Epigenetic regulation of chemosensitivity to 5-fluorouracil and cisplatin by zebularine in oral squamous cell carcinoma

MAIKO SUZUKI[1], FUMIAKI SHINOHARA[2], KENTARO NISHIMURA[1,2], SEISHI ECHIGO[2] and HIDEMI RIKIISHI[1]

Departments of [1]Microbiology and Immunology and [2]Oral Surgery, Tohoku University Graduate School of Dentistry, Aoba-ku, Sendai 980-8575, Japan

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Abstract. Epigenetic alterations such as histone acetylation and DNA methylation play an important role in the regulation of gene expression for cell cycles and apoptosis that may affect the chemosensitivity of cancers. Previously, we have reported that the combination of suberoylanilide hydroxamic acid (SAHA), a newly developed histone deacetylase inhibitor, with cisplatin (CDDP) possessed synergistic cytotoxicity against human oral squamous cell carcinoma (OSCC) cell line HSC-3. In this study, we used a novel DNA methyltransferase inhibitor, zebularine (Zeb), to investigate the epigenetic influence on the sensitivity of carcinoma cell lines to 5-fluorouracil (5-FU) or CDDP by evaluating apoptotic inducibility. Treatment with CDDP or 5-FU either alone or in combination with Zeb or SAHA continued for 48 or 72 h. In HSC-3 cells, Zeb had chemosensitive efficacy with CDDP, but not 5-FU, whereas SAHA showed efficacy with both CDDP and 5-FU. We showed that Zeb has strong anti-proliferative activity against HSC-3 cells, shown by decreased cellular growth and G_2/M cell cycle phase accumulation. Furthermore, DNA methylation could be a regulatory mechanism for dihydropyrimidine dehydrogenase (DPD), known to be a principal factor in 5-FU resistance. CDHP (5-chloro-2,4-dihydroxypyridine), an inhibitor of DPD, had an enhancing effect on the apoptotic ability of 5-FU alone or 5-FU/Zeb combination. In conclusion, the present study suggests that low-dose (IC_{20}) Zeb may sensitize cancer cells to CDDP, which may be an important characteristic for solid cancer treatment, and that DPD and other agents activated by Zeb in cancer cells could be an inhibitory factor in the response to apoptosis induced by 5-FU.

Correspondence to: Dr Hidemi Rikiishi, Department of Microbiology and Immunology, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
E-mail: riki@mail.tains.tohoku.ac.jp

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Introduction

Combination chemotherapy remains the predominant treatment approach before and after surgery for advanced malignant cancers. The combination of cisplatin (cis-diamminedichloroplatinum II, CDDP) and 5-fluorouracil (5-FU) has been the most active regimen in oral squamous cell carcinoma (OSCC) (1). However, the presence of cancer cells with intrinsic or extrinsic resistance is a severe impediment to successful therapy. As the majority of conventional anti-cancer drugs exert their effects by inducing apoptosis, such resistant cells can often up-regulate anti-apoptotic oncogenes and/or down-regulate pro-apoptotic tumor suppressors (2). This mis-regulation may be caused by coding sequence mutations, gene deletions, or epigenetic aberrations (3). Aberrations in DNA methylation are now well established in the process of carcinogenesis and the acquisition of drug resistance. In particular, tumor suppressors are often rendered non-functional and, in many cases, loss of function is associated with epigenetic modifications, primarily DNA methylation. In this regard, recent work has demonstrated that epigenetic transcriptional silencing by promoter methylation of tumor suppressor genes is relatively common in OSCC and is an important feature of this disease (4). Thus, re-expression of tumor suppressors and apoptosis-related genes is one proposed strategy for the sensitization of drug-resistant cancers to conventional anti-cancer drugs.

Virtually all types of human cancer have epigenetic abnormalities which collaborate with genetic changes to drive the progressive stages of cancer evolution (3). Primarily, DNA methylation and histone modification are the principal driving forces behind the phenomenon of epigenetics (5). Compared to genetic mutations, epigenetic events are not accompanied by changes in the DNA sequence itself and are potentially reversible, which make them attractive targets for therapeutic intervention. Recent advances in the understanding of epigenetic processes in cancer have led to the development of several beneficial inhibitors of DNA methyltransferase (DNMT) and histone deacetylase (HDAC). The methylation of cytosines, usually located within dinucleotide CpG-rich promoters, is catalyzed by enzymes known as DNMTs. DNMT inhibitors, such as 5-aza-cytidine (5-aza-CR) and 5-aza-2'-deoxycytidine (5-aza-CdR, clinically referred to as decitabine), resulting in genomic

