Sequence-dependent interaction between cisplatin and histone deacetylase inhibitors in human oral squamous cell carcinoma cells

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Abstract. Chemotherapeutic treatment with combinations of drugs is front-line therapy for many types of cancer. Combining drugs that target different signaling pathways often lessens adverse side-effects while increasing the efficacy of treatment and reducing patient morbidity. Histone deacetylase (HDAC) inhibitors represent a novel class of anti-neoplastic agents that act by promoting acetylation of core histones, leading in turn to the uncoiling of chromatin and activation of a variety of genes implicated in the regulation of cell survival, proliferation, differentiation, and apoptosis. A defined scheduling protocol is described by which HDAC inhibitors facilitate the cytotoxic effectiveness of cisplatin (CDDP) in the killing of carcinoma cells. An oral squamous cell carcinoma cell line (HSC-3) was treated with sodium butyrate (NaB), suberoylanilide hydroxamic acid (SAHA) or MS-275 on the day of, the day before, or the day after addition of CDDP. The IC_{50} (48-h assay) value of 3.48 μg/ml CDDP could be lowered to 0.41 μg/ml CDDP when concurrently combined with an HDAC inhibitor (MS-275). The percentage of apoptosis by treatment with CDDP for 24 h, followed by NaB for an additional 24 h without washing was significantly greater than that observed in the reverse order. Depending on the time of addition of HDAC inhibitors, CDDP-treated cells displayed varying degrees of apoptotic responses, indicating the critical nature of timing in the use of HDAC inhibitors. Interestingly, experiments suggested that cells arrested at the G_1/S checkpoint by CDDP were more sensitive to subsequent treatment with an HDAC inhibitor. Moreover, these events were associated with an enhancement of reactive oxygen species (ROS) generation and caspase-3 activation by HDAC inhibitors. They raise the possibility that combining these agents may represent a novel anti-neoplastic strategy.

Introduction

Chemotherapy has an important role to play in the multi-disciplinary treatment of advanced head and neck cancer (1). Recently, neoadjuvant chemotherapy was introduced in an attempt to improve survival rates and organ preservation. Although the benefits of chemotherapy, in terms of survival rates, for patients with head and neck cancer are controversial, evidence has been accumulating to show that patients who respond well to chemotherapy have a further beneficial response to subsequent radiotherapy, and a better survival rate after radical therapy than patients with a poor response (2). This suggests that the response to chemotherapy reflects a certain biological behavior.

Cis-diamminedichloroplatinum(II) (cisplatin, CDDP) is an effective chemotherapeutic agent against head and neck cancer, both alone and in combination with other chemotherapeutic agents or radiation therapy. Intracellular CDDP binds to DNA to form covalent platinum-DNA adducts and also acts as a DNA alkylator (3). Although the principle mechanisms that translate CDDP-induced DNA damage into cytotoxicity are not fully understood, there is considerable evidence that CDDP treatment can induce cell cycle arrest and/or apoptosis in cancer cells (4). Despite the impressive anti-neoplastic activity of CDDP, two major limitations of the drug, severe side-effects and drug-resistance, make its use difficult for cancer therapy. High doses of CDDP are more effective than low doses in cancer chemotherapy. Unfortunately, CDDP has several severe side-effects, such as nephrotoxicity and ototoxicity. The nephrotoxicity of CDDP is well documented as the most important dose-limiting factor in cancer chemotherapy, but the mechanisms of CDDP-induced nephrotoxicity are still a matter of debate (5). The toxicity of CDDP limits the dose that can be given to patients, contributing to the under-dosing of patients and failure to blunt disease. Thus, drugs that would sensitize cancer cells toward CDDP could increase their efficacy in the clinic. On the other hand, it has been shown that CDDP promotes resistance to the

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induction of apoptosis of cancer cells, suggesting that repeated use of this drug is often impedimental to successful therapy because of the appearance of CDDP-resistant cancer cells that are present *de novo* or develop in response to treatment of cancer cells. This should be considered as a limitation to its potential application in anti-neoplastic treatment. Resistance to CDDP in such cells appears to be mediated through various mechanisms, including inactivation of CDDP by thiol-containing molecules such as glutathione and metallothionein, increased DNA repair, decreased drug accumulation, increased expression of DNA topoisomerase I, and increased abundance of thioredoxin (6). One novel strategy to overcome both side-effects and anti-neoplastic drug resistance is the development of pharmacologically active compounds, designated as chemosensitizers or drug resistance modulators.

Because chromatin DNA is tightly compacted, crucial accessibility to the drug target may reduce the efficiency of these anti-neoplastic drugs targeting DNA or enzymes acting on DNA. Acetylation and deacetylation of the core histones play an important role in the regulation of gene expression. Histone deacetylase (HDAC) inhibitors are pharmacological compounds of diverse chemical structures that induce hyper-acetylation of nuclear histones, weaken the histone-DNA inter-actions, and consequently increase accessibility to DNA (7). Several structural classes of HDAC inhibitors have been identified including the following: short-chain fatty acids (butyrate and valproic acid); hydroxamic acids (trichostatin A and suberoylanilide hydroxamic acid); cyclic tetrapeptides containing a 2-amino-8-oxo-9,10-epoxy-decanoyl (AOE) moiety (trapoxin A); cyclic peptides not containing the AOE moiety (FR901228); and benzamides (MS-275) (8). Further-more, these classes of inhibitors are promising agents with low toxicity for anti-neoplastic therapy, which can induce cell cycle arrest, differentiation, and apoptosis in neoplastic cells by modifying the expression of genes involved in these events (9,10). In addition, sodium butyrate (NaB) and other deacetylase inhibitors are not growth inhibitory to normal cells (11). HDAC inhibitors are currently in early-phase clinical trials to treat hematologic and solid tumor malignancies (12). Interestingly, HDAC inhibitors have been found to have additive and even synergistic effects with a number of anti-neoplastic agents in blocking the proliferation or inducing apoptosis in cancer cells in culture (13). How-ever, little attention has been paid to the importance of scheduling the addition of HDAC inhibitors to cytotoxic therapies.

