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Enhancement of cisplatin induced apoptosis by suberoylanilide hydroxamic acid in human oral squamous cell carcinoma cell lines

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ABSTRACT

Both the resistance of tumor cells to cisplatin and dose-related toxicity remain two of the most important problems in the chemotherapy of clinical oral squamous cell carcinoma (OSCC). Researchers have been seeking a combinative treatment regimen to improve the effect of chemotherapy. As potent new anti-cancer drugs, histone deacetylase inhibitors ($HDACI_S$) have been reported to be associated with chromatin modification and display synergistic activities with some traditional chemotherapeutic agents. In this study, we evaluated the potential combinative effect of low dose cisplatin and suberoylanilide hydroxamic acid (SAHA, one of the most potent $HDACI_S$) in OSCC cell lines. Cell viability and apoptotic assay were examined. Compared with either cisplatin (4 μg/ml) or SAHA (2 μM) treated alone, co-administration of both drugs synergistically induces cytotoxicity and apoptosis in both Tca8113 and KB cell lines. Furthermore, diverse apoptosis-associated proteins, including p53, BID, cytochrome C and caspase-3 were involved in the induction of apoptosis. Our results suggest that concurrent treatment with SAHA enhances tumor cell sensitivity to subtoxic doses of cisplatin. This may be regarded as a novel strategy for treatment of OSCC.

1. Introduction

Carcinomas of the oral cavity, especially oral squamous cell carcinoma (OSCC), are one of the most leading causes of cancer related death and affect nearly 500,000 patients annually world-wide [1]. Three major modalities are currently applied in the conventional treatment of OSCC, these being surgery, radiation and chemotherapy [2]. Among these strategies chemotherapy (pre- or post-surgery) is beneficial for local control and survival improvement. In spite of this, treatment with current chemotherapeutic drugs does not always substantially induce a positive response. In fact, the lack of effective chemotherapeutic strategies results in a high death rate in patients with oral carcinoma [3]. To overcome such a problem, multiple chemotherapeutic agents with different modes of actions, either used alone or in combination have been suggested [4].

Cisplatin is an alkylating agent that targets DNA and results in bulky adducts as well as intra- and inter-strand crosslink [5,6]. It has powerful therapeutic effects against oral carcinoma

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and is thus still used as a first-line single-agent despite of its high dose-related toxicity, including renal toxicity, ototoxicity and neurotoxicity. *De novo* and acquired resistance of cancer cells to cisplatin are the two main causes of treatment failure [7]. As the molecular mechanisms of such resistance are poorly understood much research has been focused on a cisplatin-based combinative therapy, the results of which are not always satisfactory [8,9].

Histone deacetylase inhibitors ($HDACI_S$) are emerging as an attractive new class of potent anticancer drugs in the treatment of solid as well as hematological malignancies. HDACIs inhibit the deacetylation of histones and weaken the histone–DNA interactions, thereby permitting the chromatin scaffolding to assume a more relaxed conformation and increase gene transcription rate [10,11]. While there are several different $HDACI_S$, suberoylanilide hydroxamic acid (SAHA), is one of the most potent. SAHA alone has demonstrated activities causing differentiation, growth arrest and/or apoptosis in a series of tumor cells while normal cells were observed to be relatively resistant [12].

Although cisplatin and $HDACI_S$ target different sites, the close relationship and functional importance of DNA and chromosome structure in cancer development suggest the possible interaction between these two agents [13]. Since chromatin structure and DNA sequence accessibility can be regulated by DNA-associated proteins, such as histones, a more relaxed chromosome should facilitate the formation of DNA adducts that enhance the activity of cisplatin. In fact, $HDACI_S$ have been regarded as ''sensitizer drugs'' that display synergistic effects with other agents, such as DNA methylase inhibitors and retinoic acids. In addition, the activation of gene expression and induction of apoptosis have been reported in these combinative strategies [14,15]. Recently, the augmented cytotoxic effect by $HDACI_S$ was reported in brain and breast cancer [16], even though the molecular mechanisms underlying $HDACI_S$ associated combinative therapies remain elusive [17,18].

In this investigation, we aim to evaluate the possible synergistic anticancer efficiency of both cisplatin and SAHA. Molecular mechanisms underlying drug induced apoptosis and activation of apoptosis related proteins in OSCC cell lines are also examined.

2. Materials and methods

2.1. Reagents

Suberoylanilide hydroxamic acid (SAHA) was purchased from (Alexis Corp., San Diego, CA, USA), and was dissolved in DMSO as stock solution. The maximum volume (%) of DMSO in the experiment was less than 0.1%. Cisplatin was purchased from Jintai Pharmaceutical Co. Ltd. (Liaoling, PR China) and dissolved in PBS.

