Suberoylanilide hydroxamic acid enhances the antitumor activity of oxaliplatin by reversing the oxaliplatin-induced Src activation in gastric cancer cells

CHENFEI ZHOU[1], JUN JI[1], MIN SHI[1], LIU YANG[1], YINGYAN YU[1], BINGYA LIU[1], ZHENGGANG ZHU[1] and JUN ZHANG[1,2]

[1]Department of Surgery, Shanghai Institute of Digestive Surgery; [2]Department of Clinical Oncology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, P.R. China

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Abstract. Oxaliplatin and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA), also known as vorinostat, are potent antitumor agents. The aim of this study was to investigate the effect of SAHA on the antitumor efficacy of oxaliplatin in gastric cancer and the interaction between oxaliplatin and SAHA. Cell growth inhibition was evaluated using Cell Counting Kit-8 and colony formation assays. Xenografts established in nude mice were used to assess tumor growth *in vivo*. Western blot analysis was used to detect the expression of acetyl-histone H3, phosphorylated histone H2AX (γH2AX), B-cell lymphoma 2 (Bcl-2), cleaved caspase-3, cleaved poly (ADP-ribose) polymerase (PARP), phosphorylated- (p-) Src, Src, Akt and p-Akt in gastric cancer cells. The *in vitro* growth of SGC-7901, Hs746T and MKN28 gastric cancer cells was found to be dose-dependently inhibited by oxaliplatin and SAHA. Furthermore, combined treatment was observed to be more effective in inhibiting cancer cell growth and colony formation than monotherapy. Similar effects were found in the xenografts. A positive interaction was identified between oxaliplatin and SAHA (between-subject effects of oxaliplatin and SAHA, $P<0.001$). In addition, combined exposure to oxaliplatin and SAHA increased γH2AX expression and decreased Bcl-2 expression. The expression of cleaved caspase-3 and PARP was also increased with combination treatment. Oxaliplatin-induced Src phosphorylation was detected in gastric cancer cells, as we have previously reported. However, this effect was inhibited by SAHA. The oxaliplatin-induced Src phosphorylation was not impaired with Akt inhibition. In conclusion, oxaliplatin and SAHA exhibited a positive interaction when used in combination and were found to suppress gastric cancer cell survival and growth. The reversal of oxaliplatin-induced Src activation may be responsible for this positive interaction.

Correspondence to: Dr Jun Zhang, Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, P.R. China
E-mail: jun_zj10977@163.com

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Introduction

Gastric cancer is one of the most prevalent malignant diseases in China and is associated with a low early diagnosis rate and a high mortality rate (1). At present, radical surgery is the only potentially curative approach for this life-threatening disease. However, ~50% of patients are diagnosed at an unresectable stage due to locally far-advanced disease or distant metastasis (2).

Although chemotherapy has enhanced the survival rate of patients with advanced gastric cancer, the median overall survival rate remains poor (3). Combining the targeted agent trastuzumab with chemotherapy for the treatment of advanced gastric cancer has proven to be superior to chemotherapy alone and has been approved to treat human epidermal growth factor receptor 2-positive patients with gastric cancer (4). However, recent trials have revealed that other targeted agents, including bevacizumab, cetuximab and panitumumab, do not show potential for the front-line treatment of late-stage gastric cancer (5-7). Since the development of novel therapeutic candidates is limited, the combination of currently available agents that have shown theoretical or clinical efficacy may be a potential strategy for the treatment of patients with gastric cancer.

Oxaliplatin is a third-generation platinum complex with potent antitumor effects. Oxaliplatin has shown similar efficacy to cisplatin in the first-line treatment of advanced gastric cancer, as revealed by the Randomized ECF for Advanced and Locally Advanced Esophagogastric Cancer 2 study (8). Furthermore, the side-effects associated with oxaliplatin were moderate compared with those associated with cisplatin, and the drug was well tolerated (9). At present, oxaliplatin is widely used in the palliative and adjuvant treatment of gastric cancer (10). However, our previous findings demonstrated that the tyrosine kinase Src was activated following oxaliplatin

exposure in gastric cancer cells, which may serve as a potential mechanism of chemoresistance (11).

