, 5 μg of streptomycin/ml and 2 mmol/L glutamine in 5% CO₂, USA) supplemented with 10% fetal bovine serum -ture Collection (ATCC) and maintained in DMEM (Gib-

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U2OS were purchased from the American Type Cul

tumor activities in human osteosarcoma both

Cell proliferation assay

Cell proliferation was assessed by the 3-(4,5-dimeth-
ylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sul-
fophenyl)-2H-tetrazolium inner salt (MTS) assay ac-
cording to manufacturer's instructions (CellTiter 96 Aqueous Assay reagent, Promega). SaOS2 and U2OS cells were seeded in 96-well plates in culture medium in triplicate wells. After overnight incubation, cells was treated with SAHA or SB and incubated for 72 h. Then, 20 μl MTS (Sigma, St Louis, MO, USA) were added. Plates were incubated at 37 °C for 4 h. The optical density (OD) was read at 490 nm using a microplate reader. Cell growth curves were calculated as mean values of triplicates per group.

Cell cycle analysis

Flow cytometry was performed according to a standard protocol as previously described [12]. Following the incubation of cells in 10 cm dish overnight, SAHA at 5 μM as well as SB at 5 mM final concentration were added to the culture media for 72 h. Cells were harvested with Trypsin-EDTA and washed with PBS, then centrifuged at 800r/min and fixed with ice-cold 70% ethanol kept at 4 °C overnight. Cells were incubated in reagent consisting of 0.5% Triton X-100, 250 μg/ml RNase A and 50 μg/ml propidium iodide in PBS for 30 min at 37 °C. Then, they were detected by flow cytometry analysis (Becton Dickinson FACScan, CA, USA).

Western blotting

Western blotting analysis was carried out as described previously [12]. Equal amount of proteins (20 μg) were loaded onto 10% or 15% SDS-PAGE gel and transferred to PVDF membranes (Millipore, MA). The membranes were blocked with 5% non-fat dry milk in TBST containing 0.1% Tween-20 and subsequently incubated with appropriate antibodies. The immunoreactive protein complexes were detected by enhanced chemiluminescence (Amersham Bioscience, Boston, MA). β-actin served as a control for equivalent protein loading. The specific antibodies used were: anti-acetylated H3 lysine 9,14 antibody (Millipore, 06-599, 1 μg/ml), anti-H3 antibody (Millipore, 05-499, 1.0 μg/ml), appropriate HRP conjugated secondary antibodies (Santa Cruz).

Animal studies

The animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee at the Affiliated First Hospital of Shanghai Jiaotong University. The nude mice were housed in standard cages with food and water provided ad libitum. A total of 3×10⁶ SaOS2 cells in the logarithmic growth phase were injected subcutaneously into the flanks of male athymic nude mice. Treatment was initiated about 2 weeks after injection of SaOS2 cells when tumors had reached 100 mm³. SAHA (2.0 mg/kg) was dissolved in DMSO and SB (100 mg/kg) was dissolved in saline, where 0.1% DMSO and saline were vehicle controls, respectively. The mice were subjected to intraperitoneal injection of vehicle, SAHA or SB daily for 4 weeks. During the treatment period, the mice were monitored for any signs of toxicity including body weight loss (measured twice/week) or lack of activity.

Statistics

Data were expressed as mean ± SD. All statistical analyses were performed using SPSS 10.0. Significant differences among groups were determined by one-way analysis of variance (ANOVA) and p<0.05 was considered statistically significant.

Results

Effects of HDACIs on the growth of human osteosarcoma cells

In order to investigate whether HDACIs affects the proliferation of human osteosarcoma cells, SaOS2 and U2OS cells were exposed to increasing concentrations of SAHA (0–625 μM) and SB (0–625 mM). MTS assays were performed to evaluate the effects of HDACIs on growth of osteosarcoma cells. Cell proliferation assay was monitored for 72 h after HDACIs treatment. Compared with vehicle-treated cells, treatment with SAHA or SB suppressed the growth of SaOS2 and U2OS.
cells in a dose-dependent manner (p<0.01; Figure 1a). Similar results were seen in SaOS2 and U2OS cells treated with SB (p<0.01; Figure 1b). The effects of HDACIs on histone H3 acetylation were examined by western blotting assay. As shown in Figure 2a, histone H3 acetylation was increased in SaOS2 and U2OS cells treated with SAHA compared with vehicle-treated cells (p<0.01, compared with vehicle-treated SaOS2 cells; p<0.001 compared with vehicle-treated U2OS cells). Similar changes were seen in SaOS2 and U2OS cells treated with SB (p<0.01; Figure 2b).

