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ORIGINAL ARTICLE

Zebularine suppresses the apoptotic potential of 5-fluorouracil via cAMP/PKA/CREB pathway against human oral squamous cell carcinoma cells

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Abstract
Purpose During tumorigenesis, tumor suppressor and tumor-related genes are commonly silenced by aberrant DNA methylation in their promoter regions, which is one of the important determinants of susceptibility to 5-fluorouracil (5-FU) in oral squamous cell carcinoma (OSCC) cells. Here, we examine the chemotherapeutic efficacy of epigenetic agents on 5-FU cytotoxicity.
Method We investigated the effect of a DNA methyltransferase (DNMT) inhibitor, zebularine (Zeb), on the chemosensitivity of 5-FU and cisplatin (CDDP) by MTT and TUNEL methods, and compared the molecular mechanism of action with those of a GSK3β inhibitor, LiCl, and an Hsp90 inhibitor, 17-AAG.
Results A significant apoptotic effect by a combination of Zeb or 17-AAG was found in CDDP treatment; however, considerable suppression of 5-FU-induced apoptosis was observed after incubation with Zeb, 17-AAG, or LiCl. Zeb's suppressive effects were associated with activation of the cAMP/PKA/CREB pathway, differing from mechanisms of 17-AAG and LiCl. Suppression of 5-FU-induced apoptosis by Zeb was not associated with increased Bcl-2 and Bcl-xL expressions dependent on transcription factor CREB, and with the expression level of thymidylate synthase.
Conclusions In the present study, we identified a more detailed mechanism of action by which Zeb suppresses 5-FU-induced apoptosis. These results indicate that combination therapies have to be carefully investigated due to potential harmful effects in the clinical application of DNMT inhibitors.

Keywords Zebularine · Methylation · 5-Fluorouracil · Chemosensitivity · cAMP/PKA/CREB pathway · Suppressive action

Introduction

Oral squamous cell carcinoma (OSCC) represents the sixth most common type of cancer in the world. Despite multiple modalities of treatment, such as surgery, radiation, and chemotherapy, OSCC continues to have one of the lowest 5-year survival rates. 5-Fluorouracil (5-FU) and cisplatin [*cis*-dichlorodiamineplatinum (CDDP)] are frequently used in combination therapy for the treatment of OSCC [1]. Apoptosis could represent the final common step in the cytotoxicity exerted by a number of anti-cancer drugs with different mechanisms [2]. Altered expression based on gene mutations, gene amplifications, or epigenetic changes that influence apoptotic proteins can provide OSCC cells with resistance to chemotherapeutic drugs [3]. Previously, we have showed the epigenetic influence on the sensitivity of oral carcinoma cell lines to 5-FU or CDDP by evaluating apoptotic inducibility. The level of methylated cytosine in DNA was decreased to 34% of control cells in zebularine

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(Zeb, IC_{50})-treated cells. Zeb had chemosensitive efficacy with CDDP, whereas Zeb showed inhibitory effect with 5-FU [4]. Furthermore, no decrease in S-phase cells by Zeb was observed, suggesting no involvement of cell cycle arrest as a mechanism of the suppressive action of Zeb to S-phase active 5-FU. However, these results could not fully explain the suppressive action mechanism of Zeb.

The fluoropyrimidine drug 5-FU is used in the treatment of head and neck, gastrointestinal, and breast cancers [5]. 5-FU is converted intracellularly, and forms a stable ternary complex with thymidylate synthase (TS), resulting in enzyme inhibition, followed by DNA damage. In addition, non-TS-directed mechanisms of cytotoxicity have also been described for 5-FU, such as mis-incorporation of fluoronucleotides into DNA and RNA [6]. The major limitation to the clinical use of 5-FU is acquired or inherent resistance, which is thought to mediate therapeutic efficacy in cancer chemotherapy. One of the most proximal regulators of the apoptotic program is Bcl-2, which provides resistance to a wide variety of cell death stimuli, including classical chemotherapeutic drugs [7]. Furthermore, in vitro and in vivo studies have demonstrated that increased TS expression correlates with increased resistance to 5-FU [8]. Other upstream determinants of 5-FU chemosensitivity include the 5-FU-degrading enzyme dihydropyrimidine dehydrogenase (DPD) [6]. To overcome 5-FU resistance in OSCC, 5-FU is usually used in combination with other agents, which quench these resistance determinants via novel mechanisms.

Screening for factors to overcome resistance to chemotherapy is vitally important, since this could avoid subjecting patients to unnecessary, ineffective, and potentially toxic treatment. Two deviating DNA methylation patterns have been observed in cancer cells: wide areas of global hypomethylation along the genome that can result in proto-oncogene activation, and localized areas of hypermethylation in CpG islands or within gene promoter regions that result in gene silencing [9]. Promoter hypermethylation has been detected in OSCC for a number of genes in pathways regulating apoptosis, proliferation, invasion, metastasis, and angiogenesis [10, 11]. This epigenetic process occurs during the S phase and is catalyzed by the DNA methyltransferase (DNMT) family of enzymes, which catalyze the transfer of methyl groups to the carbon-5 position of cytosines in CpG islands [12]. DNA methylation, although heritable, is reversible, making it a therapeutic target. The re-expression of epigenetically silenced genes following treatment with the demethylating agent could provide a means to increase sensitivity for anti-cancer drugs [13]. Several DNA cytosine methylation inhibitors, such as 5-aza-cytidine (5-CR), 5-aza-2′-deoxycytidine (5-aza-CdR), and Zeb, have been evaluated as experimental therapeutics. Among these, a cytidine analog Zeb, which is a DNMT and a cytidine deaminase inhibitor, has been shown to be more stable and less toxic than the other two [14]. Indeed, DNA in treated cells becomes progressively hypomethylated after each round of cell cycle in the presence of Zeb. The reversal of gene methylation and epigenetic silencing by DNMT inhibitors has the potential to influence tumor growth, sensitivity to anti-cancer drugs, and ultimately the clinical outcome, which provides the foundation for the use of such inhibitors in novel cancer therapy strategies [13].