2.2. Cell culture

Two oral carcinoma cell lines Tca8113 and KB were kindly provided by Min Zhou (Laboratory of Oral Medicine, Sichuan University). Cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2.

2.3. Drug treatment

First, a pilot study was performed to observe the dose response of cisplatin or SAHA when used separately and the dose causes 50% inhibition (ID_{50}) for cisplatin and SAHA were obtained. Subsequently, a subtoxic dose, 4 μg/ml for cisplatin and 2 μM for SAHA was used alone, or in combination to compare their activities against OSCC cancer cells. Cells were treated either with SAHA (2 μM) or cisplatin (4 μg/ml) or a combination of both or a sequential treatment of SAHA and then cisplatin for 4 h.

2.4. MTS assay

Cell growth and viability were assessed using MTS cell proliferation kit (Promega Inc., Madison, WI, USA). After exposure of the cells to cisplatin and SAHA as described above, the cells were incubated with 20 μl MTS solution for 4 h, and the absorbance at 590 nm monitored on a Spectra Max M5 (MDC, Sunnyrale, CA, USA).

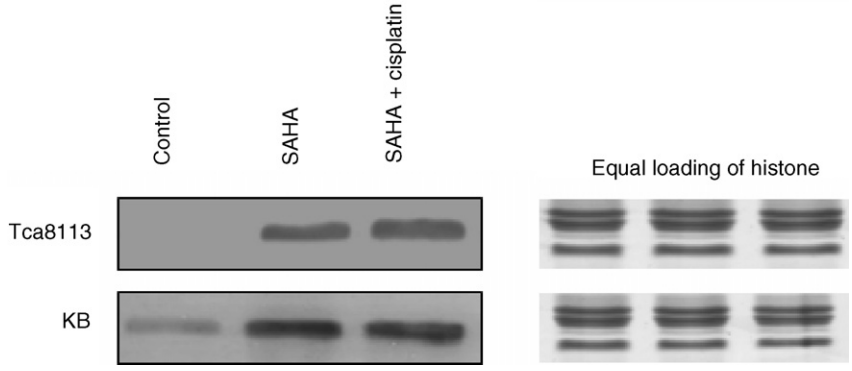


Fig. 1 – Examination of acetylated histones by Western blots. Histones extracted from Tca8113 or KB cells treated with SAHA (2 μM) or a combination of SAHA (2 μM) and cisplatin (4 μg/ml) for 4 h, were probed with anti-acetylated H3 antibody as described in Section 2. Equal loading of protein was verified by Coomassie Blue staining on 12% SDS-PAGE gel.

2.5. Colony formation assay

Cells were seeded at 3×10^2 per dish (60 mm diameter) and incubated overnight. The cells were next treated with cisplatin and SAHA alone or in combination as indicated above. After drug treatment, the cells were washed and allowed to grow (10–14 days). The colonies were then fixed with methanol and stained with Crystal Violet (Sigma, St. Louis, MO, USA).

2.6. Flow cytometry

Cells were seeded into a six-well plates at 10^6 cells/ml and incubated overnight. The cells were treated with cisplatin and SAHA alone or in combination as indicated above. After the treatment, cells were trypsinized, washed with cold PBS and fixed in 70% ethanol at 4 °C. Then the cells were washed with PBS and incubated at room temperature with staining solution (5 μg/ml propidium iodide, 20 μg/ml RNase A) for 1 h in the dark. Stained cells were analyzed by fluorescence-activated cell sorting (FACS) using EPICS Elite ESP flow cytometer (Coulter Corp., Miami, FL, USA).The percentage of sub-G_1 in each population were resolved from at least 1×10^4 cells.

2.7. TUNEL assay

Apoptosis was measured by the DeadEnd™ Fluorometric TUNEL System (Promega Inc., Madison, WI, USA). Cells were cultured on chamber slides overnight, treated with cisplatin and SAHA as indicated above. After drug treatment, cells were washed with PBS and fixed by 4% methanol-free formaldehyde solution in PBS for 25 min at 4 °C, washed with PBS and permeablized by 0.2%Triton X-100 in PBS for 5 min at room temperature. Staining was done according to the manufacture's instructions. Fluorescence was visualized with Olympus BX60 microscope (Olympus Optical Co., Hamburg, Germany).

