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ORIGINAL ARTICLE

Histone deacetylase inhibitors repress chondrosarcoma cell proliferation

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Summary

Purpose: *Due to the high resistance to conventional therapy, there is still no convincingly effective treatment for chondrosarcoma. As a promising new treatment strategy, histone deacetylase inhibitors (HDACIs) have been reported to induce cell arrest, apoptosis and differentiation in some kinds of malignancies, but how HDACi exert their effects on chondrosarcoma is not well understood yet.*

Methods: *We investigated the effects of HDACIs trichostatin A (TSA) and sodium valproate (VPA) on chondrosarcoma cells in vitro and in vivo. The cell proliferation and cell cycle were examined in two chondrosarcoma cell lines, SW1353 and JJ012, by MTS and flow cytometry assays, respectively. The in vivo effects of HDACIs were investigated by assessing the chondrosarcoma growth in a mouse xenograft model.*

Results: *Our results showed that TSA and VPA significantly repressed the proliferation of chondrosarcoma cells in a concentration-dependent manner. Flow cytometry indicated that TSA arrested the cell cycle in G2/M phase and VPA arrested the cell cycle in G1 phase. The tumor growth was markedly suppressed in mice treated with TSA and VPA.*

Conclusions: *HDACIs significantly repress the proliferation of chondrosarcoma cells in vitro and in vivo. Our findings imply that HDACIs may provide a novel therapeutic target for the treatment of chondrosarcoma.*

Key words: *chondrosarcoma, HDACIs, proliferation, TSA, VPA*

Introduction

Chondrosarcoma is the second most common malignant primary bone sarcoma characterized by its poor response to currently used chemotherapy and radiotherapy and surgical resection is currently the only primary treatment modality [1]. The prognosis of chondrosarcoma is very poor and the 5-year survival rate has not increased over the last several decades, being only about 10 to 25% [2]. Due to lack of effective therapy, high mortality accompany patients with chondrosarcoma [3]. Therefore, new therapeutic strategies are necessary to increase survival in patients with chondrosarcoma [4].

As a novel class of promising anticancer agents approved for clinical use, HDACIs have been reported to increase histones acetylation, which is required for facilitation of gene transcription, and to inhibit cell proliferation, invasion, angiogenesis, and metastasis, while they induce cell cycle arrest, differentiation and apoptosis in many kinds of cancer cells [5-10]. However, the roles of HDACIs in chondrosarcoma have been rarely reported in the past. Previous studies revealed that HDACIs block cartilage resorption and decrease collagen- and gelatin-degrading proteolytic activities [11]. Recent reports show that HDACi induce apoptosis, differentiation and autophagy-associated cell death in chondrosarcoma cells [12,13]. Though studies have confirmed that HDACIs exert antitumor effects on chondrosarcoma, still little research has been reported since. Therefore, a better understanding of the effect and mechanism of HDACIs on chondrosarcoma is needed.

The purpose of the present study was to investigate the anticancer roles of HDACIs TSA and VPA in chondrosarcoma cells. We were therefore prompted to explore the effects of HDACIs on the proliferation of chondrosarcoma cells *in vitro* and *in vivo*.

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Methods

Cell culture and reagents

The human chondrosarcoma cell lines JJ012 and SW1353 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in an atmosphere with 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 10 µg/ml gentamycin. TSA and VPA were purchased from Sigma (St.Louis, USA) and dissolved in dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) respectively, with a stock concentration of 1 mM and 1 M and stored at–80 °C until use.

Cell proliferation assay

Cell proliferation assay were performed by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. Cells were seeded onto 96-well plates at a density of 3-5×10^4 in triplicate wells and incubated overnight for attachment. Then, cells were treated with TSA (0–62.5 µMoL), VPA (0–625 mMoL) or 0.1% DMSO as control and incubated for 48 hrs. After that, cells were stained with 20 µl MTS (Sigma, USA) at 37 °C for 4 hrs. Absorbance at 490 nm was measured using a microplate reader (BioRad, USA). All data derived from at least 3 independent experiments and repeated for three times.