hypomethylation, have been widely studied. However, these inhibitors have some drawbacks in that they are quite toxic *in vitro* and *in vivo*, and are unstable in aqueous solution, making them difficult to administer both experimentally and clinically. The novel DNMT inhibitor zebularine (Zeb) has been demonstrated to be stable and minimally toxic, although higher concentrations of Zeb are needed to obtain similar levels of demethylation in cancer cells in comparison with 5-aza-CdR (6). The stability and minimal cytotoxicity of Zeb allowed normal cells to grow in the continuous presence of the agent, and led to the induction and maintenance of the expression of silenced genes in cancer cells, which thereby circumvented the problem of remethylation. Another commonly occurring epigenetic modification in silenced genes is histone hypoacetylation. Suberoylanilide hydroxamic acid (SAHA, referred to as vorinostat) is an HDAC inhibitor, currently undergoing clinical trials, which causes hyperacetylation of histones H3 and H4 (7). As histone deacetylation, in parallel with DNA methylation, is associated with gene silencing, crosstalk between their two processes has been proposed (8). Despite the promise of epigenetic therapy, there are several problems that must be considered. One relates mainly to the non-selectable activation of genes, and also to potential mutagenicity and carcinogenicity. Another potential problem with epigenetic therapy is that demethylated promoters tend to undergo slow remethylation and resilencing after the removal of DNA methylation inhibitors (9). Nevertheless, it has been established that the inhibition of DNMT and HDAC activities can strongly inhibit the formation of cancers (6,7). Thus, trials to validate the approach will be based on patients with life-threatening diseases such as cancer.

As DNA methylation has been shown to contribute to the silencing of chemosensitivity-related genes, inhibitors of methylation may enhance the sensitivity of cancer cells to anti-cancer drugs (10). Indeed, these epigenetic inhibitors have their broadest activity in chemosensitizing recalcitrant cancers in combination with conventional anti-cancer drugs. For example, the demethylating agent Zeb re-sensitized the CDDP-resistant cell line of ovarian cancer, with a 16-fold reduction in the IC_{50} of CDDP (10). 5-aza-CdR treatment of the ovarian drug-resistant cell line A2780/CP70 restored mismatch repair protein (hMLH) activity and CDDP sensitivity in both cultured cells and mouse xenografts (11). Furthermore, 5-aza-CdR restored the expression of hMLH1 and sensitivity to 5-FU in colorectal cell lines (12). Although HDAC inhibitors activate a more limited set of epigenetically silenced genes, low doses of SAHA also showed synergistic anti-cancer activity with cytotoxic agents, such as radiation, kinase inhibitors, and differentiating agents (13). In our OSCC model, combination treatment (CDDP/HDAC inhibitors) yielded a synergistic effect on apoptotic induction (14); however, the basis of the synergistic induction of anti-cancer activity by these combinations remains unclear but epigenetic therapies directed toward tumor suppressor re-expression could potentially reverse malignant phenotypes and chemosensitize recalcitrant cancers.

With the aim of improving the chemotherapeutic efficacy of OSCC, we investigated a novel combination strategy of anti-cancer drugs with DNMT inhibitor Zeb or HDAC inhibitor SAHA, and showed that these epigenetic inhibitors interact with anti-cancer drugs in a highly synergistic manner in the OSCC cell line to induce apoptosis based on their diverse effects. This suggests the possible application of Zeb in oral cancer therapy in combination with conventional drugs. With this exception, we studied the molecular basis of the unexpected degradation of anti-cancer efficacy of 5-FU by Zeb, which may be attributable to aberrant methylation of the dihydropyrimidine dehydrogenase (DPD) gene that plays a major role in 5-FU metabolism.

Materials and methods

Cell lines and reagents. Human carcinoma cell lines including HSC-3 cells (tongue squamous cell carcinoma) for principal experimental research and A549 cells (non-small-cell lung carcinoma, NSCLC) as reference resistant cell line, were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Japan). The cells were grown in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Both cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37˚C. 5-FU (Kyowa Hakko Kogyo, Tokyo, Japan) and CDDP (Nippon Kayaku, Tokyo, Japan) were used in this study. Zebularine [1-(ß-D-ribofuranosyl)-1,2-dihydropyrimidin-2-1] and SAHA were purchased from Sigma (St. Louis, MO) and Alexis Biochemicals (San Diego, CA), respectively. CDHP (5-chloro-2,4-dihydroxypyridine) was generously provided by Taiho Pharmaceutical Co. Ltd. (Tokyo, Japan). All other chemicals used were commercially available.

MTT assay. Cells were seeded into 96-well plates at a density of $1x10^4$ cells per well, so that the cells were in a log growth phase. Twenty-four hours after plating, cells were treated with CDDP (48 h) or 5-FU (72 h). For combination treatments, cells were treated with Zeb for 48 h, followed by treatment with anti-cancer drugs, or with SAHA concomitantly with anti-cancer drugs. A colorimetric MTT/WST-8 assay (Dojindo Laboratories, Kumamoto, Japan) was then performed to measure cell growth. The untreated controls were assigned a value of 100%.

Trypan blue exclusion assay. Total cell number and cell viability (cytotoxicity) after drug treatment were determined by the mean of the trypan blue exclusion assay. Cells were incubated without (controls) or with drug(s) for 48 or 72 h. Floating and adherent cells were collected and then incubated with trypan blue solution (0.1% final concentration) for 1 min, and the numbers of trypan blue-positive and -negative cells were determined using a haematocytometer.