It is of interest to find pharmacological agents that can be used in combination with CDDP to complement its anti-neoplastic activity. The use of HDAC inhibitors, probably in association with classical chemotherapy drugs, could be promising for cancer patients. In the present study, we examined whether suppression of histone deacetylation was capable of enhancing the sensitivity of oral squamous cell carcinoma cells to CDDP. Furthermore, this study examined whether the scheduling of the addition of low doses of HDAC inhibitors and CDDP altered the effectiveness of *in vitro* cancer cell cytotoxicity by this combination. Then, the potential cellular mechanisms underlying the enhanced anti-neoplastic activity were examined.

Materials and methods

Cell culture and reagents. HSC-3 cells (human oral squamous cell carcinoma cell line) were obtained from the Institute of Development, Aging and Cancer, Tohoku University. HSC-3 cells were grown in RPMI-1640 medium (Nissui Pharma-ceutical, Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum and 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2 atmosphere at 37˚C. We used a commercial preparation of CDDP (Nippon Kayaku, Tokyo, Japan). MS-275, *N*-acetyl-L-cysteine (NAC), z-DEVD-fmk and z-FA-fmk were obtained from Calbiochem (La Jolla, CA). NaB was purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell viability assays. Cell viability was evaluated by trypan blue exclusion assay. Then, the cytotoxic effects of anti-neoplastic agents were also determined in WST-8 [2-(2-methoxy-4-nitro-phenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetra-zolium, monosodium salt] colorimetric assay kit (Dojindo Laboratories, Kumamoto, Japan) (14). Cells were plated into 96-well flat-bottomed plates in triplicate wells ($5x10^3$ cells/well) and incubated overnight prior to drug exposure, and then incubated with various concentrations of CDDP and/or other drugs. After exposure at the indicated concentrations and times, WST-8 reagent was added to each well, followed by incubation for 2 h at 37˚C. Absorbance at 450 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA). Results were expressed as a percentage of the absorbance of control (untreated) cells. Furthermore, IC_{50} values were graphically defined as the inhibitory concentration of drugs at which 50% cell death occurs after 48 or 72 h of treatment.

Cell cycle analysis by flow cytometry. The cell cycle distribution was determined by analyzing the DNA content after propidium iodide staining. Adhering and floating cells were collectively harvested, fixed in 70% ethanol for 24 h, and washed with PBS. Cells were then stained with 10 μg/ml propidium iodide containing 10 μg/ml RNase A. The DNA content of the cells (10^4 cells/sample) was analyzed using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA). The fractions of cells in the G_0/G_1, S, and G_2/M phases, including the population in the sub-G_1 phase, were evaluated using a DNA software program.

TUNEL assay. The TUNEL assay was performed using a Mebstain apoptosis kit from MBL (Nagoya, Japan). Cells were collected by centrifugation, washed twice with washing buffer (PBS containing 0.2% BSA), and fixed in 4% paraformal-dehyde in phosphate-buffer on ice for 30 min. The cells were collected, washed with washing buffer, and fixed in 70% ethanol for 30 min at -20˚C. Cells were resuspended in 30 μl of reaction buffer containing 1.5 μl of FITC-dUTP and 1.5 μl of TdT. The reactions were carried out at 37˚C for 1 h. After the reactions, cells were washed with washing buffer. FITC-positive cells are counted as apoptotic cells using FACSCalibur.

Measurement of reactive oxygen species (ROS). HSC-3 cells were treated with CDDP (5 μg/ml) and/or NaB (0.5 mM). At
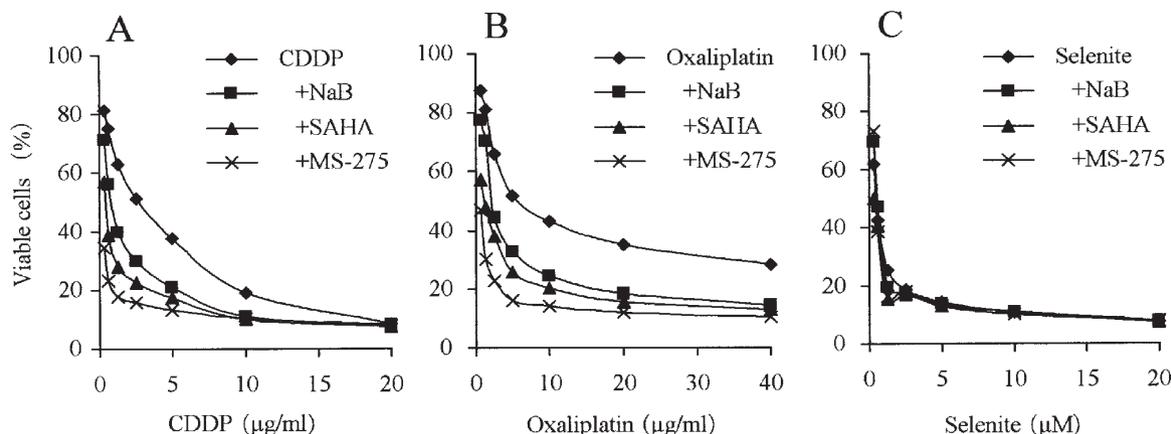
Figure 1. HDAC inhibitors potentiate the cytotoxic activity of platinum analogues in an oral squamous cell carcinoma cell line. HSC-3 cells were treated for 48 h with CDDP (A) or selenite (C) or for 72 h with oxaliplatin (B) at increasing concentrations in the absence or presence of 0.5 mM NaB, 1.5 μM SAHA, or 0.75 μM MS-275. Viable cell numbers were determined using the WST-8 assay. Viable cell numbers for the treated cultures were plotted as a percentage of the untreated control. The means of triplicate wells are shown.