Flow cytometry

After treated with HDACi (0.8 µMoL TSA or 5 mMoL VPA) for 48 hrs, chondrosarcoma cells were harvested with Trypsin-EDTA, washed with PBS and fixed with 70% cold ethanol for 30 min at 4 °C. Cells were then incubated with 50 µg/ml propidium iodide solution and 50 units of RNase for 30 min at 37 °C. The stained cell nuclei were detected by flow cytometry analysis (Becton Dickinson FACScan, CA, USA).

Western blotting

Cells treated with HDACIs were solubilized and equal amount of proteins were loaded on SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked with 4% BSA for 1 h at room temperature, and then incubated with appropriate antibodies. The signals were detected by enhanced chemiluminescence (Amersham Bioscience, Boston, MA). The signal strength of each blot band was normalized to β-actin and quantified using ImageJ software (Natl Institutes of Health, USA). The antibodies used were: acetylated H3 lysine 9,14 antibody (Millipore), H3 antibody (Millipore), appropriate HRP conjugated secondary antibodies (Millipore).

Animal studies

Animal experimentation was approved by the Institutional Animal Care and Use Committee at the Affiliated First Hospital of Shanghai Jiaotong University. JJ012 and SW1353 cells were harvested from the monolayer cultures at 80% confluence, and resuspended in DMEM at 1×10^6 and 1×10^7 in 0.05 ml, respectively. Then, cells were subcutaneously injected into the flanks of nude mice. Treatment was initiated about 3 to 4 weeks after injection when tumors had reached about 100 mm^3. The mice were administered an intraperitoneal injection of vehicle (0.1% DMSO or PBS), 2 mg/kg TSA or 400 mg/kg VPA daily for a total treatment period of 21 days. Tumor volume and body weight were measured once every 3 days using vernier calipers and scale. The formula of tumor volume was: $width^2$×length×0.52.

Statistics

Data were expressed as mean±standard error of the mean (SEM). Student's t-test was used for all statistical analyses. Differences were considered as significant if $p < 0.05$.

Results

HDACIs reduce proliferation of human chondrosarcoma cell lines

To examine the growth inhibitory effect of HDACIs on human chondrosarcoma cells, JJ012 and SW1353 cells were treated with various concentrations of TSA and VPA for 48 hrs and evaluated by MTS assay to determine the drug effect on cell proliferation. As shown in Figure 1a, the growth of JJ012 and SW1353 cells treated with TSA was significantly inhibited in a dose-dependent manner compared with vehicle-treated cells. Similarly, cells treated with VPA also had a profound dose-dependent inhibition of cell growth (Figure 1b). To investigate the activities of HDACIs, acetylated histone H3 of chondrosarcoma cells was detected using Western blotting assay after 48 hrs incubated with 0.8 µMoL TSA and 5 mMoL VPA. Compared with vehicle-treated cells, an increase of acetylated histone H3 was found in JJ012 and SW1353 cells treated with TSA (Figure 1c). Similar results were seen in JJ012 and SW1353 cells treated with VPA (Figure 1d).

HDACIs induce cell cycle arrest in human chondrosarcoma cells

After establishing that HDACIs inhibit cell proliferation in human chondrosarcoma cells, we further investigated whether the growth inhibitory effect was associated with cell cycle arrest using DNA flow cytometric analyses. For this purpose, the effect of HDACIs on cell cycle progres-

