

Combination of Pan-HDAC Inhibitor and COX-2 Inhibitor Produces Synergistic Anticancer Effects in Human Salivary Adenoid Cystic Cancer Cells

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Objective: To evaluate whether the combination of the pan-histone deacetylase (HDAC) inhibitor, suberanilohydroxamic acid (SAHA), and the cyclooxygenase-2 (COX-2) inhibitor, celecoxib, could produce synergistic anticancer effects in human salivary adenoid cystic cancer (SACC) cells.

Methods: SACC cells were treated with the COX-2 inhibitor celecoxib or the pan-HDAC inhibitor SAHA, or a combination of celecoxib and SAHA, for 24 hours. Cell proliferation, apoptosis, migration and invasion were evaluated using the cell counting kit 8 (CCK-8) assay, and the 4',6-diamidino-2-phenylindole staining assay, transwell migration or invasion assays, respectively. The protein expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and protein kinase B or AKT1 (PKB/AKT) were evaluated using western blot.

Results: The combinational treatment with SAHA and celecoxib synergistically inhibited cell proliferation, migration and invasion, and synergistically induced apoptosis, whereas the treatment with SAHA or celecoxib alone only slightly inhibited cell proliferation, migration and invasion, and slightly induced apoptosis. Meanwhile, the combinational treatment synergistically upregulated the membrane-bound PTEN (activated form) and downregulated phospho-AKT (activated form).

Conclusion: The combination of pan-HDAC and COX-2 inhibitors produced synergistic anticancer effects at least partially via activating PTEN and inactivating AKT in the SACC cells.

Key words: HDAC, COX-2, celecoxib, SACC, SAHA

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Chemotherapy is one of the three major strategies for cancer treatment, and targets cell proliferation, angiogenesis, apoptosis, invasion and migration¹. Clinically, cisplatin², pingyangmycin³ and Taxol (paclitaxel)⁴ are widely applied in chemotherapy for cancer treat-

ment. However, drug resistance and severe side-effects still impede the effects of chemotherapy⁵. Searching for new anticancer agents or new combinations of anticancer agents to better fight against cancer, is still an important clinical strategy for cancer treatment.

Histone deacetylases (HDACs) inhibitors as potential anticancer agents, have been receiving more and more attention^{6,7}. Acetylation is one of the major post-translational modification mechanisms and is responsible for regulating various cellular processes, including cell proliferation and cell survival⁸. Acetylation is regulated by histone acetyltransferases (HATs) and HDACs. HATs transfer acetyl groups to lysine residues, whereas HDACs remove acetyl groups from lysine residues⁹. In total, 11 human classical HDACs have been identified, i.e., HDAC1 to HDAC11. Their activity is inhibited by pan-HDAC inhibitors, such as trichostatin A¹⁰. HDACs are overexpressed in many types of tumour,

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such as head and neck cancer, liver cancer and lung cancer, which suggests that anticancer effects might be achieved by inhibiting HDACs¹¹. Recently, three pan-HDAC inhibitors have been cleared by the US Food and Drug Administration (FDA)⁶ to be applied in clinical cancer treatment; one of them is vorinostat (also known as suberanilohydroxamic acid, SAHA), which has been cleared for the treatment of T-cell lymphoma. The mechanisms underlying the antitumour effects of pan-HDAC inhibitors are complex. Our previous study¹² showed that pan-HDAC inhibitors can inhibit cell proliferation through enhancing membrane-translocation of PTEN (activation) and decreasing AKT phosphorylation (inactivation) by inhibiting HDAC6. However, the clinical application of the pan-HDAC inhibitors has shown several side effects such as fatigue, diarrhoea, bone marrow toxicity and thrombocytopenia^{13,14}. Therefore, searching for agents that can synergistically enhance the anticancer effects of pan-HDAC inhibitors remains an important strategy to improve their use in chemotherapy.