1, 3 or 6 h after the beginning of the treatment, the cells were harvested and treated with 20 μM of 2',7'-dicholorodihydro-fluorescein diacetate (H$_2$DCF-DA, Calbiochem) for 30 min at 37˚C and fluorescence was measured by flow cytometry.

Assessment of mitochondrial membrane potential (ΔΨm). At specific points in time, cells were harvested, and incubated with 0.1% of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanin iodide (JC-1, Trevigen, Gaithersburg, MD) for 20 min at 37˚C. Analysis was then carried out on a FACScalibur. For the positive control, cells were activated with 50 μM SeO$_2$ (Sigma-Aldrich) (15).

Measurement of caspase activity. Caspase-3 activity was measured using a Colorimetric assay kit from MBL. In brief, cells were scraped into PBS, pelleted at low speed, and resuspended in lysis buffer for 10 min at 4˚C. Cell lysates were cleared by centrifugation and assayed for caspase-3 activity using Ac-DEVD-pNA peptide substrate, by incubation for 1 h at 37˚C. The activities were quantified spectrophoto-metrically at a wavelength of 405 nm. Caspase activity was calculated as the change in absorbance at 405 nm and expressed as nmoles of pNA produced per mg cell lysate protein per hour.

The combination of CDDP with HDAC inhibitors. For the combination experiments, three different schemes were used to investigate the interaction between CDDP and HDAC inhibitors: a) CDDP/HDAC inhibitors were exposed concur-rently for 48 h (C+H), b) CDDP was added for 24 h followed by HDAC inhibitors for an additional 24 h without washing (C→H), or c) HDAC inhibitors were added for 24 h followed by CDDP for 48 h without washing (H→C). Immediately after these treatments, the apoptotic effects were evaluated by TUNEL assay. Net increases in apoptotic cells after subtracting the apoptotic cells in each control group are shown: with CDDP for 48 h in HDAC inhibitor-free medium (CF), and in inhibitor-free medium for 24 h followed by CDDP for 48 h (F→C). Moreover, to study the importance of caspase activation and ROS generation in the induction of apoptosis, various

inhibitors, such as NAC, z-DEVD-fmk, and z-FA-fmk, were added 1 h before the CDDP and/or NaB treatment. All experiments were performed in triplicate wells.

Statistical analysis. Values are given as means ± SD. Multiple comparisons were performed using the Bonferroni/Dunn test. P<0.05 was regarded as significant.

Results

Combined treatment with anti-neoplastic drugs/HDAC inhibitors. In search for novel strategies to enhance the efficacy of chemotherapy, we investigated the effects of the HDAC inhibitors on cytotoxicity induced by anti-neoplastic drugs. We performed a WST-8 assay to evaluate the viable cell numbers and determine the IC$_{50}$ of the cell viability as a function of the concentration of anti-neoplastic drugs, in the absence or presence of HDAC inhibitors. In this study, we applied three HDAC inhibitors belonging to different structural classes, the short-chain fatty acid NaB, the hydroxamic acid SAHA (suberoylanilide hydroxamic acid) and the benzamide MS-275. Oral squamous cell carcinoma HSC-3 cells (mutated *p53*) (16) were concurrently treated for 48 h with increasing concentrations of anti-neoplastic drugs and suboptimal concen-trations of structurally different HDAC inhibitors (0.5 mM NaB, 1.5 μM SAHA, or 0.75 μM MS-275). Concurrent treatment of HSC-3 cells with one of the HDAC inhibitors significantly increased the cytotoxic efficiency of CDDP at every dose compared to that of CDDP alone (Fig. 1A). Each dose of HDAC inhibitor when used alone minimally affected the viability of HSC-3 cells in the WST-8 assay (data not shown). IC$_{50}$ values calculated from data in Fig. 1 are shown in Table I. The mean IC$_{50}$ value of CDDP for 48 h of culture without treatment with HDAC inhibitors was 3.48±1.32 μg/ml for HSC-3 cells. Combination with NaB, SAHA and MS-275 produced IC$_{50}$ values in the range of 0.41-0.91 μg/ml, which was a significant reduction compared to the IC$_{50}$ value of CDDP alone. These results indicate that HDAC inhibitors potentiate the CDDP-induced cytotoxicity.

Table I. IC$_{50}$ of chemotherapeutic drugs in combination with HDAC inhibitors.

| Drugs[a] | IC$_{50}$ (mean ± SD) | | | |
| | HDAC inhibitors[b] | | | |
	None	NaB	SAHA	MS-275
CDDP (μg/ml)	3.48±1.32	0.91±0.57	0.69±0.17	0.41±0.16
Oxaliplatin (μg/ml)	5.66±0.42	2.12±0.03	1.09±0.08	0.57±0.13
Selenite (μM)	0.47±0.03	0.58±0.02	0.50±0.02	0.48±0.06

[a]Period of incubation with CDDP or selenite, 48 h; oxaliplatin, 72 h. [b]Concentration of NaB, 0.5 mM; SAHA, 1.5 μM; MS-275, 0.75 μM.