Cell cycle analysis. Cell cycle distribution was measured with or without treatment with Zeb for 48 h. After culture, cells were harvested (including detached cells), suspended in PBS and fixed in 70% ethanol, and routine DNA staining was performed by the addition of propidium iodide and RNase to each sample. Cellular DNA content was measured by a FACSCalibur (Becton-Dickinson, San Jose, CA). Analysis of

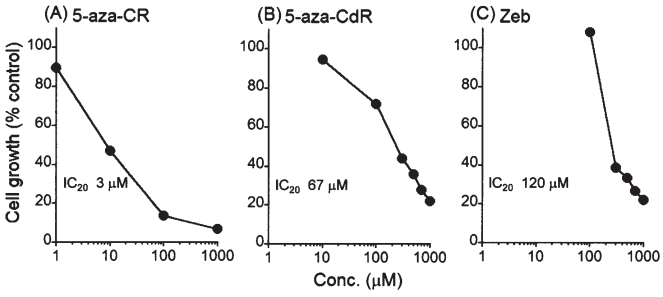

Figure 1. Effects of DNMT inhibitors on growth of OSCC cell line. HSC-3 cells were treated with increasing concentrations of 5-aza-CR (A), 5-aza-CdR (B), or Zeb (C) for 48 h, and cell growth was measured by MTT assay. The concentrations required to inhibit 20% of cells (IC_{20}) were calculated from dose-response curves. Results represent the mean of triplicate wells.

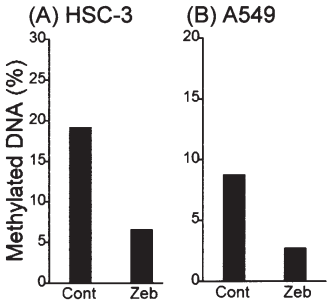

Figure 2. Reduction of global DNA methylation in Zeb-treated cells. HSC-3 (A) or A549 cells (B) were treated with Zeb (IC_{50}) for 48 h. DNA was extracted from untreated and treated cells, immobilized to the wells, and incubated with 5-methylcytosine antibodies. Global DNA methylation was quantified through an ELISA reaction. Results are representative of triplicate experiments, and are expressed as an absolute percentage of 5-methylcytosine.

the percentage of cells in G_0/G_1, S and G_2/M phases of the cell cycle was made using CellQuest software (Becton-Dickinson). Cell debris and fixation artifacts were gated out.

Flow cytometric analysis of apoptotic cells using TUNEL staining. Apoptotic cells were assayed by the TUNEL method using the MEBstain apoptosis kit direct (MBL, Nagoya, Japan) for flow cytometric analysis. Cells were treated with anti-cancer drugs with or without Zeb or SAHA. In addition, cells were treated with Zeb for 48 h, followed by treatment with CDHP 1 h before the addition of 5-FU. Cells were fixed with 4% paraformaldehyde for 30 min at 4˚C, washed with BSA-PBS, and permeabilized with 70% ethanol for 30 min at -20˚C. After washing cells with BSA-PBS, they were incubated with terminal deoxynucleotidyl transferase and FITC-dUTP for 60 min at 37˚C. Cells were then rinsed with BSA-PBS and evaluated by flow cytometry.

Global DNA methylation. To quantify the global DNA methylation of cells, a DNA methylation quantification kit (Epigentek Inc., Brooklyn, NY) was used. In this assay, DNA was immobilized on a strip with high affinity to the DNA. The methylated fraction of DNA was recognized by 5-methylcytosine antibody and quantified through an ELISA reaction. Results are expressed as the absolute percentage of 5-methylcytosine.

Statistical analysis. All assays were in principle performed in triplicate. Values are given as the means ± SD. Data were analyzed by the Bonferroni/Dunn multiple comparison test. A p-value of <0.05 was considered statistically significant.

Results

Effects of DNMT inhibitors on growth of OSCC cells. Initially, to determine the inhibitory efficacy of DNMT inhibitors (5-aza-CR, 5-aza-CdR, and Zeb) on the growth of carcinoma cells, we used the MTT assay after 48 h of incubation using the OSCC cell line (HSC-3). The growth inhibition by each compound in HSC-3 cells was dose-dependent and inhibition by Zeb occurred at doses as high as 100 μM, in contrast to the prominent inhibition observed with 5-aza-CR or 5-aza-CdR (Fig. 1). The results in Fig. 1 revealed a strong variability in IC_{20} concentrations between individual compounds, ranging between 3 μM (5-aza-CR) and 120 μM (Zeb). We designed a method to determine the chemosensitizing properties of Zeb because of its favorable pharmacological properties, including its chemical stability and low toxicity observed during continuous treatment. The IC_{20} that caused 20% inhibition of growth was chosen to examine the effects of Zeb on the cytotoxicity exerted by anti-cancer drugs (CDDP and 5-FU).