The use of drug combinations has numerous benefits for cancer therapy, since it enhances the therapeutic effects and reduces the required dosages of each drug; thus reduces the severity of adverse effects¹⁵. It has been reported that the combination of the pan-HDAC inhibitor trichostatin A and the cyclooxygenase-2 (COX-2) inhibitor celecoxib can synergistically inhibit prostaglandin E2 (PGE2) synthesis in lung cancer¹⁶; the combination of SAHA and COX-2 inhibitors synergistically inhibited the proliferation of pancreatic cancer cells¹⁷. COX-2 is an important rate-limiting enzyme in the process of PGE2 synthesis. PGE2 induces the proliferation, invasion and migration of cancer cells via several signalling pathways, including the β -catenin and AKT pathways^{18,19}. COX-2 is overexpressed in cancer cells²⁰. Usage of COX-2 inhibitors can induce the inhibition of cell proliferation and apoptosis in several cancer cell lines including lung cancer²¹, prostate cancer²², and breast cancer²³. COX-2 inhibitors are mainly applied in clinical anti-inflammation therapy and have also been reported to be used in the prevention of tumours^{24,25} and in enhancing the sensitivity of cancer cells to radiation^{20,26}. We have also previously found that the treatment with celecoxib completely rescued the radiation-induced decrease in the PTEN membrane translocation (activation) and correspondingly inactivated AKT²⁶. Considering that the pan-HDAC inhibitors and the COX-2 inhibitor celecoxib can activate PTEN, we hypothesised whether the combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib could achieve synergis-

tic anticancer effects in human salivary adenoid cystic cancer (SACC) cells.

In this study, we explored whether the combination of SAHA and celecoxib could produce synergistic effects on proliferation, apoptosis, migration and invasion in human SACC cells.

Materials and methods

Cell culture and treatments

A SACC cell line, SACC-83, was derived from human SACC²⁷ and cultured in RPMI 1640 medium (Gibco) with 10% foetal bovine serum (FBS) at 37°C, with 5% CO₂. Cells were treated with 4 μ M SAHA or 20 μ M celecoxib alone, or a combination of SAHA and celecoxib, for 24 h. Cell cultures were examined and photographed under phase-contrast light microscopy.

Reagents and antibodies

SAHA and celecoxib were purchased from Selleck Chemicals (Houston, TX, USA). Anti-PTEN (#9552), anti-AKT (pan) rabbit monoclonal antibodies (#4685) and anti-phospho-AKT (S473) (#4058) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti- β -actin (I-19) antibody (TA-09) was purchased from ZSGB-BIO Company (Beijing, China).

Cell proliferation assay

The cell proliferation assay was performed using the cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. In brief, cells were seeded into 96-well plates (1.5 \times 10³ cells per well) and treated with 20 μ M celecoxib or 4 μ M SAHA, or a combination of 20 μ M celecoxib and 4 μ M SAHA, for 24 h. Subsequently, 10 μ l of the CCK-8 reagent was added to each well. Cells were further incubated at 37°C for 3 h, and then the optical absorbance of each well was measured at 450 nm (OD₄₅₀). Data were presented as the mean \pm standard deviation (SD) of at least three independent experiments.

Analysis of the drug interaction in vitro

The coefficient of drug interaction (CDI) was used to analyse the synergistic inhibitory effect of the drug combination. CDI was calculated as follows: CDI = AB / (A \times B). AB was the OD₄₅₀ ratio of the two-drug combination group to the control group; A and B were the

OD₄₅₀ ratios of each of the single-drug groups to the control group. CDI < 1 indicated a synergistic effect, CDI < 0.7 indicated a significant synergistic effect, CDI = 1 indicated additivity and CDI > 1 indicated antagonism²⁸.