Histone deacetylase inhibitors (HDACIs) are a group of novel antitumor agents that target HDACs. The overexpression of HDACs, a group of enzymes that are responsible for the modification of lysine acetylation, is observed in various types of cancer, including gastric cancer, and participates in the regulation of malignant biological behaviors, including growth, resistance to apoptosis, angiogenesis and metastasis (12,13). Suberoylanilide hydroxamic acid (SAHA), also known as vorinostat, is the first HDACI to be approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma. Preclinical research has also demonstrated that SAHA may show antitumor activity in solid tumors (14,15). Recently, a phase I trial of SAHA in combination with cisplatin and capecitabine was conducted in patients with advanced gastric cancer. The median overall survival time was reported to be 18 months and the toxicity was manageable (16).

The promising findings for SAHA in combination with cisplatin suggest that SAHA may have potential in combination with oxaliplatin for the treatment of gastric cancer. The antitumor mechanisms of these two agents also support a potential synergistic effect (17-19). However, to date, the combination of oxaliplatin and SAHA in gastric cancer is yet to be investigated. Therefore, the aim of the present study was to investigate the antitumor effect of oxaliplatin and SAHA in gastric cancer and to explore the potential molecular mechanisms.

Materials and methods

Cell lines and cell culture. SGC-7901 and MKN28 gastric cancer cells were preserved in the Ruijin Hospital (Shanghai, China). The Hs746T cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium with 10% fetal calf serum at 37˚C and with 5% CO_2.

Reagents. SAHA (S1047; Selleckchem, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 mM. Oxaliplatin (Sanofi, Paris, France) was dissolved in 5% dextrose solution at a concentration of 10 mg/ml. Ly2940092 (Selleckchem) and dasatinib (Selleckchem) were dissolved in DMSO at concentrations of 20 and 100 mM, respectively. All reagents were divided into aliquots and stored at -80˚C.

Cell growth inhibition assay. A total of 5,000 cells/well were seeded onto 96-well plates and were allowed to adhere overnight. Various concentrations of oxaliplatin (2.5, 5, 10, 15, 20 and 25 μg/ml) or SAHA (0.5, 1, 2, 4, 6 and 8 μM) were added to the medium. DMSO solution was used as a blank control. After 48 h of treatment, the optical density of each well was detected using the Cell Counting Kit-8 assay and the survival rate was calculated. In the combination treatment experiments, the concentrations of oxaliplatin and SAHA were 5 μg/ml and 4 μM, respectively.

Colony formation assay. A total of 500 cells/well were seeded onto six-well plates. Oxaliplatin was added to the culture medium at a final concentration of 5 μg/ml and exposed for 3 h. The oxaliplatin was then washed off and cells were treated with 4 μM SAHA for 24 h. For monotherapy, the cells were incubated with either 5 μg/ml oxaliplatin for 3 h or 4 μM SAHA for 24 h. The cells were subsequently washed with fresh medium and allowed to grow for between 7 and 10 days. Cell colonies were fixed using 10% neutral formalin and stained using crystal violet. The number of colonies was counted at a low-power field using an Olympus BX50 Microscope (Olympus, Tokyo, Japan).

Subcutaneous xenografts. SGC-7901 cells were collected and diluted to a concentration of $1x10^7$ cells/ml. Twelve four-week-old male Balb/c nude mice (Institute of Zoology Chinese Academy of Sciences, Shanghai, China) were subcutaneously inoculated with $1x10^6$ cells and were randomly divided into four groups (control, oxaliplatin, SAHA and oxaliplatin plus SAHA). Treatment commenced when the length of the tumor nodules reached 4 mm. Either oxaliplatin (2.5 mg/kg every four days) or SAHA (50 mg/kg every two days) monotherapy, or combination therapy was administered using intraperitoneal injection. Intraperitoneal injection of phosphate-buffered saline (200 μl every two days) was administered to the control group. Mouse weight and tumor nodule size were measured following treatment. Xenograft volume (V) was calculated using the following formula: $V = (width)^2 \times length/2$. The study was approved by the ethics committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China.

