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ORIGINAL ARTICLE

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 of chemotherapeutic 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 anititumor 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% CO_2 saturated humidity, at 37 ^{0}C. Histone deacetylase inhibitors SAHA and SB were purchased from Sigma (USA) and Sangon Biotech, Co., Ltd (Shanghai, China), respectively. SAHA was dissolved in DMSO for a stock concentration of 20 mM. SB was dissolved in saline for a stock concentration of 20 mM.

Cell proliferation assay

Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay according 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 ^{0}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 µMoL 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 ^{0}C overnight. Cells were incubated in reagent consisting of 0.5% Triton X-100, 230 µg/ml RNase A and 50 µg/ml propidium iodide in PBS for 30 min at 37 ^{0}C, then 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 3×10^{6} 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 mm^3. 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. Tumors were measured using a vernier caliper and tumor volume was determined twice weekly using the formula: $V=length\times width^2 / 2$. 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 µMoL) and SB (0–625 mMoL). 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

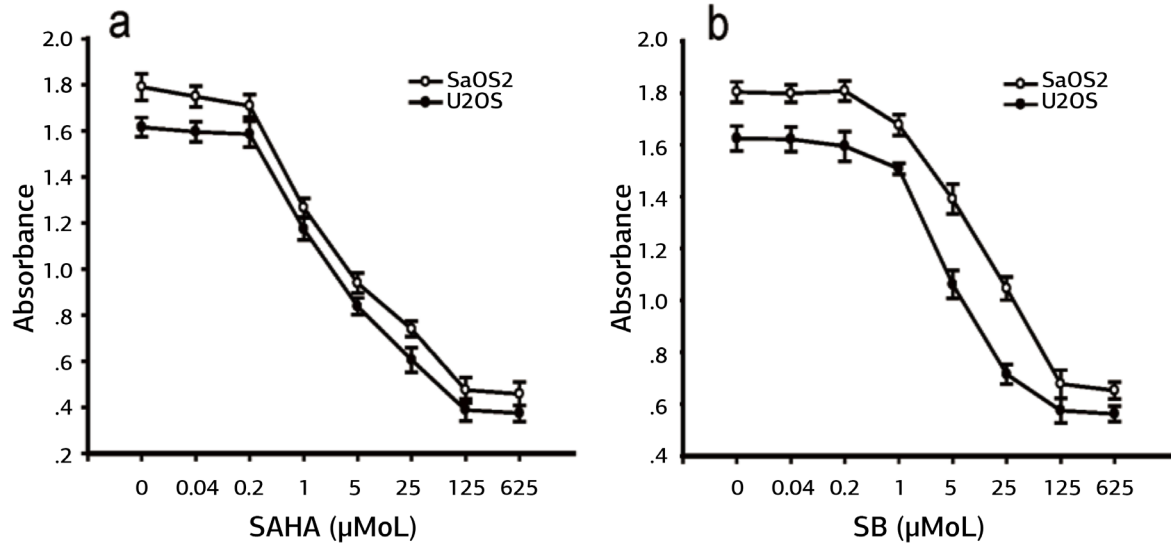

Figure 1. Human osteosarcoma cells proliferation was evaluated by MTS assays. Cells were seeded at a density of 1000 (SaOS2) or 3000 (U2OS) cells per well of 96-well plate and treated with SAHA or SB for 72 hours. **(a)** SAHA and **(b)** SB significantly suppressed the growth of SaOS2 and U2OS osteosarcoma cells (p<0.01).