Although most reports on these inhibitors assume high target specificity, many of these inhibitors in fact have pleiotropic effects. In addition, 5-aza-CdR has been shown to be capable of transcriptionally activating genes with unmethylated promoters [15], suggesting that this agent can induce chromatin remodeling independently of its effects on cytosine methylation. Similar studies have also been performed using Zeb [16]. It is likely that downstream events that are unrelated to demethylation also play key roles in determining the cellular response to epigenetic inhibitors. Thus, the identification of these defects would greatly facilitate the development of new therapeutic strategies to improve the efficacy of conventional chemotherapy.

The use of combination chemotherapy is accepted as the standard for most human malignancies, but little attention has been paid to negative interactions. The actual mechanisms of action of 5-FU and Zeb in combination in OSCC have not yet been elucidated in detail. Here, we examine the anti-apoptotic effects of Zeb on 5-FU cytotoxicity, which is involving in apoptosis, apoptosis-related proteins, and the cAMP/PKA/CREB pathway in the well-established OSCC cell line HSC-3. The results of the study provide strong molecular evidence that Zeb works as a suppressive agent in combination with 5-FU, but not with CDDP.

Materials and methods

Reagents

5-FU (Kyowa Hakko Kogyo, Tokyo, Japan) and CDDP (Nippon Kayaku, Tokyo, Japan) were used in this study. Zebularine and 17-allylamino-17-demethoxygeldanamycin (17-AAG) were purchased from Sigma (St Louis, MO, USA). The PI3K inhibitor LY294002, GSK3β inhibitor LiCl, and dibutyryl (db)-cAMP were obtained from Calbiochem (La Jolla, CA, USA). All other chemicals used in this study were commercially available.

Caspase inhibitors

Inhibitors for pan-caspase (z-VAD-fmk), caspase-3 (z-DEVD-fmk), caspase-9 (z-LEHD-fmk), caspase-8 (z-IETD-fmk) and

caspase-12 (z-ATAD-fmk) were purchased from MBL (Nagoya, Japan), and 20 μM of these inhibitors was added to dimethyl sulphoxide (DMSO). Cells were pretreated with these inhibitors for 1 h at 37°C. 5-FU was then added, followed by incubation for 72 h, after which apoptosis was evaluated. A DMSO control was also included as a control for the given concentration of each inhibitor.

Cell culture

HSC-3 cells (human oral squamous cell carcinoma) were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell viability assays

Cell viability was evaluated by the trypan blue exclusion assay. Cytotoxic effects were determined with the MTT (WST8) assay (Dojindo Lab, Kumamoto, Japan). Absorbance (450 nm) was directly proportional to the number of living cells in culture.

TUNEL assay

Apoptotic cells were assayed by the TUNEL method using the Mebstain apoptosis kit direct (MBL) for flow cytometric analysis (FACSCalibur, Bectone-Dickinson, San Jose, CA, USA).

Measurement of intracellular caspase activity

Intracellular caspase activities were measured using carboxyfluorescein-labeled fluoromethyl ketone caspase inhibitors (Cell Technology, Mountain view, CA, USA). Treated cells were labeled and analyzed by flow cytometry to determine the percentage of intracellular active cells for caspase-3, -8 or -9.

Assay for phosphorylated CREB and activated NF-κB

Phosphorylated (activated) CREB (p-CREB) was measured with a p-CREB assay kit specific for the Ser^{133}-phosphorylated segment of amino acids 128–141 of CREB (Active Motif, Carlsbad, CA, USA). Nuclear cell extracts (5 μg/well) were added to wells coated with an oligonucleotide containing the CRE site (5′-TGACGTCA-3′) and incubated for 3 h. After washing, p-CREB antibody was added and incubated for 1 h, followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody. Colorimetric reactions were measured at 450 nm. The nuclear cell extract of db-cAMP treatment was used as a positive control. Activated NF-κB was measured with an NF-κB assay kit specific for the p65 subunit (Active Motif). Nuclear cell extracts were added to 96-well plates coated with an oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′). A nuclear extract of Jurkat cells treated with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and calcium ionophore (CI) was used as a positive control.

Measurement of GSK3β phosphorylation

The amount of phosphorylated glycogen synthase kinase 3β (GSK3β) in cells was measured with a cell-based ELISA kit (Active Motif). After stimulation, cells were fixed with 4% formaldehyde in PBS for 20 min and washed three times with PBS containing 0.1% Triton X-100. Endogenous peroxidase was quenched with 1% H_2O_2 and 0.1% NaN_3, blocked with antibody-blocking buffer for 1 h, and incubated overnight with the primary antibody at 4°C. Cells were incubated with the secondary antibody for 1 h and washed. Absorbance was measured at OD_{450}.

Measurement of protein kinase A (PKA) activity

An ELISA kit for PKA activity (Assay designs, Ann Arbor, MI, USA) was used. Cells were stimulated and total cell lysates were prepared. Cell lysates were incubated in a 96-well plate pre-coated with the substrate, readily phosphorylated by PKA. ATP was then added to initiate the reaction. An HRP-conjugated phospho-specific antibody was added to wells. Absorbance was measured at 450 nm. Results were normalized to the amount of total protein (1 μg).

Western blotting

After the indicated treatments, equal amounts of cell lysates (15–20 μg) were mixed with Laemmli sample buffer (2% SDS) and placed in a heat block at 95°C for 5 min. Samples fractionated by SDS-PAGE (12–15%) were electrotransferred to PVDF membranes. The membranes were blocked with blocking agent in TBS-T buffer, and blotted with the indicated antibodies. Immunoblots were developed using HRP-conjugated secondary antibodies, followed by detection with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA). The antibodies against p16, Hsp90, Bcl-2 and Bcl-xL (BD Biosciences, San Jose, CA, USA), Hsp70 (Santa Cruz Biotech, Santa Cruz, CA, USA), TS (Ana Spec, San Jose, CA, USA), β-actin (BioVision, Mountain View, CA, USA), and HRP-conjugated sheep

anti-mouse IgG (GE Helthacre), were used for immunoblotting experiments.