Western blot analysis. Total cell protein was extracted using radioimmunoprecipitation assay lysis buffer (Beijing Solarbio Science and Technology Co., Ltd, Beijing, China) after 24 h exposure to mono- or combination therapy. Protein concentration was determined using a DC™ protein assay (Bio-Rad, Hercules, CA, USA). Samples containing 100 μg protein were separated using SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked using 5% skimmed milk for 1 h at room temperature and incubated overnight at 4˚C with the following primary antibodies: Anti-acetyl-histone H3, caspase-3, cleaved poly (ADP-ribose) polymerase (PARP), phosphorylated- (p-) Akt, Akt, p-Src (all 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), -B-cell lymphoma 2 (Bcl-2), Src and phosphorylated histone H2AX (γH2AX) (all 1:1,000; Epitomics Inc., Burlingame, CA, USA). β-actin (1:5,000; Sigma Aldrich, St. Louis, MO, USA) was used as a loading control. The membranes were then incubated with secondary antibodies (1:20,000; Li-Cor Biosciences, Lincoln, NE, USA) at room temperature for 1 h. The membranes were visualized using an infrared imaging system (Li-Cor Biosciences).

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for the statistical analyses. Quantitative data were analyzed using one-way analysis of variance. Factorial design analysis was used to analyze the interaction between oxaliplatin and SAHA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Growth inhibitory effect of oxaliplatin and SAHA in gastric cancer cells. SGC-7901, Hs746T and MKN28 gastric cancer