2.8. Histones extraction and Western blots

Isolation of histones was performed as described elsewhere [16]. Briefly, cells were scraped in ice-cold PBS and resuspended in histone lysis buffer (8.6% sucrose, 1% Triton X-100, 50 mM Sodium bisulfate, 10 mM Tris, 10 mM $MgCl_2$). The cell lysates were sonicated and centrifuged at $1000 \times g$ for 10 min. Sulfuric acid was added to a final concentration of 0.2 M and after incubation (4 °C, 1 h), the supernatant was precipitated in acetone overnight at −20 °C. The precipitate was dissolved in distilled water.

Total cell proteins were lysed in RIPA lysis buffer 24 h after drug treatment. Isolation of mitochondrial and cytosolic proteins was performed using the Mitochondria/cytosol Fractionation Kit (Beyotime Inst. Biotech, Peking, PR China). The concentration of protein was determined using the Protein Assay Kit (Bio-Rad, Hercules, CA, USA). BSA was used as a protein standard. A sample of 12.5 μg protein in each well was separated. The proteins were transferred to polyvinyliden difluoride (PVDF) membrane. After blocking with PBST (phosphate buffered saline, 0.1% Tween 20) containing 5% fat-free milk, the members were incubated with the primary antibody against acetylated histone H_3 (1:10,000; Upstate USA Inc., Chicago, IL, USA), p53, BID, procaspase-3, cleaved caspase-3,VDAC (1:500; Santa Cruz, CA, USA) and cytochrome C (1:500; PharMingen, San Diego, CA, USA).The blots were then reacted with horseradish peroxidase-conjugated antibody for 1 h at room temperature and developed with the enhanced chemiluminescence (ECL) detection system (Pierce Biotech Inc., Rockford, IL, USA).

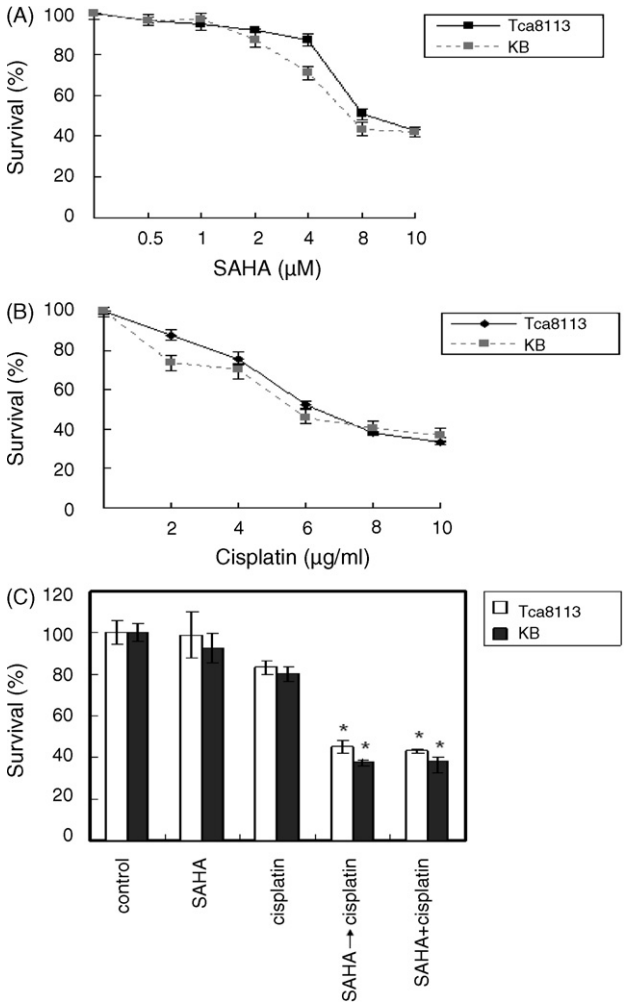


Fig. 2 – Examination of cell proliferation by MTS assay. Tca8113 and KB cells were treated with indicated concentration of SAHA or cisplatin alone. After 24 h, the MTS values were detected. (A and B) Tca8113 and KB cells were treated with SAHA (2 μM), or cisplatin (4 μg/ml) alone, in sequential treatment or administrated in combination of both agents for 4 h as described in Section 2. After 24 h, the MTS values were detected. Asterisk (*) indicates significant decrease ($P < 0.05$) in cell proliferation when compared with treatment with cisplatin alone. The sequential treatment showed no advantage over the concurrent strategy ($p > 0.05$) (C). All experiments were performed in triplicate and repeated at least three times. The results are the mean (±S.D.) of triplicate measurements of one representative experiment.

2.9. Statistical analysis

Data in different groups was analyzed using the Student's two-tailed *t* test. *P* values less than or equal to 0.05 were considered to be significant.