Statistical analysis

Data are given as the means ± SE. Multiple comparisons were made by Scheffe's test. *P* values less than 0.05 were regarded as significant.

Results

Apoptosis of OSCC cells induced by 5-FU

OSCC cell line HSC-3 was treated with increasing concentrations of 5-FU for 72 h, and reduction in cell growth was determined by MTT assay. 5-FU markedly inhibited cell growth in a dose-dependent manner (Fig. 1a). The IC_{20} value was calculated to be 250 μg/ml, which was used in subsequent studies. Furthermore, we analyzed apoptotic cells by TUNEL assay to investigate the cytotoxicity of 5-FU. Although 5-FU did not significantly increase apoptosis before 48 h (data not shown), 5-FU induced the accumulation of apoptotic cells for 72 h, proportional to the growth inhibitory effect of 5-FU (Fig. 1b). We next determined that caspase-3, -8, and -9 activities were markedly increased after 48-h treatment with 5-FU, respectively (Fig. 1c). Furthermore, we confirmed the effect of caspase inhibitors on the apoptotic potential of 5-FU against HSC-3 cells. As shown in Fig. 1d, the preincubation of cells with 20 μM of z-VAD-fmk, or caspase-3, -8, -9, or -12 inhibitors significantly prevented the frequency of apoptosis induced by treatment of cells with 250 μg/ml 5-FU for 72 h, respectively. In contrast, death ligands, such as FasL and TRAIL, were not involved in apoptotic death, because the use of CH11, a cross-linking antibody to Fas receptor, and of recombinant human TRAIL, did not induce any changes in the growth of HSC-3 cells (Figures S1A and B in Electronic supplementary material). These results suggest that the cytotoxic effects of 5-FU may depend on a caspase-driven pathway derived from cellular stress signals in OSCC cells.

Suppressive effects of Zeb on apoptotic potential of 5-FU

Next, we studied the apoptotic modulation of Zeb in combination with 5-FU or CDDP, focusing on agents effective against OSCC. Based on previous studies to obtain the demethylation status and the minimum cytotoxic response [4], we treated cells with 120 μM Zeb for 48 h, followed by treatment with 250 μg/ml 5-FU for 72 h. Figure 2a shows representative histograms of apoptotic cells for HSC-3 cells after exposure to 5-FU with or without Zeb pretreatment. Unexpectedly, Zeb showed suppressive effects on the apoptotic potential of 5-FU (apoptotic cells; 43.0% in 5-FU, and 26.8% in 5-FU with Zeb, respectively) (Figs. 2a, 3a), whereas Zeb showed enhancing effects on CDDP-induced apoptosis for 48 h (Fig. 3b), indicating the presence of negative interaction between 5-FU and Zeb.

Suppressive effects of LiCl and 17-AAG on apoptotic potential of 5-FU

It is known that LiCl confers resistance to apoptosis induced by conventional chemotherapeutic drugs [17]. To compare the mechanisms underlying Zeb's suppression with that of LiCl, cells were treated with 5-FU or CDDP in the presence or absence of LiCl (10 mM) before being analyzed for TUNEL assay. Co-treatment with LiCl was found to drastically decrease the apoptotic potential of both 5-FU and CDDP (Figs. 2b, 3a, b). The addition of solvent alone had no effect on 5-FU or CDDP sensitivity; therefore, we conclude that Zeb and LiCl conferred survival advantage through their properties affecting apoptosis.

The Hsp90 inhibitor 17-AAG causes the induction of Hsp70, and inducible Hsp70 has been suggested to be involved in cytoprotection against apoptosis [18]. HSC-3 cells were also treated with 17-AAG in combination with 5-FU or CDDP. Apoptotic cells were then measured with TUNEL assay. The combination (17-AAG/5-FU) caused a profound reduction in apoptotic cells (Figs. 2c, 3a), whereas 17-AAG had significant enhancing effects on CDDP-induced apoptosis (Fig. 3b), suggesting that cytoprotective mechanism by Hsp70 may be informative in the Zeb-induced suppression (Fig. 7c).

Role of signaling pathway in apoptotic suppression of 5-FU by Zeb

We further studied the signaling processes controlling the suppression of 5-FU-induced apoptosis by Zeb or LiCl. First, to study the involvement of cAMP/PKA signaling in the suppressive effect, we used the H89 compound, a selective and potent inhibitor of PKA, for its ability to affect 5-FU-induced apoptosis. H89 is known to bind the ATP-binding site of PKA in competition with ATP. As shown in Fig. 4a, H89 caused complete recovery of Zeb-induced suppression, whereas H89 failed to do so in LiCl-induced suppression. When PKA activity was monitored in Zeb-treated HSC-3 cells, its activity was higher than in untreated cells (Fig. 4b). In addition, we studied the effect of a membrane-permeable PKA activator, db-cAMP, on the apoptotic activity of 5-FU. The db-cAMP apparently inhibited 5-FU-induced apoptosis in HSC-3 cells (Fig. 4c). LY294002, a PI3K inhibitor, significantly enhanced 5-FU-induced apoptosis (Fig. 4d), further supporting that Zeb's