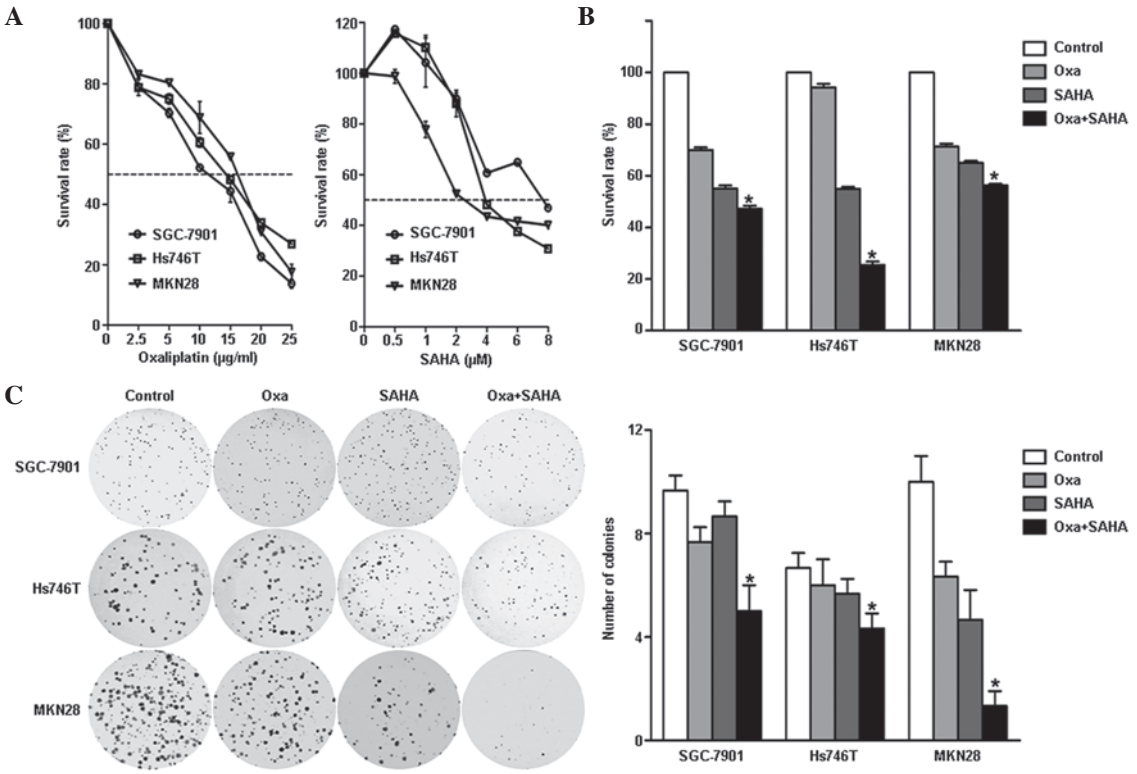

Figure 1. Oxa and SAHA inhibit gastric cancer cell growth *in vitro*. (A) Gastric cancer cells were treated with various doses of Oxa or SAHA for 48 h and cell survival was detected. (B) Gastric cancer cells were treated with Oxa or SAHA monotherapy or combination therapy for 48 h. (C) Colony formation was counted subsequent to treatment with Oxa and/or SAHA. *P<0.001 versus all other groups. Oxa, oxaliplatin; SAHA, suberoylanilide hydroxamic acid.

cells were treated with various doses of oxaliplatin or SAHA. A dose-dependent inhibition of cell growth was observed in each treatment group. Although the MKN28 cells were observed to be more sensitive to SAHA than the SGC-7901 and Hs724T cells, the survival rates of the three cell lines following treatment with 4 μM SAHA were all ~50% (Fig. 1A). Based on these findings, oxaliplatin and SAHA were used at concentrations of 5 μg/ml and 4 μM, respectively, in the subsequent experiments.

Oxaliplatin plus SAHA inhibits gastric cancer cell growth in vitro. Growth inhibition and colony formation assays were used to assess the inhibitory effect of oxaliplatin plus SAHA combination treatment. Cell survival was observed to be significantly impaired in the combination group compared with that in the monotherapy groups (Fig. 1B; P<0.001). Factorial design analysis revealed a positive interaction between oxaliplatin and SAHA in all cell lines (between-subject effects of oxaliplatin and SAHA, P<0.001). The colony formation assay showed that the number of colonies was significantly lower in the combination group than that in the other groups (Fig. 1C; P<0.001).

Oxaliplatin plus SAHA suppresses xenograft growth in vivo. SGC-7901 gastric cancer xenografts were established in nude mice, prior to the administration of oxaliplatin, SAHA or combination therapy. After two weeks of treatment, tumor growth was observed to be reduced in the mice in the mono- and combination therapy groups, compared with that in the control group (Fig. 2). The xenograft tumor volume was lower in the combination group than that in the oxaliplatin (622.2±79.3 vs. 1,680.0±291.8 mm^3, P=0.003) and SAHA (622.2±79.3 vs. 1,087.0±523.8 mm^3, P=0.098) groups. Furthermore, the xenograft tumor volume in the SAHA group was lower than that in the oxaliplatin group (1,087.0±523.8 vs. 1,680.0±291.8 mm^3, P=0.044). The body weight of the mice in the four groups was similar prior and subsequent to treatment (P>0.05; Fig. 2).

Oxaliplatin plus SAHA enhances DNA damage and cell apoptosis. The expression of acetyl-histone H3, γH2AX, Bcl-2, cleaved caspase-3 and cleaved PARP was assessed following oxaliplatin, SAHA or combination treatment using western blot analysis (Fig. 3). Acetyl-histone H3 expression was found to be elevated in the SAHA monotherapy and SAHA plus oxaliplatin treatment groups, while oxaliplatin monotherapy had no impact on the acetylation of histone H3. γH2AX expression was observed to be significantly upregulated in the combination group compared with that in the monotherapy groups, and Bcl-2 expression was reduced. Cleaved caspase-3 expression was elevated following combination treatment in Hs746T and MKN28 cells and PARP cleavage expression was increased in Hs746T cells.