3. Results

3.1. SAHA induces accumulation of acetylated histones in OSCC cell lines

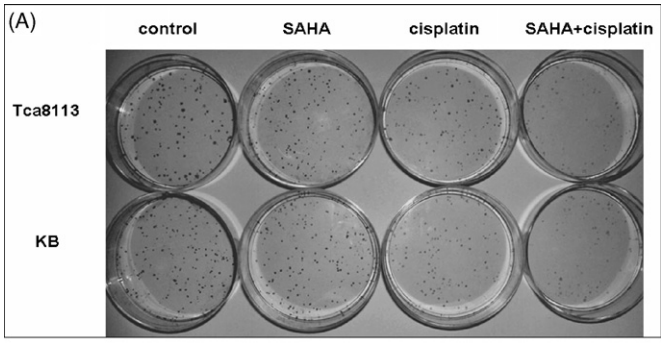
Consistent with previous observations, our pilot study demonstrates that a subtoxic dose (2 μM) SAHA rapidly induces acetylated histones within 4 h in both OSCC cell lines. Treatment with low dose SAHA within 4 h was sufficient to cause obvious histone acetylation to confer an open chromatin structure without triggering apoptosis or genome instability [19]. Coadministration of cisplatin and SAHA resulted in no further increase on the acetylated histone level (Fig. 1).

3.2. Coadministration of subtoxic dose of SAHA and cisplatin results in a marked increase in cytotoxicity of both OSCC cancer cell lines

MTS assays were carried out to evaluate whether SAHA can enhance the cytotoxicity of cisplatin, and to assess the cell viability upon treatment with SAHA, cisplatin, or in combination of both agents. The dose used in our experiments (2 μM SAHA, and 4 μg/ml cisplatin) were based on our pilot studies, which demonstrated minimal toxic against both OSCC cells when they are administered alone (Fig. 2A and B). A pronounced increase in growth inhibition occurred after combinative treatment with both agents. Compared with untreated cells, the cell number in the combinative treatment group decreased by about 57% in Tca8113 and by 62% in KB cells (Fig. 2C).This represents a significant increase in killing efficiency compared with cisplatin alone ($p < 0.05$). Sequential treatment (treatment with 2 μM SAHA for 4 h, followed by 4 μg/ml cisplatin) showed no advantage to a concurrent strategy although any combined therapy was superior to either drug alone ($p > 0.05$) (Fig. 2C).To further confirm our observations, a colony formation assay was performed to examine the long-time OSCC cell survival and to evaluate cancer cell killing efficiency in the combinative therapy. Consistent with data in MTS assay, either SAHA or cisplatin had little effect on the cell survival. However, concurrent administration of both agents results in a marked decrease in the number of colonies with statistic significance ($p < 0.05$) (Fig. 3A and B).

3.3. Enhanced apoptosis was observed in concurrent treatment of SAHA and cisplatin

Because cytotoxicity to HDACIs and cisplatin is often correlated with apoptosis, and because our data indicate that concurrent treatment of OSCC cells with SAHA increases cisplatin induced cytotoxicity (Fig. 2), it was necessary to determine whether the increased cytotoxicity affected apop-



(B)

Group	Cloning efficiency (%)	
	Tca8113	KB
Control	0.438±0.026	0.536±0.033
SAHA	0.434±0.019	0.503±0.014
Cisplatin	0.360±0.010	0.370±0.004
SAHA+cisplatin	0.210±0.024*	0.227±0.009*

Fig. 3 – Long-time OSCC cancer cell survival was examined by colony formation assay. Drug treatment was performed as described in Fig. 1. (A) Cells were allowed to grow for 10–14 days before staining with Crystal Violet. The experiments were repeated twice and similar results were obtained. (B) Percentages of colony formation efficiency in different groups were displayed. Colonies were counted and are expressed as percent cell survival ± SD. Asterisk (*) indicates significant decrease ($P < 0.05$) of survived cells in the combinative treatment group, compared with those cells treated with SAHA or cisplatin alone.