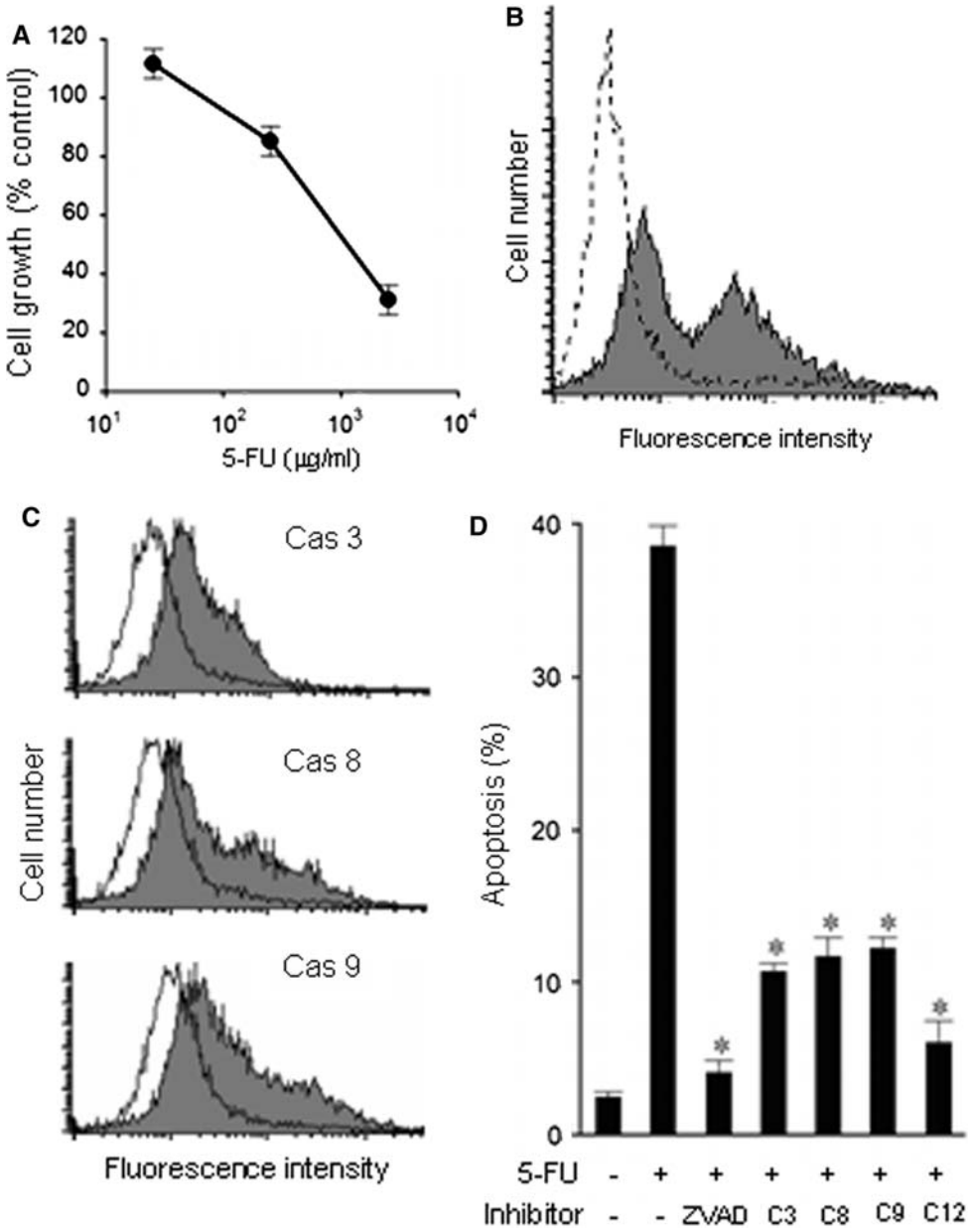

Fig. 1 Effects of 5-FU on cell growth and apoptosis in HSC-3 cells. **a** Cells were treated with increasing concentrations of 5-FU for 72 h, followed by MTT assay. Results in triplicate represent the percentages (means ± SE) of cell growth to the untreated control. **b** Cells were treated with 5-FU (250 μg/ml) for 72 h, followed by the TUNEL method. Results show flow cytometric histograms depicting apoptotic cells in untreated (*unshaded*) or treated cells (*shaded*). **c** Cells were treated with or without 5-FU, and intracellular caspase activities (caspase-3, -8, and -9) were analyzed by flow cytometry. Results show flow cytometric histograms depicting caspase active cells. **d** Cells pretreated for 1 h with each caspase inhibitor (20 μM) or DMSO alone were treated with 5-FU and evaluated by TUNEL assay. Fluorescence-positive cells, including the apoptotic subpopulation, were quantified. Results are expressed as the means ± SE of triplicate experiments. * $P < 0.05$ versus 5-FU alone

suppressive effect on 5-FU-induced apoptosis is in part mediated via cAMP-dependent PKA activation as a putative downstream effector of the PI3K signaling pathway. These results are supported by studies using interfering pharmacologic agents that either stimulated (db-cAMP) or repressed (H89) cAMP accumulation in target cells [19].

Induction of phosphorylated CREB by Zeb

One of the main downstream effectors of the cAMP/PKA signaling pathway is CREB, which is indicative of increased CRE-dependent transcription. To further study the mechanism of the suppressive activities of Zeb, we examined the effect of Zeb, LiCl, and cAMP on p-CREB protein expression. Activation of CREB in total cell extracts was assessed by ELISA using the anti-p-CREB antibody raised against Ser^{133}-phosphorylated CREB. The results showed that Zeb treatments caused a significant increase in the expression of p-CREB protein (Fig. 5a), despite being in the presence of 5-FU (Supplementary Fig. S2). In contrast, Zeb treatment did not result in the activation of another important transcription factor, NF-κB (Fig. 5c). In the presence of LiCl or db-cAMP, a substantial increase of p-CREB was induced, compared with untreated cells (Fig. 5b).

To confirm whether GSK3 is involved in apoptotic suppression induced by Zeb or LiCl, we examined the modulation of the phosphorylation of GSK3β by Zeb or LiCl.