To determine whether the enhancing effect of HDAC inhibitors was restricted to CDDP, we further tested other anti-neoplastic drugs, including a new platinum analogue, oxaliplatin (Fig. 1B), and selenite with different modes of action from that of platinum analogues (Fig. 1C) in combination with HDAC inhibitors. As shown in Table I, treatment of HSC-3 cells with oxaliplatin alone for 72 h resulted in an IC$_{50}$ value of 5.66±0.42 μg/ml. Similar to the case of CDDP, marked activity of oxaliplatin was observed with an IC$_{50}$ considerably lower than that of oxaliplatin alone when combined with each HDAC inhibitor. However, no significant enhancement by HDAC inhibitors was found regarding selenite cytotoxicity (Fig. 1C and Table I). These results indicate that the combination of platinum agents with HDAC inhibitors is selectively toxic to HSC-3 cells.

Effects of CDDP/NaB on cell cycle arrest. To elucidate whether this enhancement of cytotoxicity by HDAC inhibitors is associated with an alteration in cell cycle progression, the cell cycle profile was examined by flow cytometric analysis of propidium iodide-stained cells. Representative proportions of HSC-3 cells in each phase are shown in Fig. 2. CDDP mediated cell cycle arrest at the G$_1$/S checkpoint (increase of cells in the G$_0$/G$_1$ phase and reduction of cells in the S phase) 15 h after treatment, while NaB (0.5 mM) either alone or in combination did not affect the proportion of cells in G$_0$/G$_1$ phase (Fig. 2A), indicating that CDDP might have a dominant effect in cell cycle arrest. The population shift to G$_0$/G$_1$ was not substantial in selenite-treated cells (Fig. 2B). This G$_0$/G$_1$ phase increase may be associated with an enhancement of the CDDP-induced cytotoxicity mediated by NaB or HDAC inhibitors.

Enhancement of CDDP-induced apoptosis by HDAC inhibitors. The sub-G$_1$ population, indicating the presence of apoptotic cells, increased from 3.9 to 12.8% after 15 h in HSC-3 cells treated with CDDP alone. Furthermore, an enhancing effect (22.8%) was observed when cells were treated with CDDP/NaB. We further performed a more sensitive assay
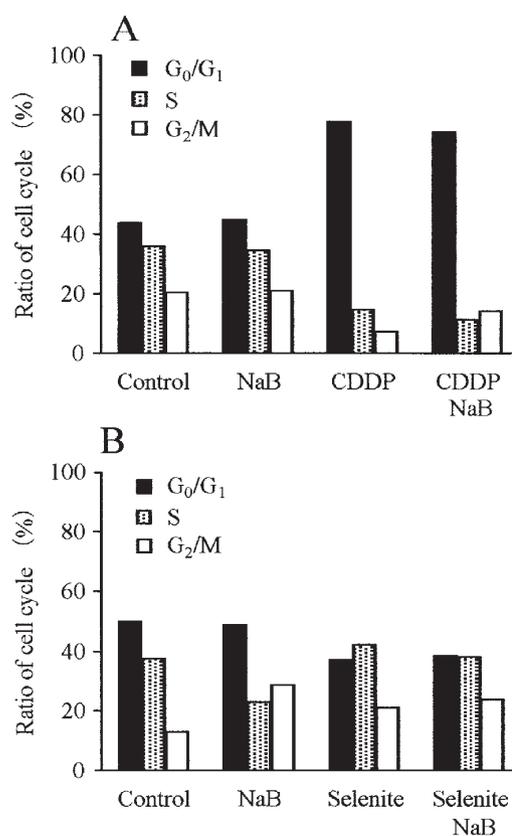
Figure 2. Cell cycling effects of CDDP or selenite/NaB combinations in HSC-3 cells. Cells were treated for 15 h with 5 μg/ml CDDP (A) or 10 μM selenite (B) either alone or in combination with 0.5 mM NaB before propidium iodide staining. The results of representative experiments are shown detailing cell cycle distribution (%) as measured by flow cytometric analysis of DNA content.
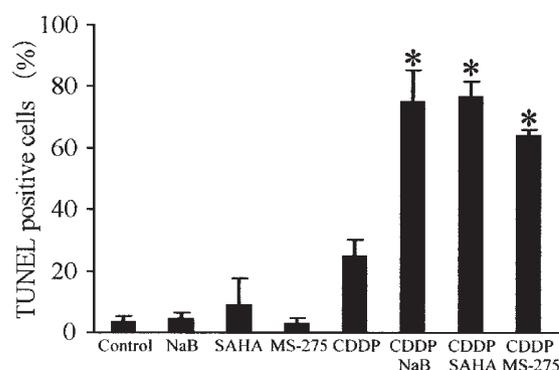
Figure 3. Combining CDDP with HDAC inhibitors increases apoptotic response. HSC-3 cells were treated concurrently with CDDP (5 μg/ml)/HDAC inhibitors (0.5 mM NaB, 1.5 μM SAHA, or 0.75 μM MS-275) for 48 h. Quantification of apoptotic cells was performed using a TUNEL assay. The data (%) shown are means ± SD of triplicate wells. *P<0.05 vs. CDDP-treated group.