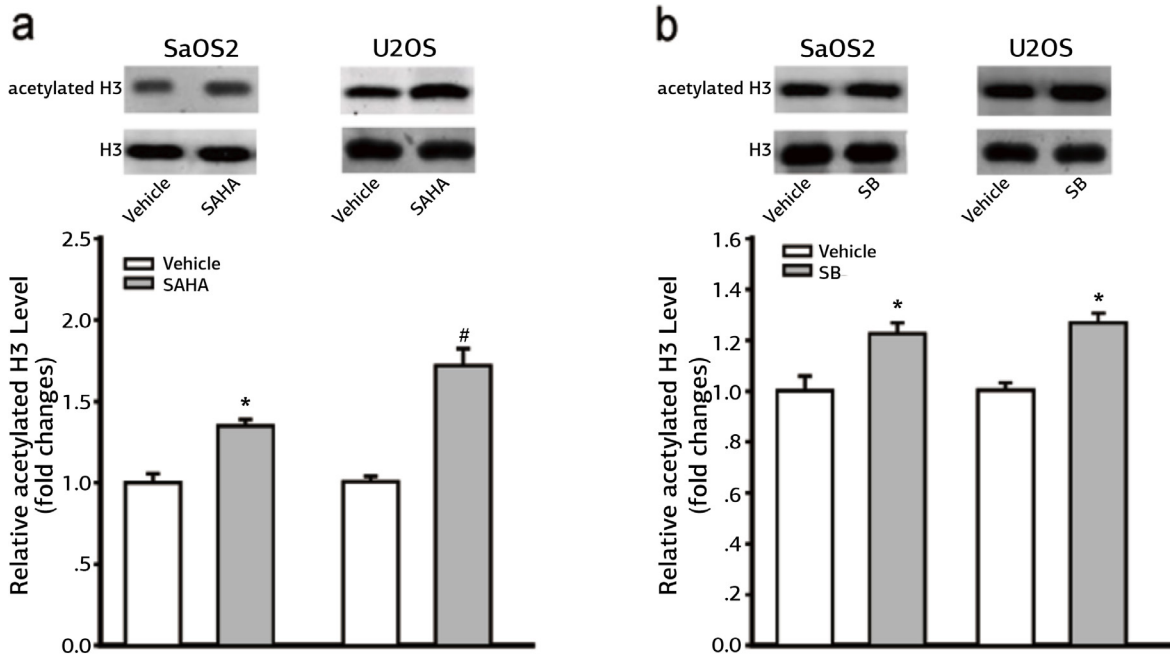

Figure 2. The levels of acetylated H3 increased after HDACIs treatment for 72 hours in human osteosarcoma cells. **(a)** SAHA treatment increased acetylated H3 in SaOS2 and U2OS cells. **(b)** SB treatment increased acetylated H3 in SaOS2 and U2OS cells. Data are presented as mean ± standard error of the mean
* p <0.01, # p <0.001, compared with vehicle treated human osteosarcoma cells.

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 the cell cycle, flow cytometry analysis was performed to