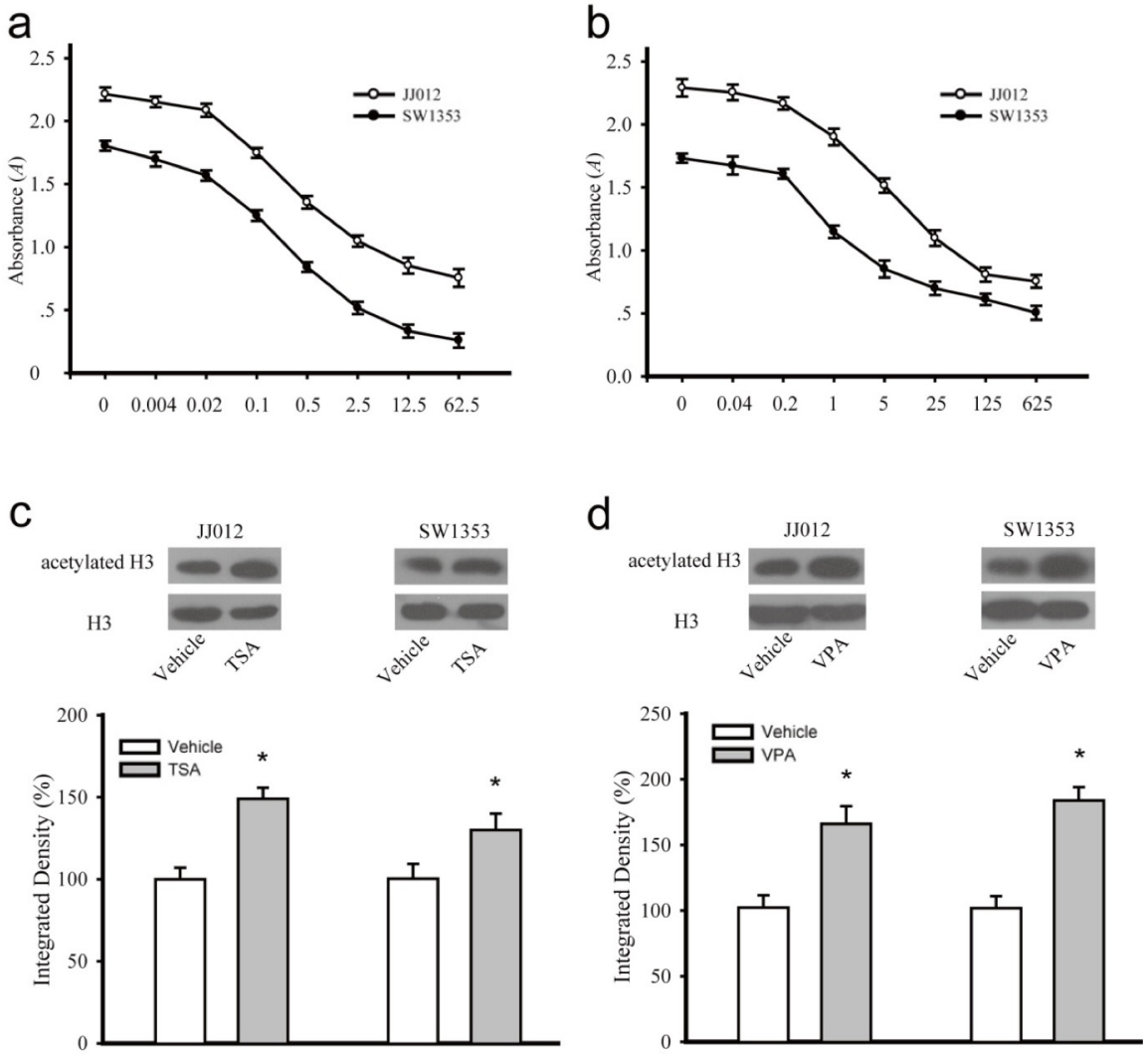

Figure 1. Antiproliferative effects of HDACi on human chondrosarcoma cells. TSA (a) and VPA (b) inhibited the proliferation of human chondrosarcoma cell lines JJ012 and SW1353 in a dose-dependent manner. (c) TSA treatment increased acetylated H3 in JJ012 and SW1353 cells. (d) VPA treatment increased acetylated H3 in JJ012 and SW1353 cells. Data are presented as mean ± standard error of the mean; * p <0.01, compared with vehicle-treated cells.

sion in chondrosarcoma cells was examined following treatment of cells incubated with HDACIs for 48 hrs. TSA (0.8 μMoL) treatment of JJ012 and SW1353 cells resulted in a significant increase in the percentage of cells in S phase of the cell cycle, accompanied by a decrease in cells in the G2/M phase (Figure 2a and 2b, p<0.01). Meanwhile, JJ012 and SQ1353 cells treated with VPA (5 mMoL) revealed an increase in the percentage of G1 phase cells with a concomitant fall in the percentage of S phase cells (Figure 2b and 2d, p<0.01). These results indicate that HDACI TSA induces S phase arrest while VPA induces G2/M arrest in human chondrosarcoma cells.