(TUNEL system) that allows detection of DNA strand breaks by labeling free 3'-OH termini. Treatment with each HDAC inhibitor alone at the doses tested did not increase the levels of DNA strand breaks in HSC-3 cells (Fig. 3). When cells were incubated with CDDP alone, cellular apoptosis was induced in a dose- and time-dependent manner (data not shown). Concurrent treatment with each HDAC inhibitor for
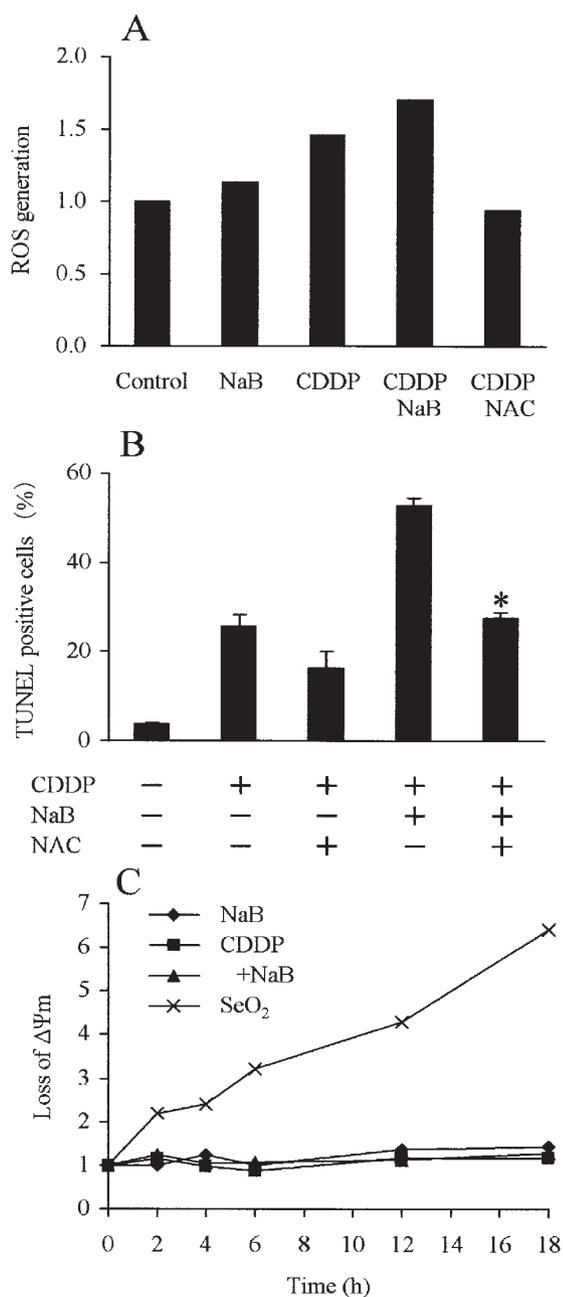
Figure 4. CDDP/NaB-induced ROS accumulation in HSC-3 cells. (A) Cells were treated for 1 h with CDDP (5 μg/ml)/NaB (0.5 mM) or NAC (5 mM). For the measurement of intracellular ROS accumulation, cells were incubated with H_2DCF-DA (20 μM) for 30 min at 37°C and analyzed by flow cytometry. Representative results are expressed as a ratio to the untreated group. (B) Cells were treated with CDDP and/or NaB with or without NAC for 48 h. Quantification of apoptotic cells was performed using a TUNEL assay. The data (%) shown are means ± SD of triplicate wells. *P<0.05 vs. CDDP/NaB-treated group. (C) Cells were treated with CDDP and/or NaB, or SeO_2 (50 μM) for the indicated periods. The harvested cells were incubated with JC-1 for 20 min, and then subjected to flow cytometric analysis. Cells with loss of ΔΨm are plotted as a ratio to the untreated group.

48 h increased the numbers of apoptotic cells several-fold above the background level (Fig. 3). This analysis revealed that CDDP in combination with HDAC inhibitors produced a significant apoptotic effect, which was larger than the sum of the effects of each drug alone.

ROS generation in apoptotic enhancement by CDDP/NaB. Studies were subsequently undertaken to characterize the effects of the CDDP/NaB combination on mitochondrial events. We first examined whether oxidative stress contributes to the apoptotic effect of this combination. As shown in Fig. 4A, following 1 h of CDDP (5 μg/ml) treatment, ROS levels rapidly increased in HSC-3 cells when compared to controls. This increase was blocked by pretreatment with anti-oxidant NAC (5 mM). Because HDAC inhibitor-induced lethality has been associated with induction of ROS (17), the possibility arose that the enhancing effects of apoptosis by the CDDP/NaB combination stem from enhanced oxidative stress. To test this possibility, cells were exposed to these agents. NaB (0.5 mM) alone had little effect on the generation of ROS in HSC-3 cells, but combined treatment resulted in a marked increase in oxidative stress (Fig. 4A). It was also found that the CDDP/NaB-induced apoptosis was significantly inhibited by the anti-oxidant, NAC. NAC was more effective in inhibiting the apoptotic effect in combination with NaB than CDDP alone (Fig. 4B). These results indicate that ROS play causative roles in the enhancement of apoptosis by CDDP/NaB in HSC-3 cells. We next determined whether these agents were acting at the mitochondrial level to reduce ΔΨm. Cells were treated with CDDP and/or NaB until 18 h before staining with JC-1. Fig. 4C shows no increase in the percentage of cells that emitted only green fluorescence after each treatment. In contrast, treatment with SeO_2 as a positive control resulted in a sustained reduction of ΔΨm in a time-dependent manner. Thus, a close correlation between loss of ΔΨm and the extent of CDDP/NaB-induced apoptosis was not observed.