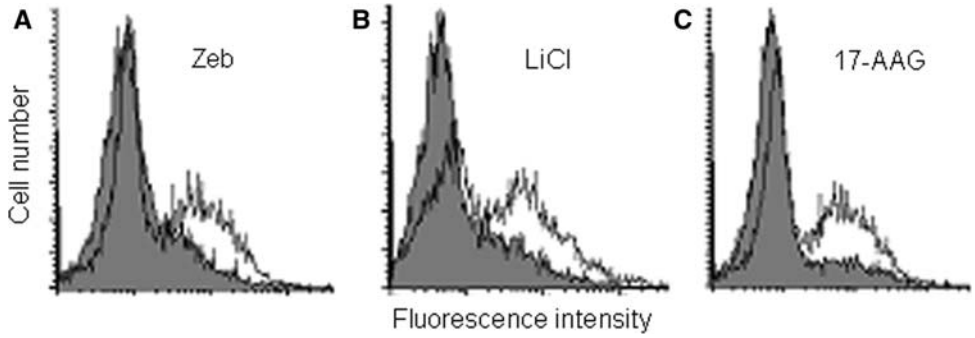

Fig. 2 Suppressive effects of Zeb on apoptotic potential of 5-FU. HSC-3 cells were treated with 5-FU alone (*unshaded*) or combination treatments (*shaded*) for 72 h. Zeb (120 μM) (**a**) was added 48 h before 5-FU treatment, and 10 mM LiCl (**b**) or 10 μM 17-AAG (**c**) was added 1 h before 5-FU treatment. Apoptotic cells were evaluated by TUNEL assay. Results show flow cytometric histograms depicting apoptotic cells

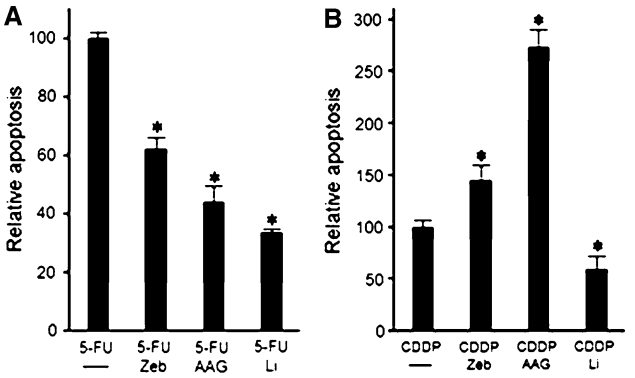

Fig. 3 Effects of each compound on 5-FU- or CDDP-induced apoptosis. HSC-3 cells were treated with 5-FU for 72 h (**a**) or CDDP (5 μg/ml) for 48 h (**b**) alone or in combination with Zeb, 17-AAG or LiCl, following by TUNEL assay. Fluorescence-positive cells, including the apoptotic subpopulation, were quantified. The effects of combination treatments are shown as ratios relative to 5-FU or CDDP alone. Results represent the means ± SE of triplicate experiments. * $P < 0.05$ versus each drug alone

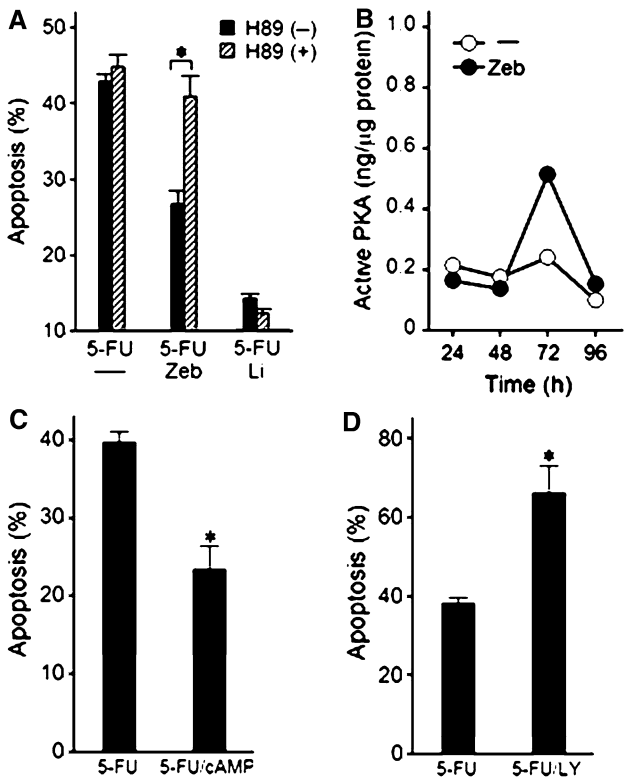

Fig. 4 Role of cAMP/PKA pathway in apoptotic suppression by Zeb and LiCl. **a** HSC-3 cells were pretreated with H89 (10 μM) for 1 h prior to 5-FU. Treatments with indicated combinations were performed as in Fig. 3. Results show the percentage of apoptotic cells and represent the means ± SE of triplicate experiments. * $P < 0.01$ versus 5-FU/Zeb. **b** Cells were treated with or without Zeb (220 μM), and PKA activity was measured at the indicated times. Results are expressed as the amount (ng) of active PKA per 1 μg of total protein. **c**, **d** Cells were treated with db-cAMP (500 μM) or LY294002 (30 μM) for 1 h prior to 5-FU treatment. Results show percentage of apoptotic cells, and represent the means ± SE of triplicate experiments. * $P < 0.01$ versus 5-FU alone

Incubation with LiCl for 4 h increased p-GSK3β (Fig. 6a), while Zeb did not increase p-GSK3 (Fig. 6b). This observation is consistent with previous findings that LiCl directly or indirectly inhibited GSK3 [20].