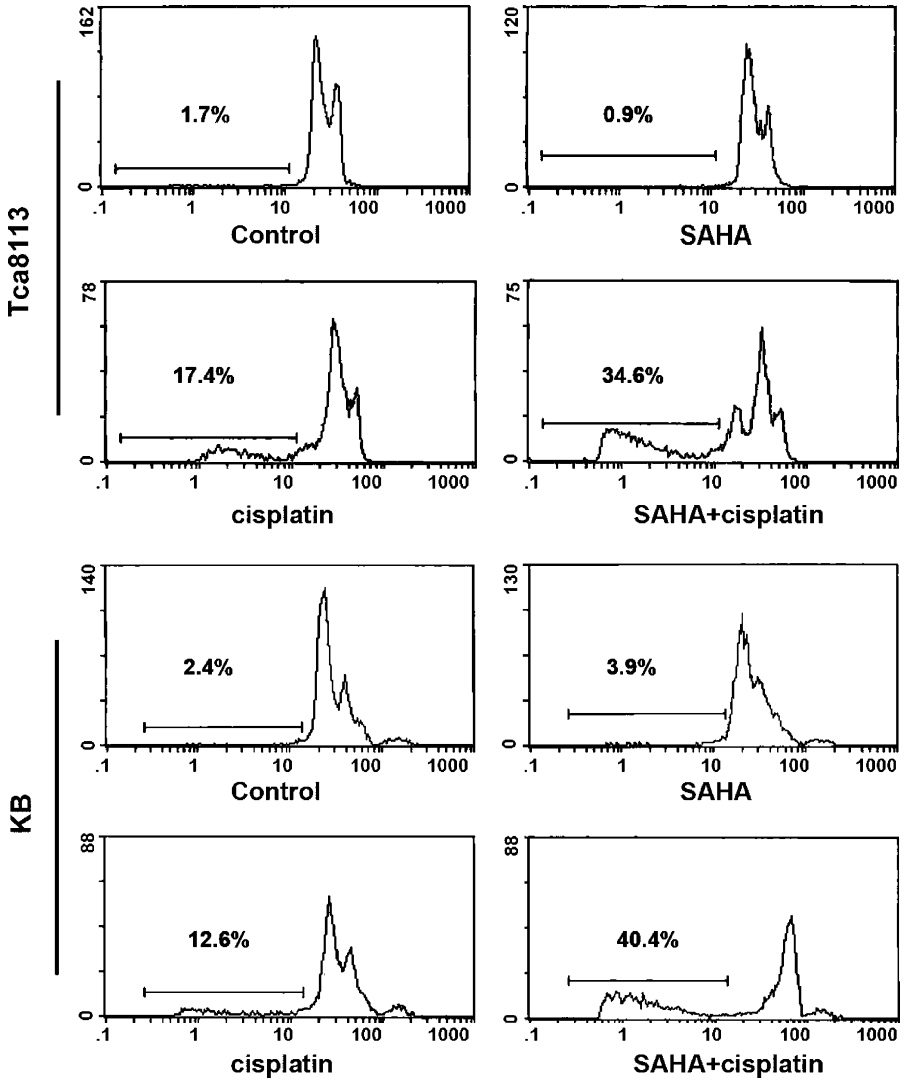


Fig. 4 – Quantitative analysis of apoptotic cells (sub-G_1 cells) by FACS. OSCC cancer cells, Tca8113 and KB, were treated with cisplatin, SAHA, or in combination. Both floating and adherent cell were collected and stained with propidium iodide. Apoptotic cells were indicated by the percentage of cells in sub-G1 (Fig. 1). In the control group, cells were cultured in the presence of 0.1% DMSO vehicle. Three independent experiments were performed, and similar data were obtained.

tosis. The levels of apoptotic cells in each of the treatment groups were first measured by FACS analysis. Our data indicated that treatment with 2 μM SAHA had little effect to induce apoptosis, while 4 μg/ml cisplatin caused a moderate apoptotic effect on both OSCC cell lines. Nevertheless, a remarkable increase of apoptotic cells was detected in both Tca8113 and KB cell lines coadministrated with SAHA and cisplatin (34.6% for Tca8113 and 40.4% for KB) (Fig. 4). As the sub-G1 value, measured by FACS, represents cells from both necrosis and apoptosis, a more sensitive assay (DeadEnd; fluorescence TUNEL system) was performed to allow detection of DNA strand breaks by labeling free 3′-OH termini. Consistent with the FACS assay, a low dose (2 μM) SAHA treatment does not induce detectable apoptosis examined by the TUNEL assay in Tca8113 cells whereas 4 μg/ml cisplatin can cause a low level of DNA strand breaks. Intriguingly, much enhanced apoptosis was observed in both OSCC cancer cell lines in response to combination of SAHA and cisplatin (Fig. 5A). Quantitative data showed that a percentage of TUNEL-positive cells in combination treatment was as high as 60% in Tca8113 cells, whereas individual treatment of SAHA and cisplatin only reached 2% and 18% respectively (Fig. 5B). These data correlate well with the cytotoxicity data presented in Fig. 2 and suggest that acetylation of the core histone can increase cisplatin cytotoxicity against OSCC.