Requirement for caspase-3 in apoptotic enhancement by CDDP/NaB. Since the activity of caspase-3 was considered a suitable measure of apoptotic responsiveness, the requirement for caspase-3 in CDDP-induced apoptosis was determined by the capacity to cleave its substrate, Ac-DEVD-pNA. Caspase-3 activity was evaluated after 24 and 48 h of treatment in HSC-3 cells. Fig. 5A shows that CDDP alone was able to detect significant caspase-3 activity 48 h after incubation although, at 24 h of incubation, an increase in caspase-3 activity was not observed. However, cotreatment with CDDP/NaB caused a strong activation of caspase-3 at 24 h of incubation, whereas NaB alone induced only a weak activation at 48 h. We used a pharmacological inhibitor of caspase-3 to confirm its involvement in the NaB-induced enhancement of apoptosis. The apoptosis caused by CDDP either alone or in combination with NaB was partly attenuated in the presence of the caspase-3 inhibitor, z-DEVD-fmk (Fig. 5B). The only incomplete decrease by z-DEVD-fmk might be explicable by residual caspase activities due to the relatively low dose (20 μM) of z-DEVD-fmk applied. When the cathepsin inhibitor, z-FA-fmk, which lacks inhibitory activity for caspases, was used as a negative control, no inhibitory effect was observed. These results pointed toward the involvement of the caspase-3 pathway in the enhancement of CDDP-induced apoptosis by NaB.

Enhancement of CDDP-induced apoptosis by HDAC inhibitors in a sequence-dependent manner. The results described above did not clarify whether the combination was improved by a
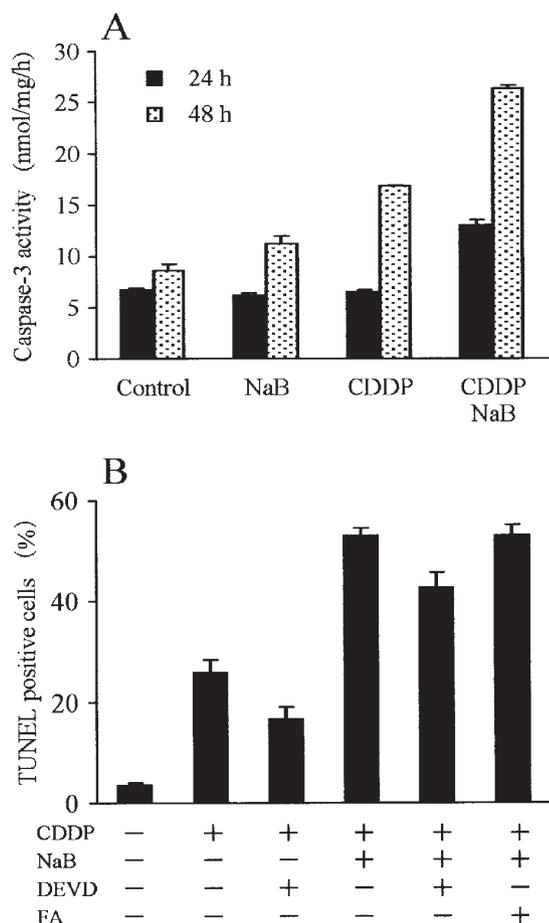
Figure 5. Effects of CDDP/NaB on caspase-3 activation in HSC-3 cells. (A) Cells were treated for 24 or 48 h with CDDP (5 μg/ml) and/or NaB (0.5 mM), after which cytosolic cell lysates were prepared and then assayed for DEVDase (caspase-3) activity. Results are expressed as the amount (nmol) of pNA released by 1 mg of protein in 1 h and as means ± SD of three trials for measurement. (B) Cells were pretreated for 1 h with 20 μM of z-DEVD-fmk or z-FA-fmk and then treated with CDDP and/or NaB. After 48 h of treatment, apoptotic cells were determined by TUNEL staining. The data (%) shown are means ± SD of triplicate wells.

Figure 6. Sequential treatment with CDDP/HDAC inhibitors and the induction of apoptosis in HSC-3 cells. Cells were treated with CDDP (5 μg/ml)/0.5 mM NaB (A) or 1.5 μM SAHA (B) in different ways (H→C, C+H, or C→H) as described in Materials and methods. Cells were then stained by the TUNEL method and subjected to flow cytometric analysis. Net increases (%) in apoptotic cell numbers after subtracting the number of apoptotic cells in each control group (F→C and CF) are shown as means ± SD of triplicate wells.

defined sequence of addition. An analysis was conducted to determine the nature of drug interactions and whether the interactions were sensitive to the timing of the addition of the two drugs. HSC-3 cells were treated sequentially with CDDP (5 μg/ml) for 24 h, followed by NaB (0.5 mM) for an additional 24 h without washing (C→H) or with the same drugs given in sequence of NaB for 24 h, followed by CDDP for 48 h (H→C). The apoptosis percentage with C→H was significantly greater than that observed with CDDP for 48 h in NaB-free medium (CF). The net increase after subtracting the apoptotic cells in CF was 24.2% of apoptotic cells (Fig. 6A). When the cells were treated in the reverse order such that NaB treatment preceded CDDP treatment, only 9.7% of the cells underwent apoptosis. The results of the sequential treatment C→H were not so different from the effects of concurrent treatment C+H (24.6%). Similar effects were seen with 1.5 μM SAHA (Fig. 6B), demonstrating that the sequence of drug addition is important to the response. These findings suggest that HDAC inhibitors are even active when added after CDDP, and cells might have more of a chance of being affected with C→H.