Assessment of cell apoptosis

Cells were washed in phosphate-buffered saline (PBS) three times, fixed with 10% formaldehyde for 5 m, and then incubated with 5 mg/ml of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) in the dark for 5 m, at room temperature. Following three washes in PBS, cells were examined under a fluorescence microscope (Nikon). Cells presenting features of nuclear condensation and fragmentation under fluorescence microscopy were considered to be apoptotic cells. The apoptotic cells were counted within five randomly selected fields, and their rate was presented as the mean \pm SD of at least three independent experiments.

Transwell migration and invasion assay

The cell migration and invasion assays were performed in Transwell chambers (Corning Costar, Corning, NY, USA) with polycarbonate membranes. For the migration assays, cells were seeded into the upper chambers of each well (1×10^5 cells per well) in serum-free culture medium, while culture medium containing 10% FBS was added to the lower chambers. The cells were incubated for 12 h, after which the cells on the top surface of the membrane were wiped off, and the cells on the bottom surface were fixed with 4% paraformaldehyde and stained with 0.01% crystal violet. Cells on the bottom surface of the membrane were examined under a light microscope, counted and averaged from six randomly selected fields.

The transwell invasion assays were performed in the same manner as the migration assays, except that the upper chambers were coated with 20 μ g extracellular matrix gel (BD, #354234) prior to seeding the cells.

Western blotting assay

Whole cell lysates were extracted with the radioimmunoprecipitation (RIPA) lysis buffer (Applygen Technologies, Beijing, China). The membrane proteins were extracted using the nuclear-cytosol extraction kit (Applygen Technologies). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of samples were run on a 10% sodium

dodecyl sulphate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% fat-free milk in tris-buffered saline with Tween 20 (TBS-T) (50 mmol/l Tris, pH 7.5; 150 mmol/l NaCl; 0.05% Tween 20) for 1 h. After incubation with the primary antibodies diluted at 1:1000 in TBS-T overnight at 4°C, the membrane was washed extensively with TBS-T for 5 m at room temperature, and then, washed again two times until a total of three washes, and incubated with a secondary antibody conjugated with fluorophore for 1 h at room temperature. After extensive washing with TBS-T, the membrane was visualised with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

All statistical examinations were performed using SPSS 21 for Windows. All data were presented as the mean \pm standard deviation (SD). Differences between multiple groups were analysed by one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib showed synergistically inhibitory effects on the proliferation of SACC-83 cells.

The concentrations of SAHA (4 μ M) and celecoxib (20 μ M) were determined on preliminary experiments, in which they individually just slightly inhibited the proliferation of SACC-83 cells, 24 h post-treatment; however, the combination of SAHA and celecoxib showed synergistic inhibition of proliferation (CDI = 0.64, indicating significant synergistic inhibitory effect) (Fig 1).

The combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib synergistically induced the apoptosis of SACC-83 cells.

As shown in Figure 2a, the use of DAPI staining revealed that apoptotic cells exhibited nuclear condensation or fragmentation. The rate of apoptotic cells in the SACC-83 cells treated with SAHA or celecoxib alone was about or less than 20%, whereas the rate of apoptotic cells in the SACC-83 cells treated with the combination of celecoxib and SAHA was about 60%; significantly higher than the sum of the individual ones (Fig 2b).

The combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib showed synergistically inhibitory effects on the migration and invasion of SACC-83 cells.