3.4. *Role of apoptosis-related proteins in mediating SAHA-induced sensitization to cisplatin*

To further explore the cellular basis of the apoptotic response observed in OSCC cell lines, the analysis of selected apoptosis-related proteins was then performed. Tumor suppressor p53 was examined because it is considered to be functionally important in cellular response to DNA-damage and affects

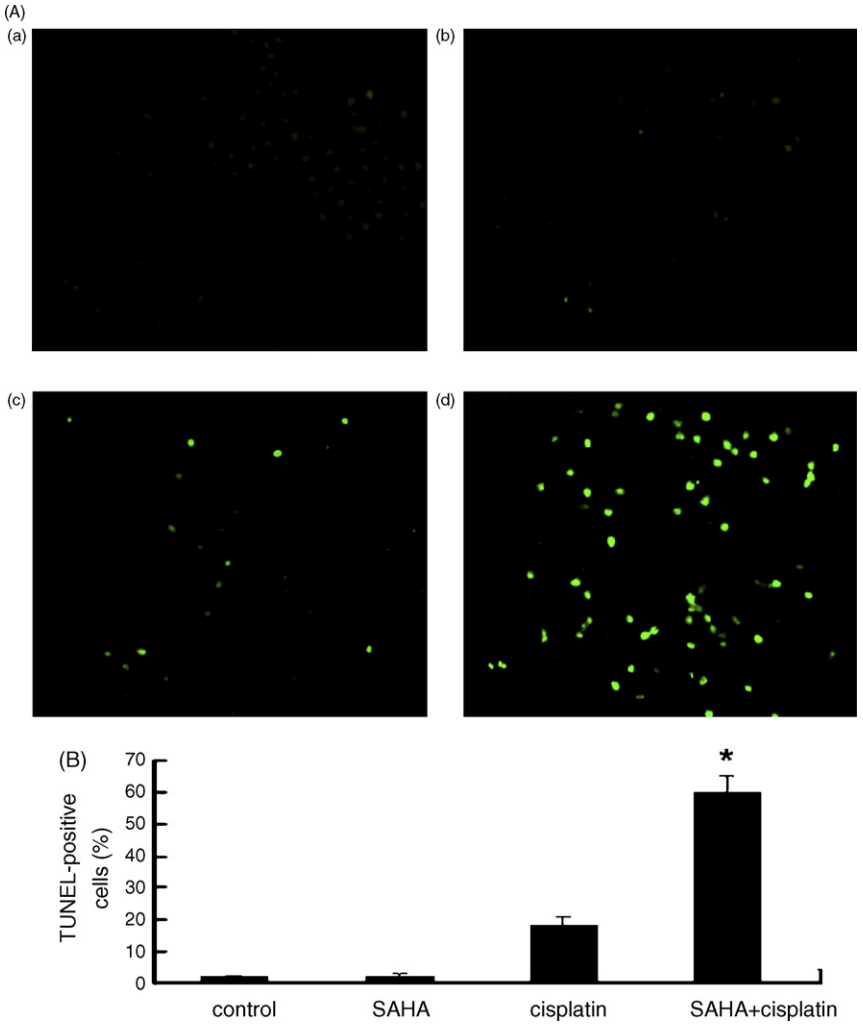


Fig. 5 – Examination of apoptosis by TUNEL assay. (A) Tca8113 cells were cultured in chamber slides and allowed to attach for 24 h. Cells were incubated with (a) no agent, (b) SAHA, (c) cisplatin, or (d) both agents as described in Section 2. Apoptotic effect was detected by TUNEL assay (×200). (B) TUNEL-positive cells were presented. For each coverslip, ≥500 cells were counted. Asterisk (*) denotes that the increased TUNEL-positive cells in the combinative treatment group are significant ($p < 0.05$, $n = 3$), compared with those cells treated with cisplatin alone.

drug sensitivity. Western blots analysis revealed that expression of p53 is undetectable in both OSCC cell lines, and treatment with 2 μM SAHA can not induce obvious p53 expression either. Even though cisplatin (4 μg/ml) is sufficient to induce a weak to moderate p53 expression in both cancer cell lines, coadministration of SAHA and cisplatin cause a much higher p53 expression (Fig. 6).The activation of p53 paralleled the apoptosis induction and consequently part of this effect, is mediated by a p53-dependent apoptotic pathway. Since it has been found that p53 may play a functional role in SAHA-mediated sensitization to cisplatin against OSCC cancer cells, the alteration of the key p53 effectors that modulate drug sensitivity, pro-apoptotic protein BID, cytochrome C and pro-caspase-3 were examined and no significant changes in both OSCC cancer cells administered with SAHA or cisplatin alone were noted. Co-treatment with SAHA and cisplatin, however, resulted in a marked decrease of inactive BID, increase of cytochrome C in cytosol, as well as activation of caspase-3 (Fig. 6). These data indicate that the increased drug sensitivity observed with co-treatment of the cells with $HDACI_S$ is also mediated by increased activation of pro-apoptotic protein BID, release of cytochrome C to cytosol, and increased activation of caspase-3.