**Effects of HDACIs on cell cycle of human osteosarcoma cells**

To evaluate the effects of HDACIs on cell cycle, flow cytometry analysis was performed to...
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**Figure 3.** Analysis of the effects of HDACIs on human osteosarcoma cell cycle progression. (a,b) The G1 and G2/M phase fraction of the SAHA treated SaOS2 and U2OS cells were markedly increased compared with the vehicle-treated cells. (c,d) The G2/M phase fraction of the SB-treated SaOS2 and U2OS cells was markedly increased compared with the vehicle-treated cells. Data are presented as mean ± standard error of the mean *P* <0.01, #P <0.001, compared with vehicle-treated human osteosarcoma cells.

**Figure 4.** The antitumor activity of HDACIs against human osteosarcoma cells in nude mice. (a) SAHA (2.0 mg/kg) significantly inhibits the tumor growth of SaOS2 (p<0.01). (b) SB (50 mg/kg) significantly inhibits the tumor growth of SaOS2 (p<0.01). Tumor volumes are presented as mean ± standard error of the mean.
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examine whether cell cycle arrest was induced by treating human osteosarcoma cells with HDACIs. Consistent with the MTS assay, SAHA (5 μM) induced a G1 and G2/M phase cell cycle arrest in SaOS2 and U2OS cells and the percentage of cells treated with SAHA in the G1 and G2/M phase significantly increased, while the percentage in the S phase decreased significantly compared with the vehicle-treated cells (Figure 3a and 3b; p<0.01 or p<0.001). Analysis of cell cycle distribution after SB (5 mM) treatment in SaOS2 and U2OS cells revealed an increase in the percentage of G2/M phase cells, while producing a concomitant fall in the percentage of G1 and S phase cells (Figure 3c and 3d; p<0.01 or p<0.001). There results indicated that the cytostatic effect of HDACIs is characterized by block of the cell cycle in the G1 and/or G2/M phase and suggested that HDACIs lowered cell growth rates probably via cell cycle arrest.

In vivo antitumor effects of HDACIs on human osteosarcoma cells

The mice treated with SAHA (2.0 mg/kg) showed markedly suppressed tumor growth compared with the vehicle-treated control (Figure 4a; p<0.01). Similarly, the tumor volume of mice treated with SB (100 mg/kg) was also significantly decreased compared with the vehicle-treated group (Figure 4b; p<0.01). No significant differences in body weight or activity were detected between SAHA or SB and the vehicle-treated group (data not shown).

Discussion

As one of the most heterogeneous human bone tumors, osteosarcoma generally affects adolescents under the age of 20 [17]. Osteosarcoma accounts for 7% of the annual incidence of all primary bone malignancies and is a highly metastatic tumor [18,19]. Patients who present with metastatic disease at diagnosis have poor prognosis with 5-year survival rate of only 20-30% [19,20]. An increasing body of evidence supported the notion that HDACIs play a critical role in cancer cell therapy and are novel promising antitumor agents [5,8,11,21-23]. For example, HDACi SAHA inhibited the proliferation of human breast cancer and colon cancer cell lines and SB suppressed the growth of neuroendocrine gastrointestinal tumor cells [22,24,25]. Considered as a group of promising targets for cancer therapy, HDACIs have been recently evaluated in clinical trials [26,27]. However, the effects of HDACi SAHA and SB on human osteosarcoma cells have not been investigated. The purpose of this study was to evaluate the effect of HDACIs SAHA and SB on human osteosarcoma in vitro and in vivo. First, we sought to determine whether HDACIs SAHA and SB might affect the ability of the cell proliferation via MTS and flow cytometry assays. Our results showed that treatment with SAHA or SB caused a dose-dependent decrease in the proliferation for SaOS2 and U2OS cells. Consistent with this, flow cytometry analysis revealed that SaOS2 and U2OS cells treated with SAHA underwent cell cycle arrest in G1 and G2/M phase, while SaOS2 and U2OS cells treated with SB underwent cell cycle arrest in G2/M phase. The inhibition of cell growth was accompanied by cell cycle arrest.

Collectively, the in vitro data described above indicated that SAHA and SB were potent inhibitors of human osteosarcoma cell proliferation. Considering the inhibitory effect on cell growth and induction of cell cycle arrest by HDACIs, we predicted that HDACIs may have therapeutic potential in blocking the growth of human osteosarcoma in vivo. To model this situation, mice xenograft models were introduced and the antitumor activity of HDACIs was examined in subcutaneous tumors of SaOS2 cells developed in nude mice. We found HDACIs SAHA and SB significantly inhibited the growth of SaOS2 tumor cells with no noticeable effect on body weight and activity of mice, proving a beneficial effect of HDACIs on human osteosarcoma therapy.

In conclusion, our data convincingly showed that HDACIs SAHA and SB treatment had a favorable effect on the proliferation of osteosarcoma cells both in vitro and in vivo. We believe that the mechanism of HDACIs antitumor activity needs further investigations.

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References