HDACIs inhibit the growth of chondrosarcoma in vivo

Based on the promising *in vitro* results obtained above, we finally evaluated the *in vivo* efficacy of HDACIs in the chondrosarcoma xenograft model. Mice bearing established chondrosarcoma tumors were treated with TSA, VPA or vehicle once a day for 21 days. Our results showed that there was no significant difference in the average body weight of mice among distinct groups with no visible sign of toxicity and the mice treated with HDACIs did not exhibit any abnormal behavior (data not shown). Mice treated with TSA and VPA showed statistically significant reduction in tumor volumes compared with vehicle-treated control group mice established with JJ012 cells (Figure 3a and 3c, p<0.01). Similar results were also obtained in the tumor volumes of mice established with SW1353 cells (Figure 3b and 3d, p<0.01). These results suggested that intraperitoneal administration of HDACIs leads to effective

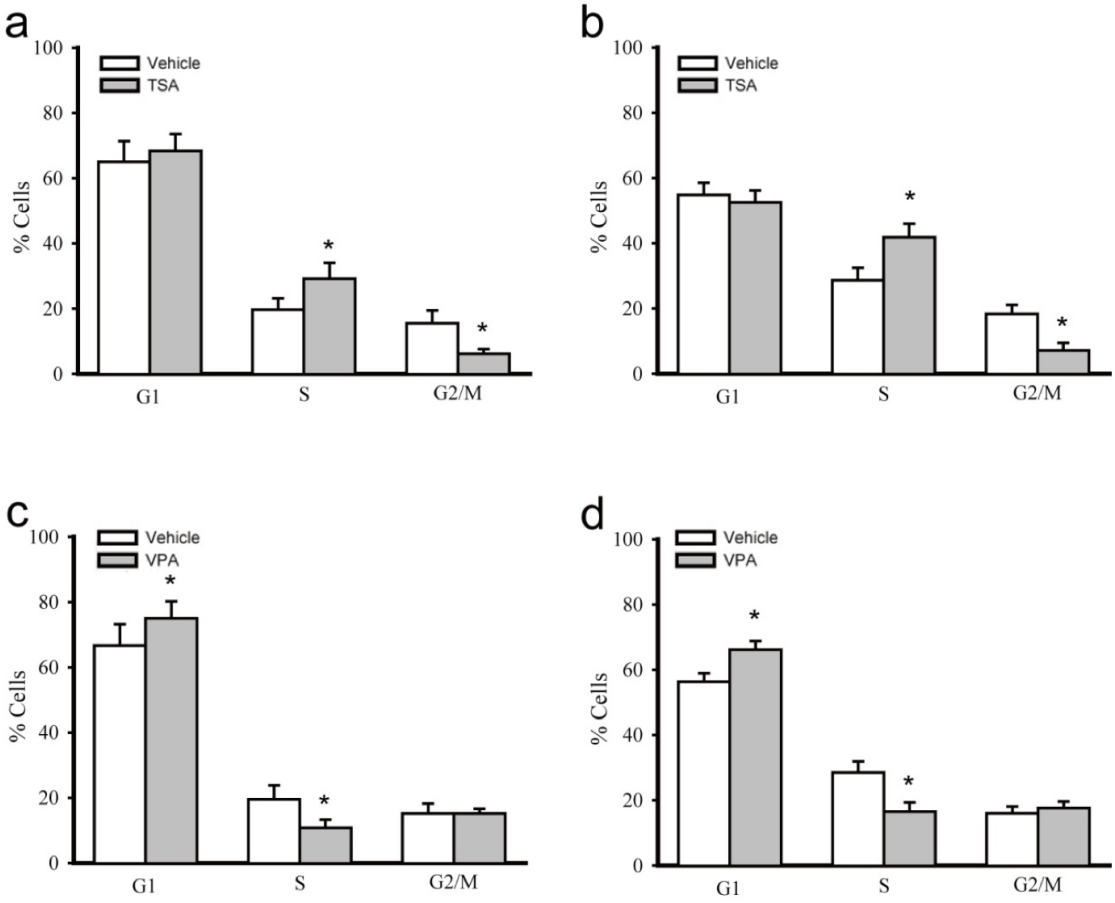

Figure 2. HDACi promoted cell cycle arrest. TSA treatment led to a significant increase in the proportion of cells in the S phase in JJ012 (a) or SW1353 (b) cells. VPA treatment led to a significant increase in the proportion of cells in the G1 phase in JJ012 (c) or SW1353 (d) cells. Data are presented as mean ± standard error of the mean; * p <0.01, compared with vehicle-treated groups.

inhibition of tumor growth in chondrosarcoma cells *in vivo*.

Discussion