Expression of apoptosis-related proteins by each inhibitor

To identify a possible mechanism by which active CREB might protect cells from 5-FU-induced apoptosis, we analyzed the effect of these apoptotic inhibitors on the expression of apoptosis-related proteins. A previous report suggested that CREB increases the expression of Bcl-2, which plays an important role in resistance to apoptosis [21]; therefore, we analyzed the expression of Bcl-2 by Western blot analysis with a specific antibody. The results showed that treatment with Zeb or 5-FU did not increase Bcl-2 protein expression, whereas Bcl-2 was higher in LiCl-treated cells than in control cells (Fig. 7a); however, each treatment did not modify the expression of a Bcl-2 family member, Bcl-xL, in cells (Fig. 7a). Kinetic studies

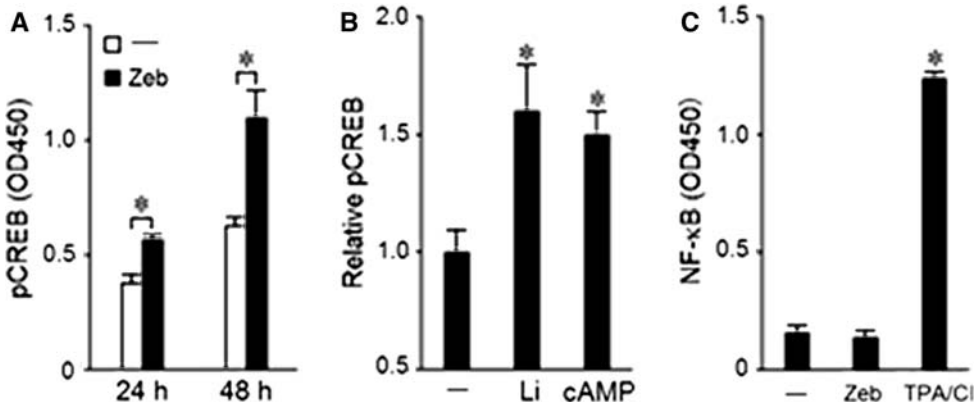

Fig. 5 Induction of p-CREB by Zeb and LiCl. **a** HSC-3 cells were treated with Zeb (220 μM) for 24 or 48 h, and nuclear proteins were extracted. Each sample (5 μg/well) was added to 96-well plates coated with oligonucleotide containing a cAMP-responsive element, and the reactions were measured at 450 nm. **b** Cells were treated with LiCl or db-cAMP for 24 h. Results are shown as ratios relative to untreated cells. **c** Nuclear proteins of Zeb-treated cells were extracted. Each sample was added to 96-well plates coated with oligonucleotide containing the NF-κB consensus site. Nuclear protein from Jurkat cells treated with TPA/CI was assayed as a positive control. Results represent the means ± SE of OD_{450} values from triplicate experiments. * $P < 0.05$ versus untreated cells

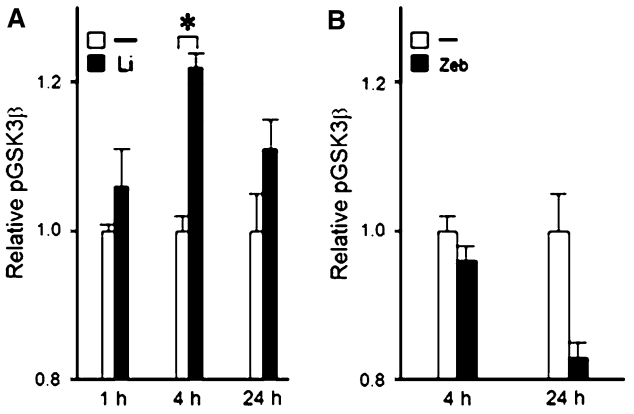

Fig. 6 Induction of p-GSK3β by LiCl. HSC-3 cells were treated with LiCl (**a**) or Zeb (**b**) for the indicated periods, and fixed. p-GSK3β was assayed in triplicate and reactions were measured at 450 nm. Results are shown as ratios relative to untreated cells in each period. Results represent the means ± SE of triplicate experiments. * $P < 0.01$ versus untreated cells

of the expression of tumor suppressor p16 demonstrated that p16 was not elevated with the addition of Zeb during periods tested (Fig. 7b).

We performed similar immunoblot analysis for the expression of chemoresistance genes. The expression of Hsp90 protein decreased in cells before or after treatment with Zeb or 17-AAG (Fig. 7c). Treatment of cells with 17-AAG markedly increased the expression of Hsp70. In addition, Zeb caused an apparent induction of Hsp70 in Zeb-treated cells. In TS expression, pretreatment of cells with 17-AAG nearly abolished their expression. These data also suggest the role of Hsp70 and Hsp90 as negative regulators of the apoptotic pathway in cells treated with 5-FU/Zeb.

Discussion

OSCC is a very aggressive tumor that is difficult to treat. In this study, we compared the action of the epigenetic inhibitor Zeb to that of the apoptotic inhibitors LiCl or 17-AAG in the regulation of apoptosis induced by the chemotherapeutic drug 5-FU. We have previously demonstrated that histone deacetylase (HDAC) inhibitor SAHA, combined with 5-FU, causes apoptotic enhancement in OSCC cell line HSC-3 [4]. In contrast, Zeb/5-FU combination exhibited lower apoptotic potential than 5-FU alone. Although we addressed DPD activity, known to be a principal factor in 5-FU resistance, these results could not fully explain the suppressive action mechanism of Zeb. Therefore, to further examine the possible mechanism underlying the suppressive action, we analyzed the perturbations induced by Zeb and/or 5-FU in the signaling pathway associated with apoptosis by specific inhibitors, and in some of its related markers by the immunoblot technique. We found that LiCl or 17-AAG did not account for the Zeb's suppression in HSC-3 cells, in spite of the observed suppression of apoptosis induced by 5-FU. Eventually, our data suggest that Zeb-induced activation of the cAMP/PKA/CREB pathway suppresses 5-FU-induced apoptosis, but Zeb-induced suppression is decisively distinct from that of LiCl or 17-AAG (Figs. 4a, 6a, b, 7c).