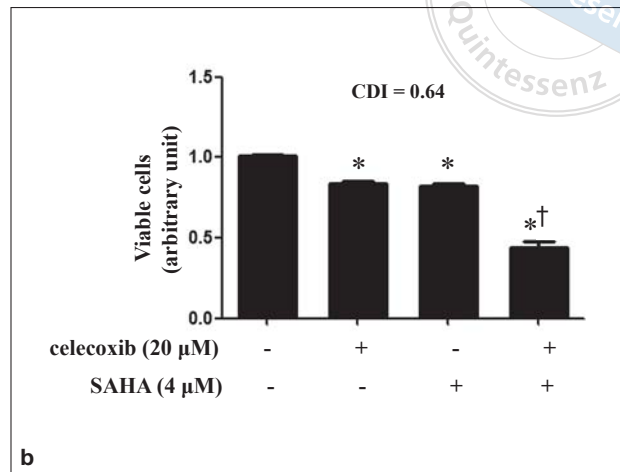
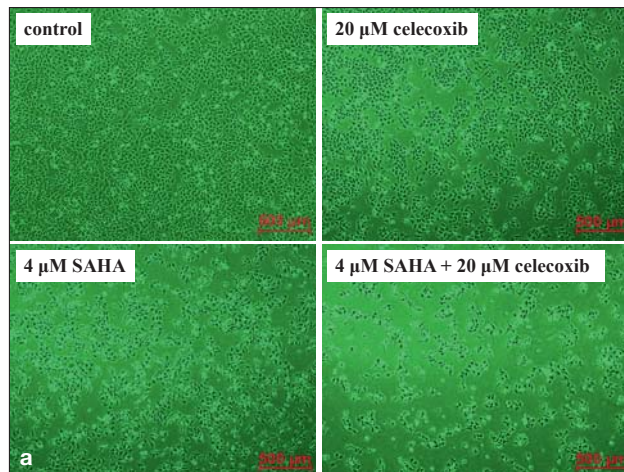


Fig 1 The combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib exerted synergistically inhibitory effects on the proliferation of SACC-83 cells. **(a)** Phase-contrast photomicrographs of SACC-83 cells after different treatments were applied for 24 h. **(b)** Cell proliferation of SACC-83 cells after different treatments were applied for 24 h. The cell proliferation was evaluated using the cell counting kit 8 (CCK-8) assay. The number of SACC-83 cells treated with a combination of celecoxib and SAHA was lower than SACC-83 cells treated with each inhibitor alone (CDI = 0.64, indicating a significant synergistic inhibitory effect). All data were presented as the mean ± SD of at least three independent experiments. **P* < 0.05 vs control group; †*P* < 0.05 vs celecoxib or SAHA group (n = 3).

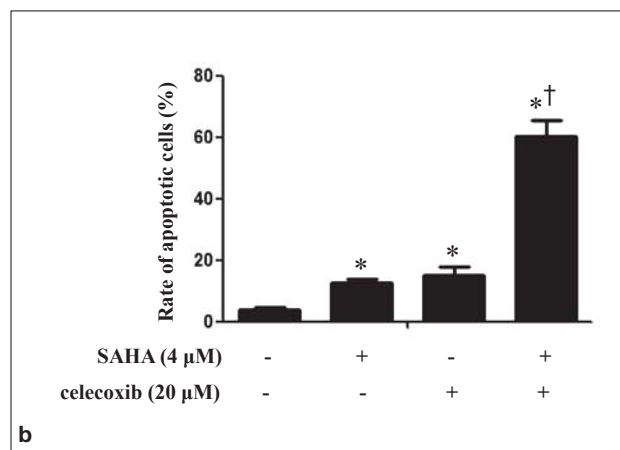
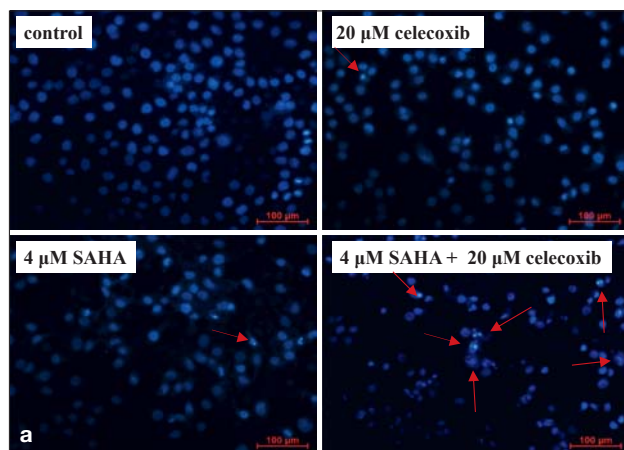