Reduction of DNA methylation in Zeb-treated cells. We then examined the effects of Zeb on the level of global DNA methylation at or close to IC_{20}. This assay showed that the level of methylated cytosine in DNA was decreased to 34% of control cells in Zeb (IC_{50}, 220 μM)-treated HSC-3 cells (Fig. 2A). The level of DNA methylation in A549 cells was lower than in HSC-3 cells. Zeb treatment also resulted in genomic demethylation in the A549 cell line (Fig. 2B). These results suggest that the inhibition of DNMTs by Zeb triggers a reduction in the fraction of methylated CpG dinucleotides in the genome under these experimental conditions.

Cytotoxic efficiency of anti-cancer drugs in combination with Zeb or SAHA. To examine whether epigenetic mechanisms affect the cytotoxic effect induced by anti-cancer drugs (CDDP or 5-FU), HSC-3 cells were treated with Zeb at IC_{20} for 48 h, followed by treatment with increasing concentrations of CDDP or 5-FU, or with SAHA (1.5 μM) concomitantly with CDDP or 5-FU. Concomitant treatment with Zeb did not show any significant effect on the cytotoxicity of both anti-cancer drugs (data not shown). After treatment, cytotoxic efficiency was measured by MTT assay, and was evaluated by comparison with the expected additive effect. Treatment of the cells with CDDP (48 h) or 5-FU (72 h) alone had cytotoxic effects in a dose-dependent manner (Fig. 3). Based on the MTT results, in the following combined experiments, we used the concentrations of CDDP (5 μg/ml) and 5-FU (250 μg/ml) to obtain an effective outcome. The dose-response curves for both anti-cancer drugs (Fig. 3) shifted to a lower concentration range when used in combination with Zeb or SAHA. These data suggest that

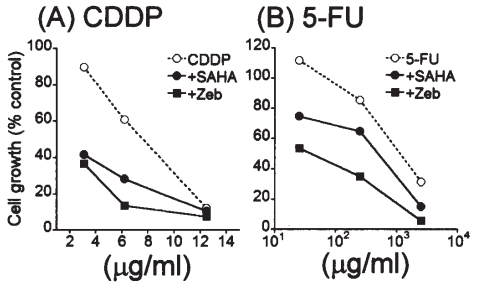

Figure 3. Inhibitory efficiency of anti-cancer drugs in combination with epigenetic agents. HSC-3 cells were treated with Zeb (IC_{20}) for 48 h, followed by treatment with increasing concentrations of CDDP or 5-FU, or with SAHA (1.5 μM) concomitantly. MTT assays were performed at 48 h for CDDP (A) or at 72 h for 5-FU (B). Depicted curves represent the percentage of cell growth to the untreated control. Each dilution was performed in triplicate and the mean values are shown.

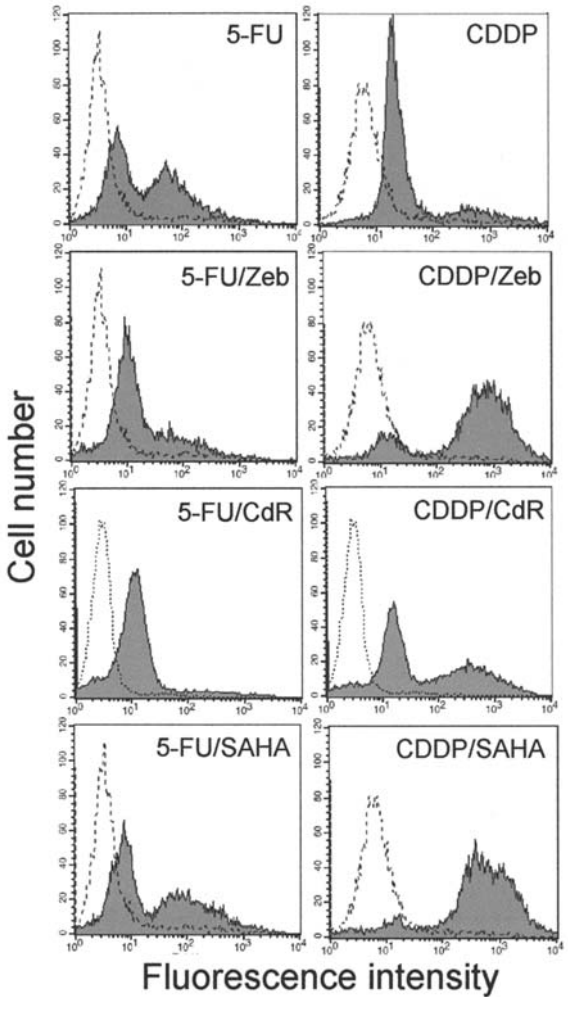

Figure 4. Flow cytometric detection of apoptosis of HSC-3 cells. Cells were treated with CDDP (5 μg/ml) or 5-FU (250 μg/ml) alone or in combination with Zeb (120 μM), 5-aza-CdR (67 μM), or SAHA (1.5 μM), after which they were stained with the TUNEL method and evaluated by cytometric analysis. Results show flow cytometric histograms depicting apoptotic cells in untreated (unshaded) or treated samples (shaded), which correspond to one representative result of three experiments.

either DNA demethylation by Zeb or histone hyperacetylation by SAHA could increase the cytotoxic efficiency triggered by CDDP or 5-FU in HSC-3 cells.