Elevation of intracellular cAMP levels can result in either the stimulation or repression of a specific gene expression. cAMP binds to the regulatory subunit of PKA and releases the active catalytic subunit. This subunit phosphorylates the transactivation domain of CREB. The transcription factor CREB was found to occupy approximately 4,000 promoter sites, depending on the presence and methylation state of consensus cAMP response elements near

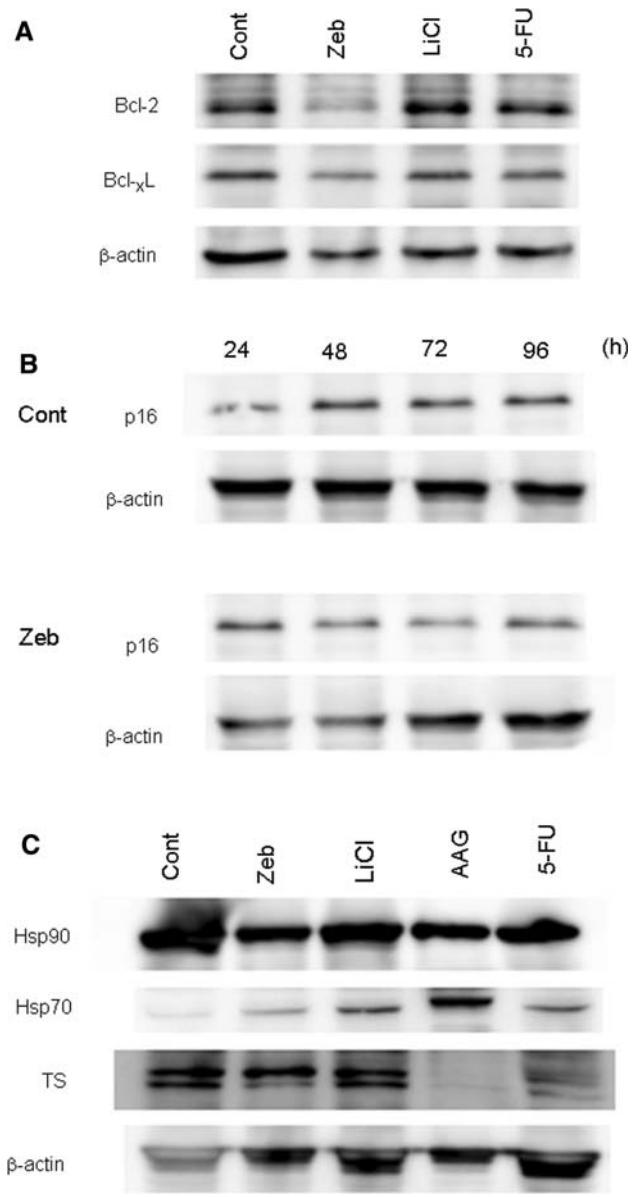

Fig. 7 Expression of apoptosis-related proteins after treatment with each compound. HSC-3 cells were treated with Zeb, LiCl, 5-FU or 17-AAG for 24 h or for the indicated periods. Cell lysates were prepared, and equal amounts of extracts were loaded for Western blotting analysis for Bcl-2 and Bcl-xL (**a**), p16 (**b**), and Hsp90, Hsp70, and TS (**c**). *β*-actin was used as a loading control

the promoter [22]. CREB and its associated proteins (Bcl-2, Bcl-xL) play an important role in the resistance of cancer cells to apoptosis induction, thus contributing to tumor growth and metastasis. The expression of dominant-negative CREB led to a decrease in the tumorigenic and metastatic potential of cancer cells in nude mice [21]. Our results not only confirmed Zeb's anti-apoptotic action, but also provided insight into how Zeb mediates negative regulation against the apoptotic inducibility of 5-FU. Phosphorylation of CREB by Zeb increased in more resistant cells compared to untreated cells against 5-FU insult. Furthermore, as a consequence of potent and specific PKA inhibitory effects, H89 accelerated the apoptotic effects induced by 5-FU when administered to cancer cells.

Prostaglandin E_2 (PGE_2) may facilitate cancer progression by stimulating cell proliferation and survival, tumor cell invasiveness, and production of angiogenic agents in cancer cells. PGE_2 stimulates the cAMP/PKA pathway, resulting in enhanced interaction between p-CREB and p300 through receptor 2 (EP_2) for PGE_2. EP_2 expression was often silenced in neuroblastoma cell lines through epigenetic mechanisms such as DNA methylation and histone modification [23]. In addition, PGE_2-dependent pro-oncogenic actions have been demonstrated to be mediated through EP_2 activation and cAMP increase [24]. PGE_2 inhibits apoptosis through the activation of cAMP-dependent PKA and stabilization of Ras-PI3K association [25]. Thus, we speculated that treatment of HSC-3 cells with Zeb may result in the up-regulated expression of several genes, including EP_2, and can activate cAMP signaling in the inhibition of apoptosis. A cAMP analog, db-cAMP, directly increased intracellular cAMP, and inhibited apoptosis by enhancing the downstream cAMP-dependent signaling pathway (Fig. 4c). However, at the present time we have had no concrete evidence as to the role of PGE_2, or its receptors in the direct protection of the OSCC cells by Zeb.

We used LiCl in a comparative study on the suppression of apoptosis in Zeb-treated cells. Lithium is known to be an anti-apoptotic agent and is widely used in the treatment of bipolar mood disorder. In addition to direct inhibition to GSK3*β*, LiCl activates PI3K/Akt signaling and subsequently inhibits GSK3*β* in order to induce binding activity of p-CREB and the CREB responsive element of various downstream anti-apoptotic genes [26]. LiCl has been shown to decrease levels of p53 and Bax while increasing levels of Bcl-2 [27]. Indeed, LiCl caused the up-regulation of CREB-dependent gene transcription, resulting in the expression of survival factor Bcl-2. However, we did not observe phosphorylation of GSK3*β* (Fig. 6b) and any increases in the expression of Bcl-2 or Bcl-xL in cells following Zeb treatment (Fig. 7a), thus showing that phosphorylation of CREB by LiCl is tightly dependent upon the GSK3*β* inhibition for inducing chemoresistance.