4. Discussion

OSCC is one of the most common malignancies that remain incurable with current therapies. Drug resistance often recurs, accompanied by distressing symptoms [20]. The response rate to most commonly used single cytotoxic agent is only about 30–40% in large studies [21]. This limitation has pointed researchers to combinative therapeutic strategies. Among the numerous clinical agents used in combinative chemotherapy, attractive synergistic effect is particularly noted in combinative agents containing cisplatin. Unfortunately, dose-related

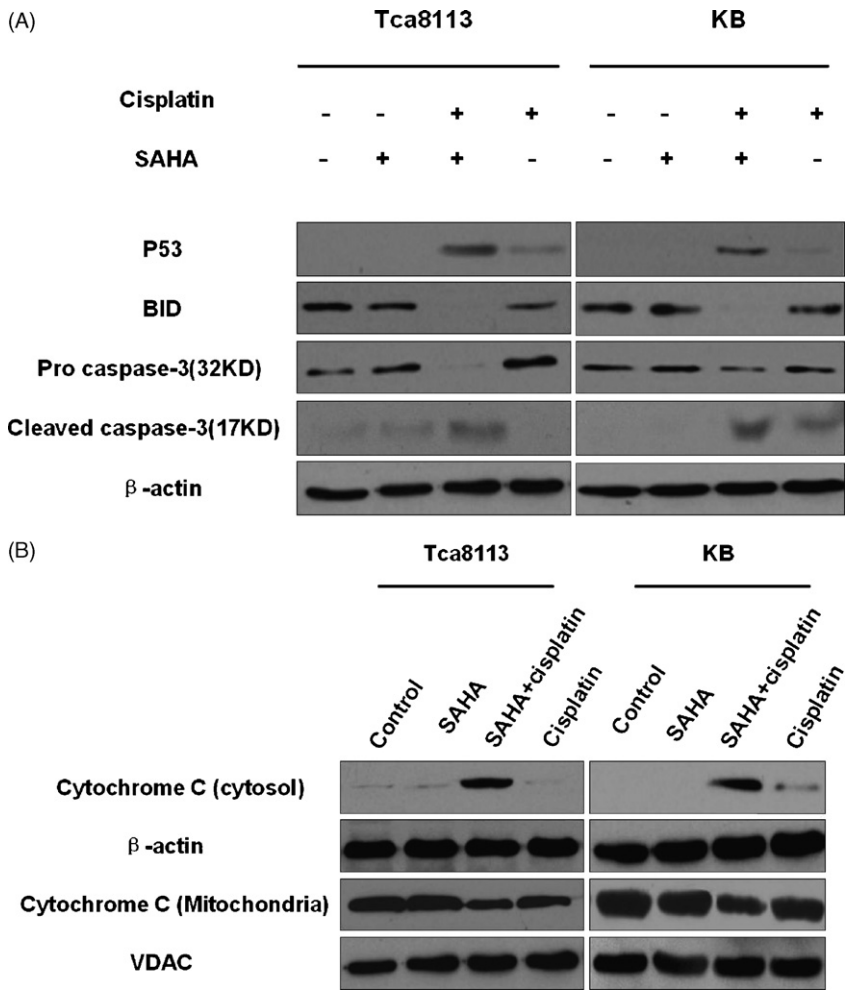


Fig. 6 – Detection of p53, BID, procaspase-3, cleaved caspase-3 and cytochrome C by Western blots. Protein extraction was performed at 24 h post-drug treatment from Tca8113 and KB OSCC cancer cells treated with either cisplatin or SAHA alone, or in combination for 4 h as described in Section 2. Proteins were probed with anti-p53, anti-BID, anti-procaspase-3 and anti-cleaved caspase-3 antibodies, respectively. β-Actin was used as equal loading control (A). Proteins were probed with anti-cytochrome C antibody. β-Actin was used as equal cytosol loading control; and VDAC, a specific mitochondrial membrane protein was used as equal mitochondria loading control (B).