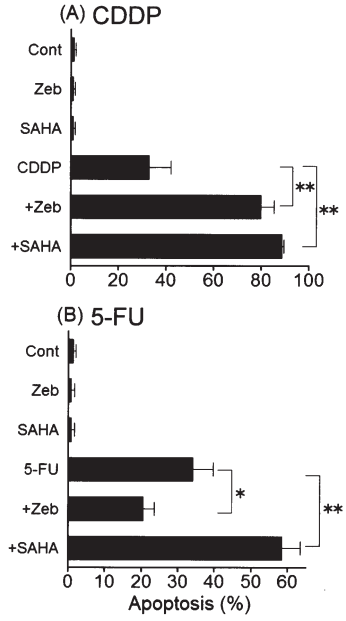

Figure 5. Effects of Zeb or SAHA on apoptosis induced by anti-cancer drugs. TUNEL assay for HSC-3 cells treated with 5 μg/ml CDDP (A) or 250 μg/ml 5-FU (B) alone or in combination with Zeb (120 μM) or SAHA (1.5 μM) was performed for DNA fragmentation. Fluorescence-positive cells representing the apoptotic subpopulation undergoing DNA fragmentation were quantified. Results are expressed as the means ± SD of triplicate experiments. *P<0.05; **P<0.01 vs. each drug alone group.

Effects of Zeb or SAHA on apoptosis induced by anti-cancer drugs. We next examined whether this observed cytotoxic enhancement was not just growth inhibition but rather represented cell death. To examine the ability of the anti-cancer drugs used in this study to induce apoptosis in HSC-3 and A549 cells, cultures were treated with CDDP or 5-FU in the absence or presence of Zeb or SAHA, after which they were stained with the TUNEL method and evaluated by cytometric analysis. Fig. 4 shows the representative cytometric profiles, showing apoptotic death of the cell population in HSC-3 cells. Furthermore, Fig. 5 indicates the percentages of apoptotic cells in drug alone or in combinations. The apoptosis rates of HSC-3 cells induced by CDDP or 5-FU alone were 32.7±9.2% and 34.0±5.6%, respectively, whereas treatment of the cells with Zeb or SAHA alone had no effect on the level of apoptosis. Combination treatments of CDDP with Zeb or SAHA resulted in a significant enhancement of apoptotic cell death after 48 h (Fig. 5A), consistent with the results obtained with MTT assay (Fig. 3A). In contrast to CDDP, Zeb reduced the apoptotic effect of 5-FU in HSC-3 cells although SAHA enhanced its effects (Fig. 5B). This is not consistent with the increase of cytotoxicity found in the MTT assay (Fig. 3B). A similar observation was made in treatment with 5-aza-CdR (IC_{20}), instead of Zeb (Fig. 4). A549 cells were primarily resistant to both drugs compared to HSC-3 cells, and did not show significant apoptotic enhancement in combination (data not shown).

Effects of 5-FU and/or Zeb on cell number and cytotoxicity. The trypan blue exclusion assay as a measure of cell number and cytotoxicity was performed to clarify the apparently discrepant results observed with 5-FU/Zeb combination

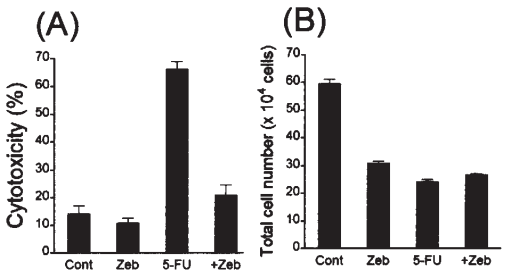

Figure 6. Effects of Zeb treatment on cell number and cytotoxicity. HSC-3 cells were treated with Zeb (120 μM) alone or in combination with 5-FU (250 μg/ml) for 72 h. Cytotoxicity (A) and total cell numbers (B) were derived from cell counts by trypan blue exclusion at each culture. Results are expressed as the means ± SD of triplicate experiments.

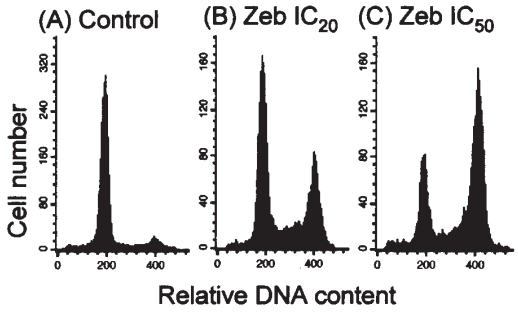

Figure 7. Effects of Zeb on cell cycle distribution of HSC-3 cells. Cells were treated with Zeb at IC_{20} (120 μM) or IC_{50} (220 μM) and incubated for 48 h. Cells were then harvested and DNA was stained with propidium iodide for flow cytometric analysis of cell cycle phase. Results show representative flow cytometric histograms depicting the cell population in G_0/G_1, S, and G_2/M phases of the cell cycle in untreated (A) or treated samples at IC_{20} (B) or IC_{50} (C).

between MTT and TUNEL methods. Only a small increase was observed in the number of trypan blue-positive cells following treatment with 5-FU/Zeb combination compared to control groups, whereas a significant increase was observed in 5-FU alone (Fig. 6A), consistent with the data obtained in the TUNEL assay. Of note, treatment with Zeb alone resulted in a significant reduction in total cell number but not in the number of viable cells (Fig. 6B). Thus, these results suggest that pretreatment with Zeb triggers strong anti-proliferative action that may be caused by cell cycle arrest, which is associated with the growth inhibition by 5-FU/Zeb combination found in MTT assay (Fig. 3B).