Discussion

In this study, we examined the effects of the exposure of oral squamous cell carcinoma cells with a mutated *p53* to both HDAC inhibitors (NaB, SAHA, and MS-275) and the anti-neoplastic agents on the induction of apoptosis. Treatment with HDAC inhibitors resulted in a timing-specific enhancement of the anti-neoplastic effects of platinum analogues, CDDP and oxaliplatin. Of particular importance to the clinician is the potential use of HDAC inhibitors to achieve a 3~10-fold increase in the effectiveness of the IC_{50} dose of platinum analogues to a level similar to the IC_{80} dose in 2 or 3-day cytotoxicity (Fig. 1A and B). In general, the toxicity of anti-neoplastic agents could be overcome by the use of rational chemotherapeutic combinations, where toxic agents are used in lower doses complemented with a non-toxic agent that has a different mechanism of action and increases the efficacy of treatment. The increase in effectiveness of the IC_{50} dose of CDDP could allow a patient to potentially receive lower levels of CDDP analogues resulting in a similar treatment outcome with reduced toxicity to the patient.

Gene silencing, associated with tumorigenesis and chemotherapeutic resistance, has recently emerged as an area of intense investigation due to recent advances in our understanding of the link between modifications of chromatin structure and the transcriptional activity of genes (18). HDAC inhibitors are a new class of drugs with anti-neoplastic potential. Currently, phase I and II clinical trials are ongoing for four different types of HDAC inhibitors, namely sodium phenylbutyrate, FK228, SAHA, and MS-275, in hematologic malignancies and various solid tumors (19). Because histone deacetylase is overexpressed in many cancers, and the death-inducing capability of different HDAC inhibitors correlates with their HDAC-inhibitory potency, it is widely accepted that the cell death-inducing function of HDAC inhibitors is due to their ability to inhibit HDAC activity (8,9). Such actions of HDAC inhibitors result in the hyperacetylation of core histones and transcriptional activation of genes involved in these processes. In addition, HDAC inhibitor-induced structural changes of the chromatin may render the DNA more accessible and HDAC inhibitors may therefore be used to potentiate DNA damaging agents (20). However, the specific downstream events responsible for the induction of cell death remain to be fully elucidated. Therefore, the optimal schedule for the drug in combination remains to be established and is the subject of ongoing clinical trials. One of the aims of this work is to illustrate the importance of the sequence in which the combination of CDDP/HDAC inhibitors is added. As shown in Fig. 6, when HDAC inhibitors were added with or after CDDP in HSC-3 cells, strong cytotoxic enhancement was observed. In contrast, weak cytotoxicity was observed when CDDP was added after HDAC inhibitors. Recently, it has been demonstrated that, in Jurkat cells, exposure to an HDAC inhibitor followed by anti-neoplastic agents such as fludarabine resulted in an additive to synergistic induction of apoptosis (21). However, in this study, we showed that HDAC inhibitors are also active when added after CDDP. This observation is consistent with a previous report in which the exposure of HL-60 cells to DNA-damaging agents, including 5-azacytidine, followed by NaB resulted in a marked increase in lethality (22). Additional studies have demonstrated that treatment of drug-resistant cells with trichostatin A (TSA) increases the cytotoxicity of a topoisomerase II inhibitor (VP-16) (23). TSA is still active and can increase histone H4 acetylation, even when added after VP-16. It is thus likely that modulation of the acetylation status generated by the post-treatment with HDAC inhibitors increased the efficiency of the anti-neoplastic drugs targeting DNA or enzymes acting on DNA.

Two responses take place when DNA is damaged by anti-neoplastic drugs. When the DNA damage is small, the cell cycle is arrested to provide time for repair. But when the DNA damage is too serious to be repaired, a failure to do so leads to the acquisition and accumulation of genetic alterations, which can ultimately cause apoptosis or tumorigenesis (24). The G_1/S and G_2/M checkpoints are crucial periods in the cell cycle when DNA damage is recognized and repaired, and failing that, apoptotic pathways are triggered. An increase in the percentage of cells in the G_0/G_1 phase was seen for HSC-3 cells when CDDP alone and in combination with HDAC inhibitors was given (Fig. 2A). This increase in G_0/G_1 phase

cells may be associated with the cytotoxic enhancement observed for concurrent and sequential C→H treatment. Checkpoints for CDDP-induced DNA damage may be positioned before the cell enters the S phase (G_1/S checkpoint). When DNA damage occurs at the G_1/S checkpoint, cell cycle arrest induced by DNA damage is usually p53-dependent. However, one study indicated that CDDP-mediated DNA damage could induce G_0/G_1 arrest through two processes: a fast p53-independent initiation of G_0/G_1 arrest caused by cyclin D1 proteolysis and a slower maintenance of arrest resulting from increased p53 stability (25). NaB (1 mM) treatment led to a major G_2/M arrest of cells in the presence of p53, while cells without wild-type p53 accumulated mainly in the G_0/G_1 phase of the cell cycle (26). Because disruption of HDAC inhibitor-induced G_0/G_1 arrest prevents differentiation, the suggestion has been made that HDAC inhibitor-induced differentiation is dependent on cell cycle arrest in G_0/G_1. TSA treatment significantly enhances topoisomerase IIα promoter activity in G_0/G_1 cells (27). Furthermore, the apparent relationship between G_0/G_1 arrest and apoptosis has also led to speculation that differentiation and apoptosis might be mutually exclusive actions of HDAC inhibition (28). A novel HDAC inhibitor, BL1521, added to neuroblastoma cells induced both arrest in the G_0/G_1 phase of the cell cycle and an increase in the number of apoptotic cells via the up-regulation of p21[WAF1/CIP1] expression and/or down-regulation of cyclin-dependent kinase (Cdk4) expression (29). These results support our hypothesis that cells are more sensitive to HDAC inhibitors in G_0/G_1 than in other phases of the cell cycle and an increase of cells re-entering the G_1 phase from the M phase due to CDDP may cause enhancement of cytotoxicity on sequential treatment with HDAC inhibitors. Alternatively, the enhancement of cytotoxicity by HDAC inhibitors may depend on the type of insult, because the action modes of anti-neoplastic drugs used in this study are distinct and, thus, the level of cytotoxicity varied (Fig. 1). CDDP and oxaliplatin form the same types of adduct at the same sites on DNA. Like CDDP, oxaliplatin induced a cell cycle block in the G_0/G_1 phase of human colorectal cancer cells although to a lesser extent than did CDDP (30). The mode of action of anti-neoplastic drugs that directly interact with DNA suggests that HDAC inhibitor-induced chromatin decondensation may be also considered to increase the accessibility to DNA of the platinum analogues. At higher concentrations, selenite induces oxidative stress and may become toxic. Selenite metabolism results in the generation of superoxide and oxidative stress through its reaction with reduced glutathione (15). DU145 human prostate cancer cells exposed to 5 μM selenite were arrested in S phase without any significant accumulation of G_0/G_1 cells sensitive to HDAC inhibitors (31). Consequently, enhanced activity was not observed with selenite (Fig. 1C).