Fig 2 The combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib synergistically induced apoptosis of SACC-83 cells. **(a)** Fluorescent photomicrographs of SACC-83 cells stained with DAPI after different treatments were applied for 24 h. Apoptotic cells exhibited nuclear condensation or fragmentation (arrows). **(b)** The rate of apoptotic cells was significantly higher in the SACC-83 cells treated with a combination of celecoxib and SAHA than the sum of both rates obtained in the other two treatments where inhibitors were used individually. All data were presented as the mean ± SD of at least three independent experiments. **P* < 0.05 vs control group; †*P* < 0.05 vs celecoxib or SAHA group (n = 3).

As shown in Figure 3, the SACC-83 cells treated with SAHA or celecoxib alone only showed a slight inhibition on cell migration or invasion, observed via the transwell assay, whereas cells treated with a combination of celecoxib and SAHA showed a synergistic inhibition on cell migration or invasion, observed via the transwell assay.

The combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib synergistically activated PTEN and inactivated AKT in SACC-83 cells.

As shown in Figure 4, SACC-83 cells treated with SAHA or celecoxib alone, only showed a slight upregulation of the membrane-bound PTEN (activated form), whereas SACC-83 cells treated with a combination of celecoxib and SAHA showed synergistic upregulation of the membrane-bound PTEN. Correspondingly, phospho-AKT (activated form) was slightly downregulated (by 10%) in the SACC-83 cells treated with celecoxib alone, or downregulated by half in the SACC-83 cells treated with SAHA alone, but downregulated by 70%