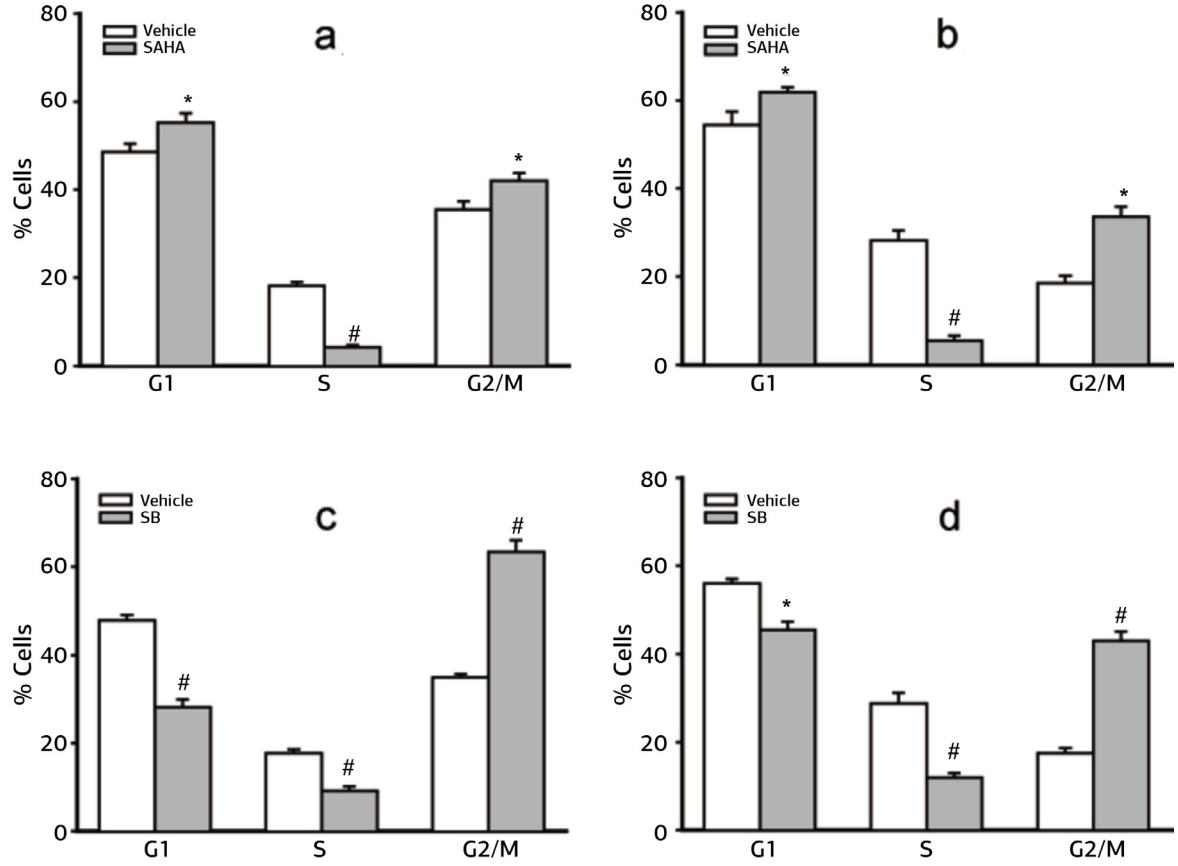

Figure 3. Analysis of the effects of HDACIs on human osteosarcoma cell cycle progression. **(a,b)** The G1 and G2/M phase fraction of the SAHA treated SaOS2 and U2OS cells were markedly increased compared with the vehicle-treated cells. **(c,d)** The G2/M phase fraction of the SB-treated SaOS2 and U2OS 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

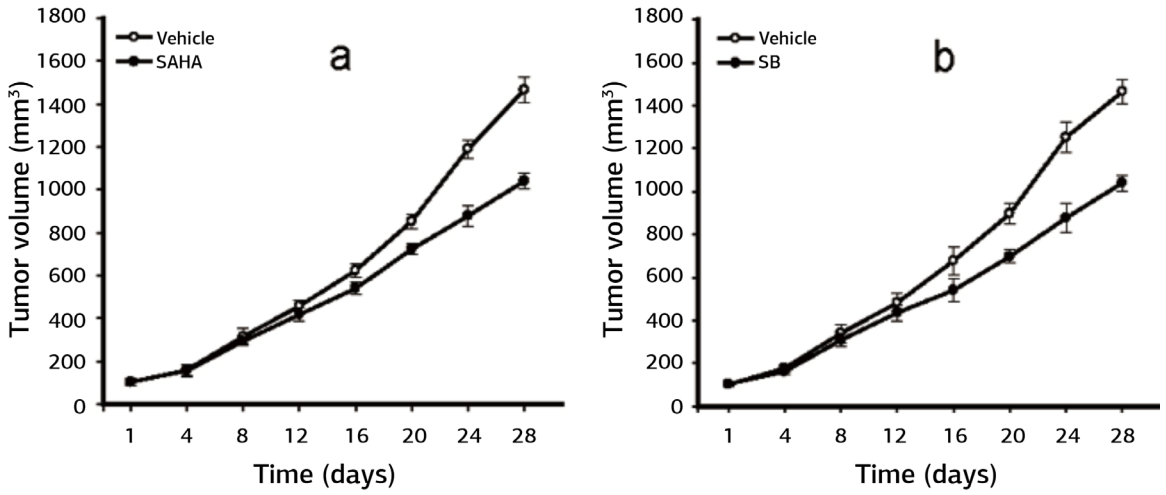

Figure 4. The antitumor activity of HDACIs against human osteosarcoma cells in nude mice. **(a)** SAHA (2.0 mg/kg) significantly inhibits the tumor growth of SaOS2 ($p<0.01$). **(b)** SB (50 mg/kg) significantly inhibits the tumor growth of SaOS2 ($p<0.01$). Tumor volumes are presented as mean ± standard error of the mean.

examine whether cell cycle arrest was induced by treating human osteosarcoma cells with HDACIs. Consistent with the MTS assay, SAHA (5 μMoL) 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 mMoL) 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 xenograph 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