There are reports that the increased generation of ROS is closely associated with CDDP-induced apoptosis (32). Functional assays using chemical inhibitors demonstrated that the phosphorylation of extracellular signal-regulated kinase (ERK) was mediated by ROS and required the activation of caspase-3 (33). On the other hand, the contribution of ROS to HDAC inhibitor-mediated lethality in neoplastic cells

has previously been described (17). The mechanism by which this phenomenon occurs is not known with certainty, but may involve alterations in the expression of redox-related genes, including the thioredoxin and superoxide dismutase genes (34). Enhanced lethality of NaB/CDDP treatment was associated with an increase in ROS generation and was significantly attenuated by the free radical scavenger, NAC (Fig. 4B). The present results strongly indicate that enhanced oxidative damage plays an important functional role in enhanced interactions between CDDP and NaB in HSC-3 cells. Interestingly, despite the increased accumulation of ROS with the combined exposure, there was preservation of the ΔΨm. It is suggested that Bax and other pro-apoptotic Bcl-2 member proteins are polymerized and insert into the outer mitochondrial membrane and allow cytochrome *c* extravasation without disrupting mitochondrial function or the ΔΨm (35).

The ERK pathway is essential for the gene expression of procaspase-3 (36). CDDP-induced apoptosis is mediated by sustained activation of the ERK signaling pathway that occurs upstream of mitochondrial signaling events including caspase-3 activation in A172 human glioma cells (37). In addition, ERK1/2 expression increases in mid-G_1 phase and ERK phosphorylation and activation occur during G_1 phase (38). Since HDAC inhibitors active at the G_0/G_1 phase markedly synergized in triggering caspase-3 activity, this apoptotic enhancement crucially relied on the activation of caspases (Fig. 5). Treatment with HDAC inhibitors led to a significant amplification of TRAIL's cytotoxic activity. This sensitization greatly relied on activation of caspases, since it was significantly reversed by the broad-spectrum caspase inhibitor, z-VAD-fmk (39). Jurkat lymphoid and LIM 1215 colorectal cancer cells primed with a low concentration of butyrate that itself did not induce activation of caspase-3 or apoptosis, were rendered highly susceptible to induction of apoptosis by staurosporine. Then, NaB and TSA induced the same program of apoptosis that is strictly dependent on new protein synthesis and that leads to the conversion of the pro-enzyme form of caspase-3 to the catalytically active effector protease and apoptotic death. It was proposed that changes at the level of chromatin structure, induced by a physiological substance, butyrate, lead to the expression of a protein that facilitates the pathway by which mitochondria activate caspase-3 and trigger apoptotic death of cancer cells (40). Furthermore, butyrate treatment activates caspase-3 and induces the accumulation of acetylated histone in MDBK cells (41). Cells were possibly arrested at the G_1/S boundary by the butyrate treatment. During the process of apoptosis, certain proteins become targets for destruction. At least two proteins, Cdc6, an essential initiation factor for DNA replication, and Cdc2/Cdk1, become targeted for proteolytic destruction on butyrate treatment. These two proteins are down-regulated by the ubiquitination/proteasome pathway interacting with the caspase-signaling pathway. Prereplicative complexes are assembled in late mitosis or early G_1 and are maintained by Cdc6 and other proteins until initiation occurs in the S phase, mediated by cyclin-dependent kinase activity. Cdc6 has six putative Cdk1 phosphorylation sites and phosphorylation by Cdk1 plays a significant role in protein stability (42). The cleavage of Cdc6 thus seems to be a general phenomenon that occurs in apoptosis (43). It is also very likely that activation of caspase-3 directly correlates with the destruction of two cell cycle regulators, strongly indicating that these two proteins may be the cause of the progression of apoptosis.

In conclusion, our studies suggest that HDAC inhibitors exhibit marked enhancing effects on cytotoxicity when added with or after CDDP to human oral carcinoma cells and provide a rationale for future clinical studies of this combination. Understanding how HDAC inhibitors work in combination with other anti-neoplastic drugs is essential to increasing their effectiveness in the clinical setting. The results of this study highlight the need to evaluate the role of scheduling in using HDAC inhibitors with platinum agents. In this context, the molecular pathways affected by the combination of CDDP/HDAC inhibitors (NaB), the mechanisms behind the enhanced cancer cell killing, and the possible influence of aberrant growth signals initiated by HDAC inhibitors are being discussed.

Acknowledgements

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