SAHA inhibits oxaliplatin-induced Src phosphorylation. The phosphorylated forms of Src (Tyrosine 416) and Akt (Serine 473) were observed to be elevated following oxaliplatin exposure. However, SAHA and SAHA plus oxaliplatin treatments were found to significantly inhibit p-Src and p-Akt expression (Fig. 4).

SAHA has been reported to attenuate Akt activation, while its effect on Src activation remains unclear (20). In

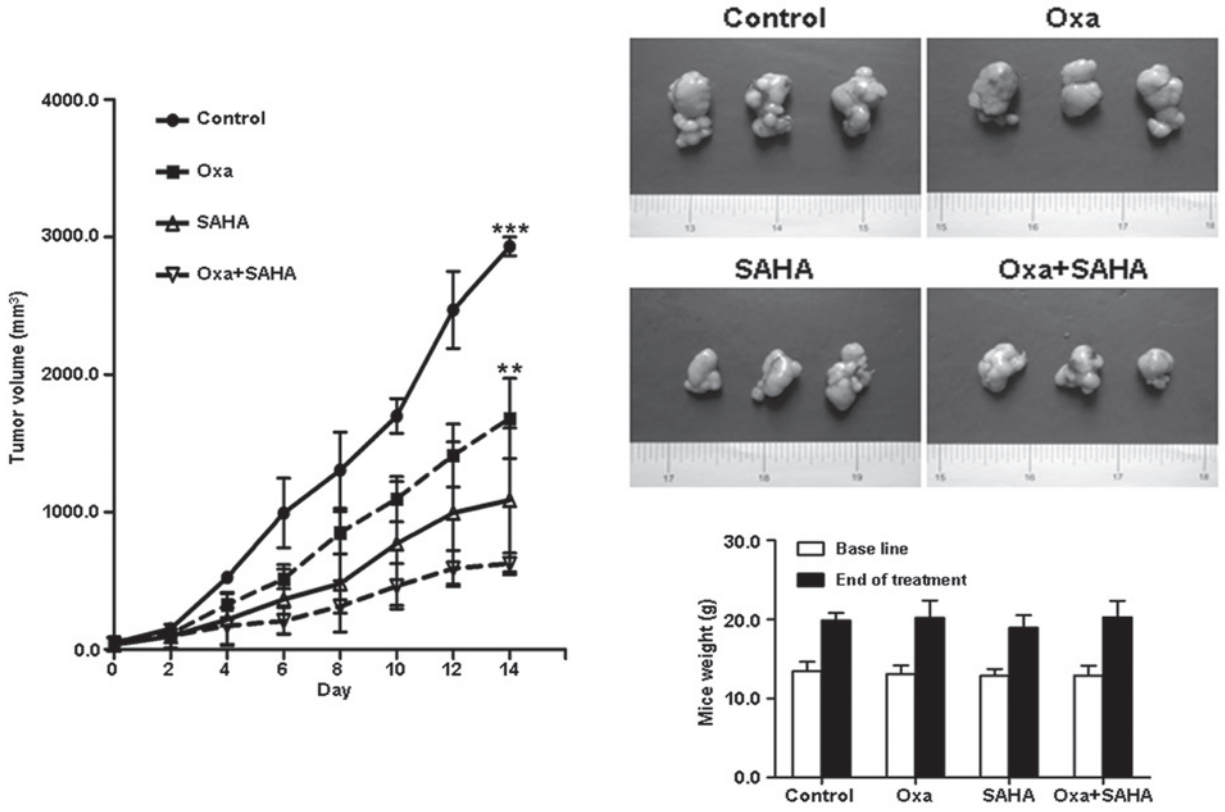

Figure 2. Oxa and SAHA suppress xenograft growth *in vivo*. Xenografts of SGC-7901 cells were established on the right-side of the backs of mice. Mice were treated with Oxa (2.5 mg/kg) or SAHA (50 mg/kg) monotherapy, or combination therapy for two weeks. Oxa, oxaliplatin; SAHA, suberoylanilide hydroxamic acid. **P<0.05 compared with the SAHA and Oxa+SAHA groups, ***P<0.05 compared with the Oxa, SAHA and Oxa+SAHA groups.