Effects of Zeb on cell cycle progression. We tested whether Zeb alters each cell cycle population of HSC-3 cells using propidium iodide staining. Cells were treated with Zeb at 120 (IC_{20}) and 220 μM (IC_{50}), and then harvested at 48 h time point. As shown in Fig. 7, Zeb increased cells arrested in G_2/M of the cell cycle at both doses. Treatment with Zeb (IC_{50}) caused a 61.7% increase in G_2/M phase cells. Although concurrent decreases in the percentage of cells in G_0/G_1 were induced, we did not observe a significant decrease in S phase cells following Zeb treatment. In the control untreated cells, a significantly higher percentage of cells in the G_0/G_1 phase was observed during this time course.

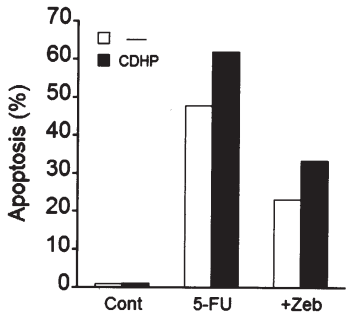

Figure 8. Apoptosis-enhancing effects of CDHP on inhibitory action by Zeb in 5-FU-induced apoptosis. HSC-3 cells were treated with 5-FU (250 μg/ml) alone or in combination with Zeb (120 μM) with or without CDHP pre-treatment (100 μM). After 72 h of incubation, an apoptosis event was assessed by TUNEL assay. Fluorescence-positive cells in the TUNEL assay were detected with a FACSCaliber cytometer. Results show the percentages of apoptotic cells with increased fluorescence intensity, which correspond to one representative result of three experiments.

Effects of CDHP on apoptotic inhibition by Zeb. Because DPD degrades 5-FU, the efficacy and toxicity of 5-FU are highly affected by DPD activities. If DPD activities in cancer cells directly influence the anti-cancer activity of 5-FU, the inhibition of DPD in cancer cells should augment the efficacy of 5-FU. In an attempt to identify the molecular mechanisms of decreased apoptotic response by Zeb, we examined the effects of DPD inhibitor on apoptosis induced by 5-FU alone or 5-FU/Zeb combination. Various inhibitors of DPD were developed to increase the anti-cancer effects of 5-FU. CDHP is a potent DPD inhibitor with no anti-cancer activity by itself (15), and CDHP (100 μM) alone had no effect on the apoptotic induction of HSC-3 cells. When cells were treated with 5-FU in the presence of CDHP, the percentage of apoptotic cells was increased in comparison with 5-FU alone. Furthermore, CDHP had an enhancing effect on the apoptotic ability of 5-FU/Zeb combination (Fig. 8).

Discussion

Epigenetic silencing of critical genes involved in tumor suppressor, DNA repair, cell cycle regulation, and apoptotic death has been demonstrated in certain cancer types (16). With advanced epigenetic technologies and an understanding of cancer cell biology, a variety of new agents capable of reactivating silenced genes in cancer cells have been developed and are being tested in clinical trials for various cancer systems. In previous reports, we described that combined treatment with HDAC inhibitor SAHA and CDDP is significantly more active than either agent alone against the human OSCC cell line, HSC-3 (14). Our studies have shown that SAHA treatment may increase chemosensitivity to CDDP, and the possible mechanisms underlying the enhancing effect of SAHA include the modulation of HDAC activity, down-regulation of intracellular reduced glutathione (GSH) levels and distinct susceptibility in respective phases of the cell cycle; however, almost nothing is known about the role of DNA methylation in the regulation of gene expression for chemosensitivity in OSCC cells. Recently, quantitative methylation status was investigated in

OSCC patients, and significant cancer specific methylation was seen (17). In addition, some methylation inhibitors or HDAC inhibitors have been shown to produce crossover effects between histone and DNA modifications (18). These data suggest that the development of OSCC is associated with histone hypoacetylation and DNA methylation. Thus, this study was designed to determine whether the combination of anti-cancer drugs (CDDP or 5-FU) with epigenetic inhibitors (Zeb or SAHA) mediates their enhancing effects against OSCC cells.