As a heterogeneous and malignant primary bone and soft tissue disease that responds poorly to both chemotherapy and radiation therapy, there are no effective therapeutic options for patients with chondrosarcomas [4,14-16]. Simultaneously, since chondrosarcoma has the capacity to invade locally and create distant metastasis, it is important to develop effective adjuvant therapies after primary surgical therapy for preventing its progression [17]. A great deal of evidence has certified that HDACIs can induce growth arrest, apoptosis or differentiation of various kinds of cancer cells including osteosarcoma, glioma, prostate, breast, pancreas, lung, colon and gastric cancer cells [18-26]. HDACI treatment may also represent a promising strategy for overcoming the limitations in chondrosarcoma therapy.

To this day, there are only few reports about how histone deacetylases affect chondrosarcoma cells. Sun et al. found that HDAC4 overexpression decreases the angiogenic activity of chondrosarcoma cells [27]. Conversely, other studies demonstrated that HDAC PCI-24781 inhibits the growth of chondrosarcoma cells and induces expression of histone acetylation, p21and PARP cleavage [28,29]. Considering previous studies, no investigations are to be found concerning the role of HDACIs TSA and VPA in human chondrosarcoma cells. The aim of the present study was to evaluate the effect of HDACIs TSA and VPA on human chondrosarcoma *in vitro* and *in vivo*. We reported for the first time the novel finding that chondrosarcoma cells treated with HDACIs TSA and VPA showed a concentration-dependent decrease in cell proliferation. To further investigate the antiproliferative mechanism of HDACIs, Western blotting and flow cytometric analysis of chondrosarcoma cells following treatment with HDACIs TSA and VPA were performed. In agreement with the discovery that