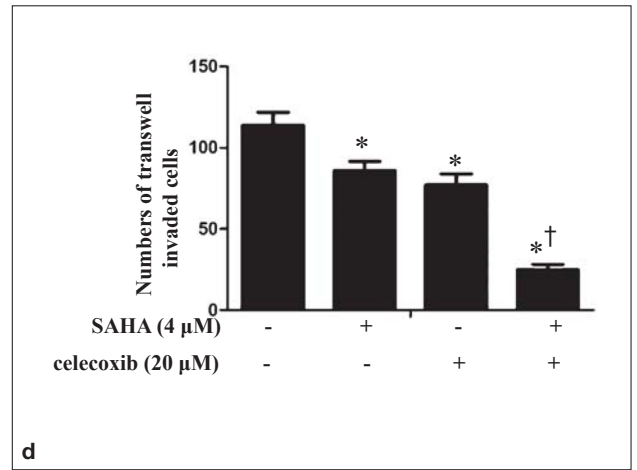
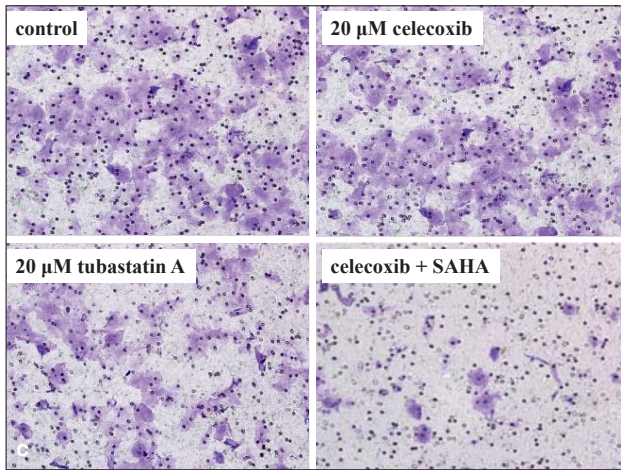
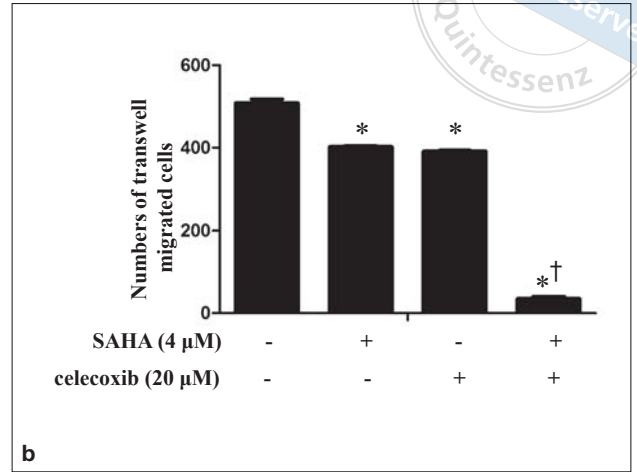
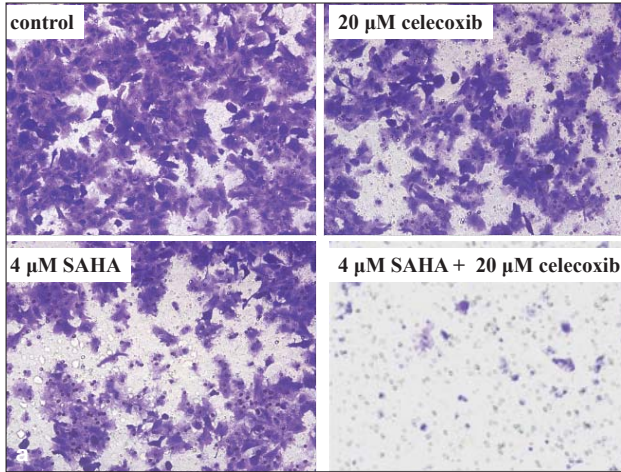
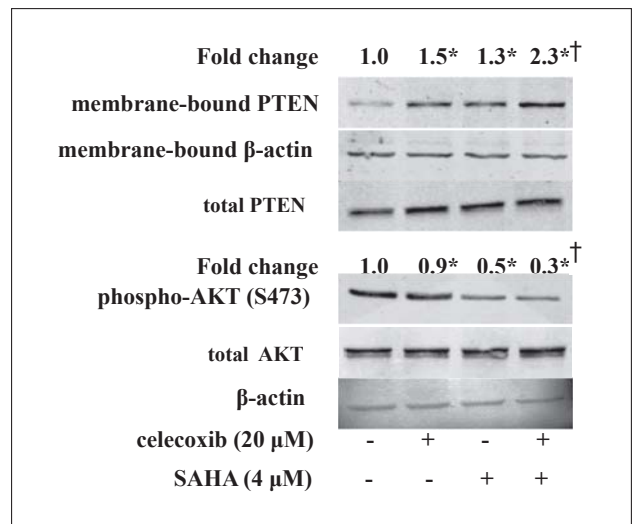


Fig 3 The combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib exerted synergistically inhibitory effects on the migration and invasion of SACC-83 cells. **(a)** Microphotographs of the transwell migration assay of SACC-83 cells after different treatments. **(b)** Quantified data of the transwell migration assay of SACC-83 cells after different treatments. **(c)** Microphotographs of the transwell invasion assay of SACC-83 cells after different treatments. **(d)** Quantified data of the transwell invasion assay of SACC-83 cells after different treatments. All data were presented as the mean \pm SD of at least three independent experiments. * $P < 0.05$ vs control group; † $P < 0.05$ vs celecoxib or SAHA group ($n = 3$).

Fig 4 The combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib synergistically activated PTEN and inactivated AKT in the SACC-83 cells. SACC-83 cells treated with SAHA or celecoxib alone only showed slight upregulation of the membrane-bound (activated form) PTEN, whereas the SACC-83 cells treated with a combination of celecoxib and SAHA showed synergistic upregulation of the membrane-bound PTEN. Correspondingly, phospho-AKT (activated form) was slightly downregulated (by 10%) in the SACC-83 cells treated with celecoxib alone, or downregulated by half in the SACC-83 cells treated with SAHA alone, but synergistically downregulated (by 70%) in the SACC-83 cells treated with the combination of celecoxib and SAHA. * $P < 0.05$ vs control group; † $P < 0.05$ vs celecoxib or SAHA group ($n = 3$).



in the SACC-83 cells treated with a combination of celecoxib and SAHA.