The restoration of aberrations in DNA methylation to allow re-expression of silenced tumor suppressors is a promising strategy for chemosensitization. The anti-cancer drugs used in this study have different mechanisms for exerting anti-cancer effects. It is generally accepted that the major pharmacological target of CDDP is DNA and its cytotoxicity is determined primarily by their DNA adducts. The effects of 5-FU have been attributed to the inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA. In this study, Zeb had chemosensitive efficacy with CDDP, but not 5-FU, whereas SAHA showed efficacy with both CDDP and 5-FU (Fig. 5). It has been previously demonstrated that HDAC inhibitors, including SAHA, TSA, and MS-275, can down-regulate the expression of TS *in vitro*: TS was the most prominently down-regulated gene, achieving 40-100-fold down-regulation at 5 and 15 μM SAHA (19). The intracellular level of TS was long ago recognized as a determinant of fluorouracil cytotoxicity *in vitro* and *in vivo*, because the high response of certain types of cancers to 5-FU has been associated with low expression levels of TS (20). Thus, by down-regulation of the TS gene, it is possible that SAHA enhances the effects of 5-FU, which targets this enzyme. Consequently, combining 5-FU with SAHA should lead to synergism of their anti-cancer effects.

The combination of CDDP/Zeb mediated its enhancing effects on apoptotic induction against OSCC cells (Fig. 5A). Although Zeb as a single agent was demonstrated to have only weak cytotoxicity against solid cancers, Zeb can re-activate genes that are unfavorable in the development and progression of various cancers (6). In addition, Zeb has been shown to be selective towards cancer cells, but is eventually less effective in normal fibroblasts in demethylating and subsequently re-expressing pro-apoptotic genes (21). These experiments are suggestive of new possibilities for the application of Zeb for chemosensitization to CDDP in OSCC. In this study, we showed that the mechanisms responsible for the synergistic cytotoxic enhancement of Zeb might be explained by strengthened apoptosis. In this regard, there are several reports on the repression of apoptosis in cancer cells by DNA methylation. It was shown that caspase-8 is silenced by methylation in neuroblastoma, and that the caspase-9 regulator Apaf-1 is silenced by methylation in melanoma cell lines (22,23). It has previously been reported that K562 cells are resistant to apoptosis induced by most DNA damaging agents, such as UV light, etoposide and daunorubicin, because of the lower level of functional Apaf-1 (24). Apaf-1 inactivation by promoter methylation is suggested as a factor responsible for the inability of cells to undergo apoptosis (23), although there is no evidence indicating a causal association between the re-expression of Apaf-1 and enhanced chemosensitivity. Furthermore, microarray analysis revealed that Zeb induced Bik, a member of the Bcl2 family that promotes apoptotic death, and several other pro-apoptotic proteins (Bad, Bak, and Bax) of the Bcl2 family (25). The imbalance between pro- and anti-apoptotic factors may obviously lead to increased sensitivity to CDDP treatment. Furthermore, because DNA demethylation is accompanied by methylation and acetylation changes on histone as well as on promoter to create a chromatin configuration compatible with transcriptional activation, it is possible that the process involving relaxation of the chromatin structure plays a role in chemosensitivity to CDDP that forms platinum-DNA adducts; however, it is also considered that the effects of Zeb are mediated by factors other than DNA demethylation. For example, the incorporation of ribonucleoside Zeb into RNA has been recently shown as 7-fold higher than DNA incorporation, which could result in altered transcription and/or ribosomal disruption (26). It is unknown whether RNA incorporation correlates with either the biological activity or the cytotoxic enhancement of Zeb.

Interestingly, the TUNEL assay showed that Zeb inhibits the apoptotic effect of 5-FU (Fig. 5B), and we therefore investigated the mechanism underlying this inhibitory function. Although 5-FU interferes with the synthesis of DNA, 5-FU does not act as an effective inhibitor of DNA repair. This might be one of the reasons for the differential apoptotic effects of combining Zeb and 5-FU or CDDP. Furthermore, at the level of molecular effectors, p53 is considered one of the main molecules involved in 5-FU cytotoxicity (27); however, HSC-3 cells retain a non-functional mutated p53 protein and demonstrate relatively high resistance to 5-FU (Fig. 3B), which may affect the poor response to 5-FU/Zeb combination. Importantly, several studies have demonstrated that catabolizing and anabolizing enzymes for 5-FU influence the sensitivity of cancer cells against this drug. Among them, DPD is the initial, rate-limiting enzyme in the catabolic pathway of 5-FU. High intra-tumoral DPD activity leads to the inactivation of 5-FU and consequently to therapeutic resistance against 5-FU. Although endogenous DPD gene (DPYD) expression was undetectable in HSC-3 cells, studies of cancer cell lines including HSC-3 cells showed the absence of genetic alterations in the DPYD promoter region with full activity. Bisulfite sequence analysis of the DPYD promoter region revealed the presence of different methylation patterns in CpG islands of these cell lines (28). Following treatment of HSC-3 cells with 5-aza-CR, DPD mRNA levels were increased up to 4.5 times, implying that methylation could be a regulatory mechanism of DPD expression. Furthermore, demethylation by 5-aza-CR treatment caused a remarkable decrease in sensitivity to 5-FU, along with a concurrent increase of DPYD expression in HSC-3 cells. In this study, DPD inhibitor CDHP had an enhancing effect on apoptotic ability of 5-FU alone or 5-FU/Zeb combination, although this effect was not complete (Fig. 8). Since Zeb is a non-specific, genome-wide inducer of demethylation, unfavorable genes for chemotherapeutic drugs are also reactivated following Zeb treatment. Further studies may lead to a more specific

drug that reactivates only a set of critical genes silenced in a specific type of cancer without affecting the methylation status of other genes.