1. Roh MS, Kim CW, Park BS et al. Mechanism of histone deacetylase inhibitor Trichostatin A induced apoptosis in human osteosarcoma cells. Apoptosis 2004;9:583-589.
2. Wittenburg LA, Bisson L, Rose BJ, Korch C, Thamm DH. The histone deacetylase inhibitor valproic acid sensitizes human and canine osteosarcoma to doxorubicin. Cancer Chemother Pharmacol 2011;67:83-92.
3. Koshkina NV, Rao-Bindal K, Kleinerman ES. Effect of the histone deacetylase inhibitor SNDX-275 on Fas signaling in osteosarcoma cells and the feasibility of its topical application for the treatment of osteosarcoma lung metastases. Cancer 2011;117:3457-3467.
4. Hamada K, Tomita Y, Inoue A et al. Evaluation of chemotherapy response in osteosarcoma with FDG-PET. Ann Nucl Med 2009;23:89-95.
5. Yang C, Choy E, Hornicek FJ et al. Histone deacetylase inhibitor (HDACI) PCI-24781 potentiates cytotoxic effects of doxorubicin in bone sarcoma cells. Cancer Chemother Pharmacol 2011;67:439-446.
6. Hancock MH, Cliffe AR, Knipe DM, Smiley JR. Herpes simplex virus VP16, but not ICP0, is required to reduce histone occupancy and enhance histone acetylation on viral genomes in U2OS osteosarcoma cells. J Virol 2010;84:1366-1375.
7. Palmieri C, Coombes RC, Vigushin DM. Targeted histone deacetylase inhibition for cancer prevention and therapy. Prog Drug Res 2005;63:147-181.
8. Maeda T, Nagaoka Y, Kawai Y et al. Inhibitory effects of cancer cell proliferation by novel histone deacetylase inhibitors involve p21/WAF1 induction and G2/M arrest. Biol Pharm Bull 2005;28:849-853.
9. Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer:causes and therapies. Nat Rev Cancer 2001;1:194-202.
10. Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. J Cell Physiol 2000;184:1-16.
11. Lopez G, Liu J, Ren W et al. Combining PCI-24781, a novel histone deacetylase inhibitor, with chemotherapy for the treatment of soft tissue sarcoma. Clin Cancer Res 2009;15:3472-3483.
12. Zhao Q, Wang C, Zhu J et al. RNAi-mediated knockdown of cyclooxygenase2 inhibits the growth, invasion and migration of SaOS2 human osteosarcoma cells:a case control study. J Exp Clin Cancer Res 2011;30:26.
13. Ellis L, Atadja PW, Johnstone RW. Epigenetics in cancer:targeting chromatin modifications. Mol Cancer Ther 2009;8:1409-1420.
14. Brehm A, Nielsen SJ, Miska EA et al. The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. EMBO J 1999;18:2449-2458.
15. Sandor V, Senderowicz A, Mertins S et al. P21-dependent g(1)arrest with downregulation of cyclin D1 and upregulation of cyclin E by the histone deacetylase inhibitor FR901228. Br J Cancer 2000;83:817-825.
16. Maeda T, Towatari M, Kosugi H, Saito H. Up-regulation of costimulatory/adhesion molecules by histone deacetylase inhibitors in acute myeloid leukemia cells. Blood 2000;96:3847-3856.
17. Lee EJ, Choi EM, Kim SR et al. Cyclooxygenase-2 promotes cell proliferation, migration and invasion in U2OS human osteosarcoma cells. Exp Mol Med 2007;39:469-476.
18. Urakawa H, Nishida Y, Naruse T, Nakashima H, Ishiguro N. Cyclooxygenase-2 overexpression predicts poor survival in patients with high-grade extremity osteosarcoma:a pilot study. Clin Orthop Relat Res 2009;467:2932-2938.
19. Bacci G, Longhi A, Versari M, Mercuri M, Briccoli A, Picci P. Prognostic factors for osteosarcoma of the extremity treated with neoadjuvant chemotherapy:15-year experience in 789 patients treated at a single institution. Cancer 2006;106:1154-1161.
20. Naruse T, Nishida Y, Hosono K, Ishiguro N. Meloxicam inhibits osteosarcoma growth, invasiveness and metastasis by COX-2-dependent and independent routes. Carcinogenesis 2006;27:584-592.
21. Okada T, Tanaka K, Nakatani F et al. Involvement of P-glycoprotein and MRP1 in resistance to cyclic tetrapeptide subfamily of histone deacetylase inhibitors in the drug-resistant osteosarcoma and Ewing's sarcoma cells. Int J Cancer 2006;118:90-97.
22. Munster PN, Troso-Sandoval T, Rosen N, Rifkind R, Marks PA, Richon VM. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res 2001;61:8492-8497.
23. Hui KF, Chiang AK. Suberoylanilide hydroxamic acid induces viral lytic cycle in Epstein-Barr virus-positive epithelial malignancies and mediates enhanced cell death. Int J Cancer 2010;126:2479-2489.
24. Baradari V, Huether A, Hopfner M, Schuppan D, Scherubl H. Antiproliferative and proapoptotic effects of histone deacetylase inhibitors on gastrointestinal neuroendocrine tumor cells. Endocr Relat Cancer 2006;13:1237-1250.
25. Jin JS, Tsao TY, Sun PC, Yu CP, Tzao C. SAHA inhibits the growth of colon tumors by decreasing histone deacetylase and the expression of cyclin D1 and survivin. Pathol Oncol Res 2012;18:713-720.
26. Al-Janadi A, Chandana SR, Conley BA. Histone deacetylation :an attractive target for cancer therapy? Drugs R D 2008;9:369-383.
27. Blum KA, Advani A, Fernandez L et al. Phase II study of the histone deacetylase inhibitor MGCD0103 in patients with previously treated chronic lymphocytic leukaemia. Br J Haematol 2009;147:507-514.