toxicity has been one of the major limiting factors in cisplatin-based therapies [22]. To design a better combinative chemotherapeutic regimen there should be a better focus on cell killing and lower systemic toxicity. Recently, Sato et al. reported the synergistic effects of cotreatment with $HDACI_S$ and cisplatin in OSCC cancer cells [23]. Due to the fact that the cell lines examined in our studies were found very sensitive to cisplatin, to minimize the side effect of cisplatin to the most degree, we focused on a subtoxic dose in the combinative treatment strategy. Our data demonstrated that administration of a low dose of SAHA and cisplatin alone had little effect on OSCC cells. In contrast, cotreatment with both drugs results in a significant synergistic effect rather than a simple additive therapeutic effect. The resulting conclusion in our studies would be more meaningful for the clinic trails. Furthermore, since SAHA alone is relatively ineffective to normal cells [11], our observations indicate that the enhanced anticancer efficacy and reduced cytotoxicity to normal cells may be achieved by $HDACI_S$/cisplatin combinative treatments. With an aim to develop a more effective drug administration strategy, we compared the cytotoxicity between sequential treatment and concurrent treatment with SAHA and cisplatin. Our data demonstrated that no significant difference could be observed (Fig. 2C), suggesting that both of the administration approaches could be considered effective. Nevertheless, further studies including generating OSCC xenograft mouse model should be conducted to confirm our results.

It is widely accepted that induction of apoptosis is the primary cytotoxic mechanism of most cancer chemotherapeutic agents, and abnormalities in the control of apoptosis can affect the sensitivity of malignant cells to multiple drugs [24–26]. Molecular events involved in HDACIs-mediated apoptosis include cleavage of BID, activation of stress-related pathway and cytoprotective pathway, etc. [27–29], whereas the apoptotic effect of cisplatin is mainly exerted by the induction of mitochondria-mediated activation of caspase [30]. Our present studies demonstrate that low dose (2 μM) SAHA used alone did not induce obvious cell apoptosis. Although 4 μg/ml cisplatin is

sufficient to trigger weak apoptotic events in both cancer cell lines, cotreatment of cells with both agents even at low dose level results in a much pronounced induction of apoptosis, which is consistent with more significant cytotoxicity as observed by MTS and colony formation assay. These results suggest that the significant cytotoxicity of OSCC cells, may due, at least partly, to the increased induction of apoptosis caused by coadminstration of subtoxic doses of cisplatin and SAHA.

Resistance to chemotherapeutic agents is known as a multifactorial phenomenon. Most factors identified so far are involved in such processes as drug uptake, target availability and interaction of drug–target to cause lethal damage [31]. Molecular mechanisms underlying cell death in response to potential lethal damage has been widely investigated. Although the results are controversial due to the complexity and diversity of apoptotic pathways in different cells, some apoptosis regulators are generally thought as crucial components of apoptosis machinery in anticancer drugs resistance. The most important regulator is tumor suppressor p53 protein and its variable expression has been implicated as determinants in sensitivity or resistance of tumor cells [32–34]. In most human cancer, the p53 apoptotic pathway are often disrupted [35]. Introduction of p53 has been reported to enhance sensitivity of cancer cells to chemotherapeutic agents [36]. Compared with single agent treatment alone, an enhanced expression of p53 could be observed in the combinative treatment group. Our observations strongly suggest that cisplatin resistance in OSCC may be circumvented by $HDACI_S$/cisplatin combinative treatment via activation of p53. To further clarify the molecular events underlying p53-mediated apoptosis, alterations of several executive components in apoptotic machinery were examined. One of them is the pro-apoptotic Bcl-2 family number, BID. BID is a novel p53 effector that has been discovered recently. The BID gene is transcriptionally regulated by p53 in response to γ-irradiation; chemosensitivity to the DNA-damaging agents, Adriamycin and 5-fluorouracil appears to be critically dependent on the presence of p53 and the cleavage of BID [37]. Cleavage of BID facilitates a conformational change in mitochondria associated Bax, which functions to initiate mitochondrial dysfunction and cytochrome C release [38]. Consistent with previous studies, the cleavage of inactive BID, and the increased cytochrome C in cytosol suggested that both BID and cytochrome C have participated in the apoptotic events (Fig. 6). Another factor examined is the apoptotic effector, caspase-3. Reduced caspase-3 activity was routinely reported to be associated with apoptosis resistance [39,40]. Consistent with these studies, our data demonstrated that caspase-3 was one of key factors mediating apoptosis in SAHA/Cisplatin cotreatment strategy (Fig. 6). Taken together, our results revealed that the alterations of diverse apoptotic factors, including p53, BID, cytochrome C and caspase-3 may contribute to sensitize OSCC cells to cisplatin via cotreatment with the histone deacetylase inhibitor, SAHA.

In summary, our study has demonstrated that cotreatment with subtoxic dose of cisplatin and SAHA synergistically increase cytotoxicity and sensitize OSCC cells to apoptosis and in this process, and we found several apoptotic components including BID, cytochrome C and caspase-3 playing important roles in p53-mediated cell death.

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