5-FU is considered to be purely an S phase-active chemotherapeutic agent. The 5-FU-resistant cell lines have the smallest S phase fractions and the largest G_2 (/M) fractions. CDDP is not cell cycle specific, although cells appear to be maximally sensitive to CDDP in G_1, just prior to the onset of DNA synthesis (29). Treatment of acute myeloid leukemia cells with Zeb at concentrations necessary to demethylate DNA caused an increase in the percentage of cells arrested in G_2/M of the cell cycle (30). Consistent with the increases in G_2/M phase cells, concurrent decreases in the percentage of cells in G_0/G_1 and S phases were observed and the decrease in S phase cells correlated with the reduction in cell proliferation following Zeb treatment. Treatment with 5-aza-CdR and 5-aza-CR produced similar results, although the magnitude of these phenotypes was lower. Both bile duct and breast cancer cell lines treated with 5-aza-CdR were accompanied by cell cycle arrest (increase in the G_0/G_1 phase and decrease in the S and G_2/M phases) (31,32). In this study, we showed that Zeb has strong anti-proliferative activity against HSC-3 cells, shown by decreased *in vitro* cellular growth and the increased G_2/M cell cycle phase, accompanied by concurrent decreases in cells in G_0/G_1; however, no decrease in S phase cells was observed (Fig. 7). These results cannot fully explain the involvement of cell cycle arrest as a mechanism of the inhibitory action of Zeb, as seen in Fig. 5B. It has long been recognized that TS protein and TS activity levels are higher in proliferating cells than in non-proliferating cells (33). For example, TS protein levels were higher in S and G_2/M cells than in G_0/G_1 phase cells. Thus, differences in TS levels in each cell cycle phase appear to be involved in the mechanism of the inhibitory action of Zeb. It is therefore possible that the position of cancer cells in the cell cycle and their ability to undergo apoptosis in response to drug treatment may together play an important role in the sensitivity of cancer cells to chemotherapy.

The epigenetic phenomenon found in this study was dependent on respective carcinoma cell lines. CDDP had highly effective anti-cancer activity in OSCC cell line HSC-3 cells, whereas 5-FU showed moderate efficacy (Fig. 3); however, these anti-cancer drugs did not show efficacy against NSCLC A549 cells used as a reference strain. The enhancing ability of epigenetic active agents (Zeb, SAHA) was also ineffective when treating with A549 cells (data not shown). Among carcinoma cells, NSCLC is primarily resistant to chemotherapeutic agents, such as CDDP and 5-FU. The CpG island of glutathione S-transferase P1 gene promoter was almost unmethylated in A549 cells, leading to high levels of GSH (34). It is believed that GSH-associated mechanisms ultimately dictate the resistance of cancer cells to CDDP *in vitro*. Regarding 5-FU derivatives, several NSCLC cell lines were characterized by high DPD and low TS activity (35). When 236 clinically removed NSCLCs were measured for DPD activity, 87% of the lung cancers had high DPD activity (36). HDAC inhibitors are also limited in their ability to induce apoptosis in NSCLC cell lines despite their ability to effectively inhibit deacetylase activity. For instance, HDAC inhibitors (TSA, butyrate) failed to induce apoptosis *in vitro* in NSCLC cells, in part by the ability of these agents to stimulate NF-κB-dependent transcription and cell survival (37). This mechanism may explain why anti-cancer effects of HDAC inhibitors are highly variable in many different cancer cell lines. In OSCCs, promoters of several tumor suppressor genes (p14, p15 and p16) are highly methylated in addition to having a rare gene mutation (38). Tumor suppressor expression was restored or significantly up-regulated by treatment of OSCC-derived cell lines with a demethylating agent. In contrast, the level of DNA hypomethylation in A549 cells is greater than in other lines (Fig. 2), although the cause of such extensive hypomethylation of the A549 genome is not fully understood (39), suggesting that A549 cells are highly resistant to Zeb treatment. Perhaps there might be a block in some methyltransferase genes or protein factors involved in targeting methylation to repeated genomic loci.

In conclusion, a novel demethylating agent Zeb is an impressive anti-cancer enhancing agent in combination with conventional chemotherapeutic drugs against cancer cells; however, Zeb was effective for apoptosis induced by CDDP, but not by 5-FU in OSCC cell line HSC-3. Although the mechanisms responsible for their differential effects are likely complex, several possible mechanisms for Zeb action were assumed from reports of other well-known epigenetic agents, highlighting the need for multiple approaches. This work lays the foundation for a novel anti-cancer approach, whereby DNMT and HDAC inhibitors may be used to modulate cytotoxic processes of anti-cancer drugs into cancers.

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