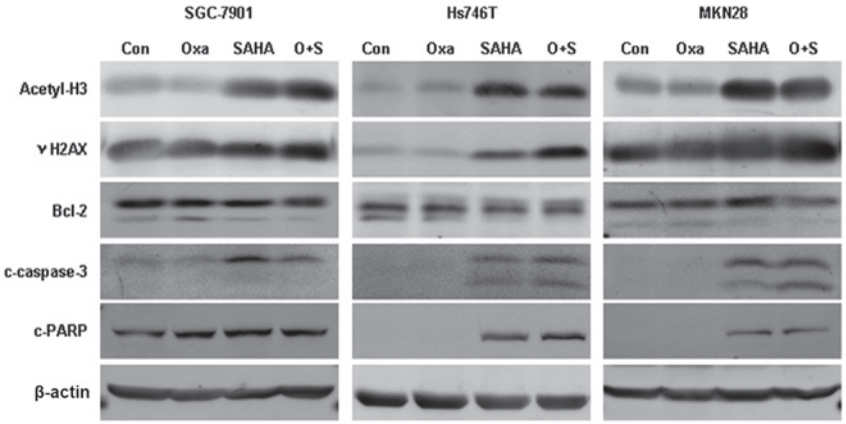

Figure 3. Expression of acetyl-H3, γH2AX, Bcl-2, c-caspase 3 and c-PARP following treatment with Oxa and SAHA monotherapy or combination therapy. After 24 h exposure to Oxa (5 μg/ml) and/or SAHA (4 μM) the expression of acetyl-H3, γH2AX, Bcl-2, c-caspase 3 and c-PARP was detected using western blot analysis. Oxa, oxaliplatin; SAHA, suberoylanilide hydroxamic acid; acetyl-H3, acetyl-histone H3; γH2AX; phosphorylated histone H2AX; Bcl-2; B-cell lymphoma 2; c-caspase-3, cleaved caspase-3; c-PARP, cleaved poly (ADP-ribose) polymerase.

order to clarify the association between Src and Akt activation following oxaliplatin exposure, gastric cancer cells were treated with oxaliplatin combined with either the phosphatidylinositide 3-kinase inhibitor Ly294002 (50 μM) or the Src inhibitor dasatinib (50 nM). Ly294002 was observed to inhibit oxaliplatin-induced p-Akt expression, but had no impact on oxaliplatin-induced p-Src expression. Dasatinib was found to impair oxaliplatin-induced p-Src expression but had no effect on oxaliplatin-induced p-Akt expression (Fig. 5A).

To investigate the interactive effect of Akt and Src inhibition with oxaliplatin, cell survival rates were assessed in Hs746T cells treated with oxaliplatin plus either Ly294002 or dasatinib. Both combinations were observed to increase the inhibition rate of Hs746T cells compared with the monotherapy. Furthermore, the cell inhibition rate was significantly increased in the oxaliplatin plus dasatinib group compared with that in the oxaliplatin plus Ly294002 group (Fig. 5B; P<0.001).