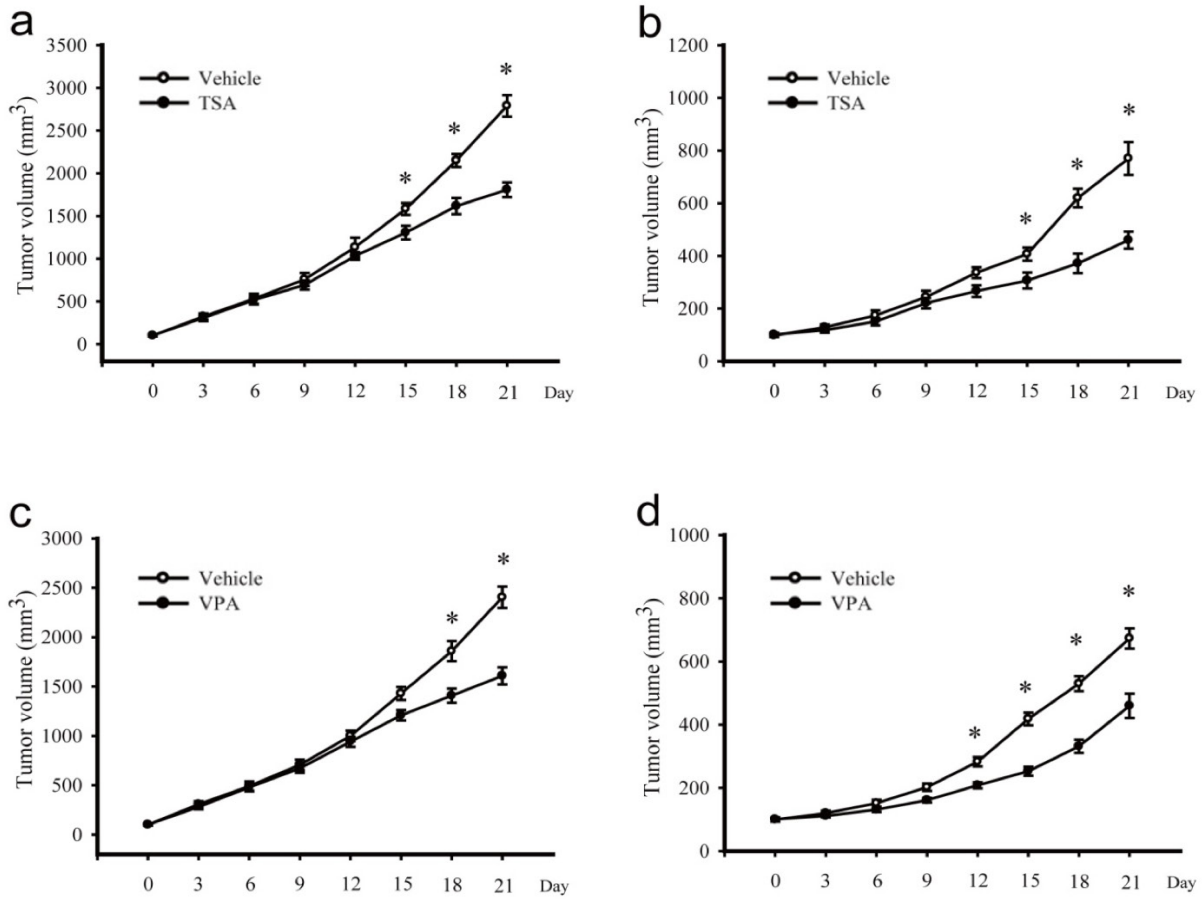

Figure 3. Effects of HDACi on tumor growth of chondrosarcoma xenografts in vivo. A significant reduction in tumor growth was observed in TSA (2.0 mg/kg) -treated mice established with JJ012 (a) or SW1353 (b) cells compared with vehicle-treated groups. A significant reduction in tumor growth was also observed in VPA (400 mg/kg) -treated mice established with JJ012 (c) or SW1353 (d) cells compared with vehicle-treated groups. Data are presented as mean ± standard error of the mean; * p <0.01, compared with vehicle-treated groups.

HDACI TSA inhibited the growth of human chondrosarcoma cells, acetylated histone H3 increase and cell cycle arrest in the S phase were also found in chondrosarcoma cells treated with TSA; meanwhile, histone H3 acetylation increase and cell cycle arrest in the G1 phase was also discovered. Therefore, we considered that the suppression of HDACIs on human chondrosarcoma cells proliferation might be the result of acetylation of histone H3 induced by HDACI treatment, leading further to cell cycle arrest. The *in vitro* inhibitory effects of HDACI on chondrosarcoma cells were also validated by the *in vivo* findings of the effective antitumor action of HDACIs. Treatment of chondrosarcoma cells with HDACIs TSA and VPA decreased the tumor volumes with no measurable toxicities (data not shown) observed in the drug-related groups. These results are consistent with previous findings on growth inhibition of tumor xenografts by HDACIs suberoylanilide hydroxamic acid and depsipeptide [12,13].

The results presented in this report revealed the growth inhibitory effects of HDACIs on human chondrosarcoma cells *in vitro* and *in vivo*. The results of the current study underscore the necessity of research of HDACIs in chondrosarcoma and future work should be directed towards the anticancer mechanism of HDACIs.

Acknowledgments

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