Discussion

In the present study, we demonstrated that the combination of the pan-HDAC inhibitor SAHA with the COX-2 inhibitor celecoxib produced synergistic anticancer effects in a SACC cell line. Our observations suggest a potential novel clinical strategy using the pan-HDAC inhibitor SAHA in combination with the COX-2 inhibitor celecoxib for SACC therapy.

The combination of the pan-HDAC inhibitor SAHA with the COX-2 inhibitor celecoxib may produce synergistic anticancer effects in a SACC cell line. Synergy usually means that the total effect is greater than the sum of the individual effects. Our results showed that the combination of both pan-HDAC inhibitor SAHA and COX-2 inhibitor celecoxib could greatly induce more apoptosis and inhibit cell proliferation, migration and invasion than the sum of their individual effect in the SACC-83 cells. These results suggest that the combination of SAHA and celecoxib produced synergistic anticancer effects, which could offer clinical advantages: one would be reducing the amount of SAHA and obtain less side effects if achieving the same anticancer effects using less SAHA; the other would be achieving additional anticancer effects if using the same amount of SAHA. Our results suggest that SAHA might be also potentially used for the treatment of SACC, although SAHA has only been cleared by the FDA for the treatment of T-cell lymphoma. In fact, SAHA or other HDAC inhibitors, have been tested for many other solid cancers and all have shown to be potentially clinical promising²⁹. Using COX-2 inhibitors as the enhancers of the pan-HDAC inhibitor SAHA in chemotherapy is rather advantageous, clinically, because COX-2 is usually overexpressed in cancer cells and is also involved in metastasis and carcinogenesis²⁰ and its inhibitors have been widely used for the treatment of inflammatory pain; furthermore, their clinical safety has been recognised^{24,25}. Therefore, the combination of pan-HDAC inhibitors, such as SAHA, with COX-2 inhibitors, such as celecoxib, is a potential new strategy for the treatment of SACC.

The mechanisms underlying the synergistic anticancer effects of the combination of SAHA and celecoxib could mainly comprise the synergistic activation of PTEN and inactivation of AKT. We observed that both SAHA or celecoxib alone could upregulate the membrane-bound PTEN (activated form) and correspondingly downregulate phospho-AKT (activated form).

However, the combination of SAHA and celecoxib could increase the upregulation of the membrane-bound PTEN and the downregulation of the phospho-AKT, compared with the sum of the inhibitory effects of SAHA or celecoxib alone. The upregulation of the membrane-bound PTEN or the activation of PTEN by SAHA, depended on the upregulation of the K163 acetylation through inhibition of HDAC6, in our previous study¹². The upregulation of the membrane-bound or the activation of PTEN by celecoxib is related to the inhibition of Sp1 expression, which is a negative regulator of PTEN transcription²⁶. To the best of our knowledge, our results obtained here demonstrated for the first time that the COX-2 inhibitor celecoxib is also an important agent for enhancing the anticancer effects of the pan-HDAC inhibitor SAHA in a SACC cell line; nevertheless, we have shown in our previous studies that celecoxib can enhance the anticancer effects of the radiation effect or the HDAC6 inhibitor. Therefore, our results also imply that the combination of the pan-HDAC inhibitor with the COX-2 inhibitor might be potentially applied in the treatment of other cancers.

Conclusion

In conclusion, we showed that the combination of the pan-HDAC inhibitor SAHA and the COX-2 inhibitor celecoxib produced synergistic anticancer effects in SACC cells. This combination might be a new clinical strategy in the treatment of SACC.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Guan Hua ZHANG carried out the experiment and drafted the manuscript; Prof. Ye Hua GAN designed the study, supervised the experiment and revised the manuscript.

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