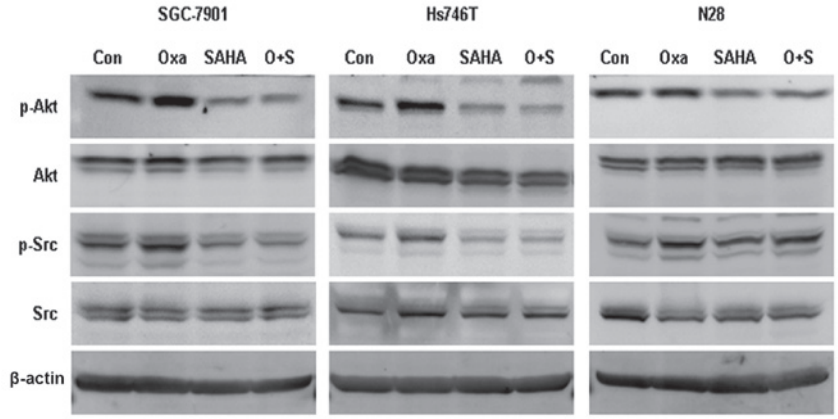

Figure 4. Phosphorylation of Akt and Src is inhibited by SAHA. Akt and Src phosphorylation was detected using western blot analysis after 24 h treatment with Oxa (5 μg/ml) and/or SAHA (4 μM). Oxa, oxaliplatin; SAHA, suberoylanilide hydroxamic acid; p-, phosphorylated-.

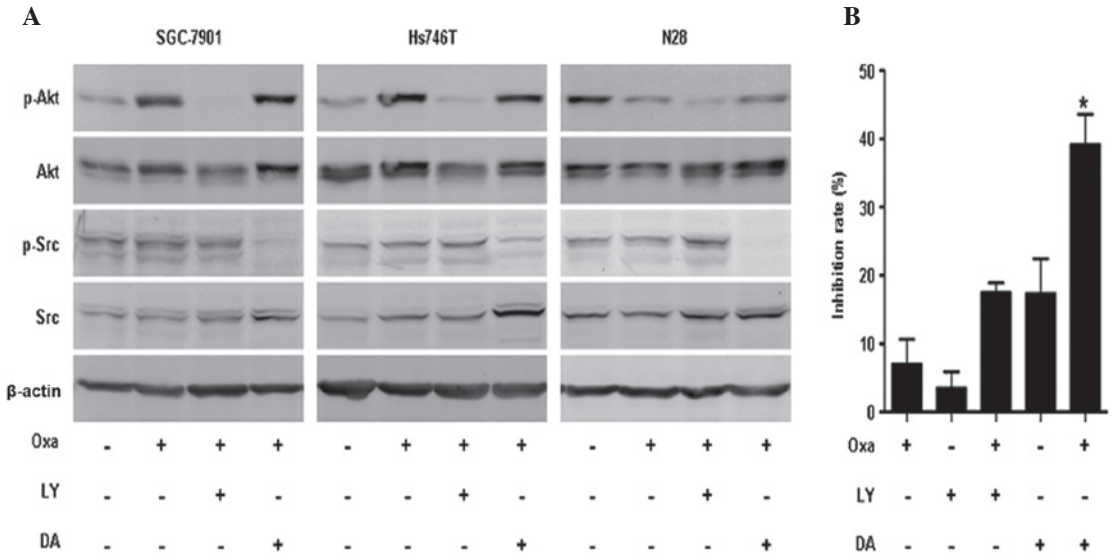

Figure 5. Association between Akt and Src activation. (A) Phosphorylation of Akt and Src was detected after 24 h treatment with Oxa combined with LY (50 μM) or DA (50 nM). (B) The inhibition rate of Hs746T cells was assessed after 48 h treatment. Oxa, oxaliplatin; LY, Ly294002; DA, dasatinib; p-, phosphorylated-. *P<0.001 compared with the Oxa + ly294002 group.

Discussion

Combination therapy, including doublet/triplet chemotherapy or chemotherapy plus targeted agents, is the primary strategy for the front-line treatment of late-stage gastric cancer. To optimize the efficacy and minimize the adverse events associated with combination therapy, translational research is required to elucidate the interactive mechanisms between different drugs. At present, oxaliplatin is frequently used in platinum complex-based regimes. In this era of targeted therapy, the optimal drug to be combined with oxaliplatin is yet to be elucidated. The present study aimed to provide preclinical evidence for agents to combine with oxaliplatin and to investigate the interaction between oxaliplatin and SAHA.

SAHA is a pan-HDACI that targets all the classical HDACs, including class I, II and IV HDACs (21). Gene signature analysis performed by Claerhout *et al* (22) suggested that SAHA may be a potential drug candidate for the treatment of gastric cancer (22). Due to its effect on histone hyperacetylation and the modification of chromosome structure, SAHA is considered to be a sensitizer of DNA damage-inducing agents (23,24). The present study showed that combining oxaliplatin with SAHA significantly increased the inhibitory effect of oxaliplatin *in vitro* and *in vivo*, and without significant toxicity. This positive interaction between oxaliplatin and SAHA in gastric cancer cells suggests that these two agents may have potential to be used in combination in gastric cancer treatment.

As a platinum-based drug, oxaliplatin causes cell cycle arrest and apoptosis primarily through inducing DNA damage. Although oxaliplatin induces the formation of fewer DNA adducts than cisplatin, the DNA damage caused by oxaliplatin is also potent (25,26). SAHA facilitates the accessibility of DNA damage factors; therefore, SAHA may induce replication-dependent DNA damage (27). These findings indicate that DNA damage may be one of the mechanisms through which oxaliplatin and SAHA interact. Following oxaliplatin plus SAHA exposure, the expression of γH2AX, an early marker of DNA double-strand breaks, was significantly

increased, indicating that the combination of oxaliplatin and SAHA potentiated DNA damage.

The regulation of apoptosis is one of the mechanisms underlying the antitumor effect of SAHA. SAHA has been reported to regulate apoptosis by decreasing the expression of Bcl-2 and Bcl-extra large, and increasing that of Bcl-2-associated X protein and Bcl-2 homologous antagonist killer. New *et al* (28) and Thompson *et al* (29) found that exogenous expression of Bcl-2 impaired SAHA-induced apoptosis in diffuse large B-lymphoma cells (28,29). In the present study, Bcl-2 expression was not changed in the gastric cancer cells in the SAHA or oxaliplatin monotherapy groups, but was reduced in the combination treatment group. However, following oxaliplatin and SAHA doublet treatment, the expression of cleaved caspase-3 and cleaved PARP was found to be increased, indicating that apoptosis was potentiated

In addition to its effects on apoptosis, SAHA was found to reverse the oxaliplatin-induced Src activation. The effects of SAHA on Src activation have been rarely reported. Trichostatin A (TSA) and butyrate, two common HDACIs, have been found to inhibit Src expression in colon cancer cell lines (30). However, in the present study, SAHA was not observed to significantly change total Src expression, indicating that the regulation of relative signaling pathways may contribute to this phenomenon.

HDACIs, including SAHA, have been reported to inhibit Akt activation. TSA has been found to increase the association between protein phosphatase 1 (PP1) and Akt through the disassembly of the HDAC/PP1 complex (20). In addition, cross-talk between Src and Akt has been reported in cancer cells (31). In present study, SAHA was observed to induce the suppression of Akt activation. However, the inhibitory effect of SAHA on Src activation appeared to be independent of its effect on Akt in the three gastric cancer cell lines.

The kinases Src and Akt are important for tumor cell survival and growth (32,33). We previously reported that Src phosphorylation was upregulated by oxaliplatin in gastric cancer cells, and that the Src inhibitor dasatinib showed a significant synergic effect with oxaliplatin (11). The inhibition of Akt activation has also been reported to potentiate the antitumor effect of oxaliplatin (34). In the present study, combining oxaliplatin with dasatinib or Ly294002 inhibited Hs746T cell growth compared with monotherapy, and oxaliplatin plus dasatinib showed a more potent efficacy than oxaliplatin plus Ly294002. These findings indicate that Src activation may have an important role in the interaction between oxaliplatin and SAHA.

In conclusion, the combination of oxaliplatin with SAHA potentiated its inhibitory effect in gastric cancer cells. The reversal of oxaliplatin-induced Src phosphorylation may be one of mechanisms by which SAHA enhances the efficacy of oxaliplatin. The present study has identified potential drug combinations for chemotherapy in gastric cancer, which may warrant investigation in clinical trials. The mechanism by which SAHA suppresses Src activation should be investigated further.

Acknowledgements

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