Several studies have indicated that TS expression or activity functions as one of the key determinants of 5-FU sensitivity, and preclinical in vitro and in vivo studies have shown an inverse relationship between TS expression in cancer cells and 5-FU sensitivity [28]. Hsp90 is a chaperone that is critically important for both tumor progression and survival. Hsp90 is an exciting target for anti-cancer drugs because most of the proteins that interact with Hsp90 are known to be in the cell cycle, signaling and chromatin-remodeling pathways. Hsp90 interacts with client TS

protein and facilitates its stabilization and activation [29]. Hsp90 inhibitor, 17-AAG, binds to Hsp90, which is a major repressor of heat-shock transcription factor 1 (HSF1) [30]. By binding Hsp90, 17-AAG via HSF1 induces Hsp70, which renders cells resistant to chemotherapy. Hsp70 can inhibit the late stages of apoptosis, blocking events downstream from caspase-3. Although these findings are important in terms of affecting 5-FU resistance, TS protein expression was not changed by Zeb treatment. Inhibition of the essential ATPase activity of Hsp90 by 17-AAG leads to the degradation of TS protein by the ubiquitin-proteasome pathway (Fig. 7c); however, these results also indicate that the key target of 5-FU cytotoxicity in this cell line was not TS inhibition. The combinations of anti-cancer drugs with 17-AAG may not always lead to enhancement of anti-cancer activity, although the mechanisms whereby 17-AAG causes down-regulation of 5-FU-induced apoptosis have been elusive.

Based on the potent pro-apoptotic and anti-angiogenic effects, epigenetic inhibitors are being tested as potentially important new chemotherapeutic agents for the treatment of solid tumors [31]. DNA hypermethylation, particularly in the GC-rich promoter region, results in transcription repression, which is often associated with a number of tumor suppressor genes, including *Rb*, *p15* and *p16* [32]. Several DNA cytosine methylation inhibitors, such as 5-CR, 5-aza-CdR, Zeb, and dietary polyphenols, are currently used to inhibit cancer progression. 5-aza-CdR (also called decitabine) treatment resulted in the up-regulation of a number of methylated genes; however, several unmethylated genes were also found to be induced [33]. In contrast, only a small number of genes were up-regulated by Zeb [34]. Among tumor suppressor genes, we did not observe Zeb's induction of *p16* as a demethylation marker in OSCC cells, and its expression did not further increase upon treatment with Zeb (Fig. 7b), confirming a report that aberrant methylation of the *p16* gene was not detected in HSC-3 cells [35]. Certainly, DNA methylation and its associated silencing have been shown to be involved in the development of drug resistance [36], prompting investigations of the use of hypomethylation therapy to re-sensitize malignant cells to classical cytotoxic drugs. Although the combination of Zeb with CDDP exerted significant anti-cancer effects against the OSCC cell line, modulation of some genes was associated with survival responses to 5-FU [4]. Such global hypomethylation by the epigenetic action in cancer cells has been proposed to cause chromosomal instability, harmful expression of endogenous viral sequences, and activation of oncogenes [37]; therefore, it will be important to extend the present studies to cancer cells and assess the consequences of hypomethylation for genomic expression.

In summary, combination treatment with both conventional chemotherapeutic drugs and DNMT inhibitors may provide a new treatment approach for cancer patients in clinical practice. Identification of the mechanisms altering chemosensitivity is important for better understanding of the role of DNMT inhibitors in the transcriptional regulation of tumor suppressor genes. Reduced levels of drug-resistant genes may contribute to improved chemosensitivity; however, in this and our previous study [4], we have described a more detailed mechanism of action by which Zeb suppresses 5FU-induced apoptosis. These findings have highlighted the clinical application of DNMT inhibitors, although several inhibitors have been shown to inhibit tumor xenograft growth in vivo.

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References

1. Wrangle JM, Khuri FR (2007) Chemoprevention of squamous cell carcinoma of the head and neck. Curr Opin Oncol 19:180–187
2. Hsu S, Singh B, Schuster G (2004) Induction of apoptosis in oral cancer cells: agents and mechanisms for potential therapy and prevention. Oral Oncol 40:461–473
3. Ha PK, Califano JA (2006) Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. Lancet Oncol 7:77–82
4. Suzuki M, Shinohara F, Nishimura K, Echigo S, Rikiishi H (2007) Epigenetic regulation of chemosensitivity to 5-fluorouracil and cisplatin by zebularine in oral squamous cell carcinoma. Int J Oncol 31:1449–1456
5. Caponigro F, Milano A, Basile M, Ionna F, Iaffaioli RV (2006) Recent advances in head and neck cancer therapy: the role of new cytotoxic and molecular-targeted agents. Curr Opin Oncol 18:247–252
6. Longley DB, Harkin DP, Johnston PG (2003) 5-Fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3:330–338
7. Lei XY, Zhong M, Feng LF, Zhu BY, Tang SS, Liao DF (2007) siRNA-mediated Bcl-2 and Bcl-xl gene silencing sensitizes human hepatoblastoma cells to chemotherapeutic drugs. Clin Exp Pharmacol Physiol 34:450–456
8. Tumber A, Collins LS, Petersen KD, Thougaard A, Christiansen SJ, Dejligbjerg M, Jensen PB, Sehested M, Ritchie JW (2007) The histone deacetylase inhibitor PXD101 synergises with 5-fluorouracil to inhibit colon cancer cell growth in vitro and in vivo. Cancer Chemother Pharmacol 60:275–283
9. Ehrlich M (2006) Cancer-linked DNA hypomethylation and its relationship to hypermethylation. Curr Top Microbiol Immunol 310:251–274
10. Hasegawa M, Nelson HH, Peters E, Ringstrom E, Posner M, Kelsey KT (2002) Patterns of gene promoter methylation in squamous cell cancer of the head and neck. Oncogene 21:4231–4236
11. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG, Sidransky D (2000) Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res 60:892–895

12. Herman JG, Baylin SB (2003) Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349:2042–2054
13. Egger G, Liang G, Aparicio A, Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. Nature 429:457–463
14. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, Jones PA, Selker EU (2003) Inhibition of DNA methylation and reactivation of silenced genes by Zebularine. J Natl Cancer Inst 95:399–409
15. Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebnik YA, Cordon-Cardo C, Lowe SW (2001) Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 409:207–211
16. Pompeia C, Hodge DR, Plass C, Wu YZ, Marquez VE, Kelley JA, Farrar WL (2004) Microarray analysis of epigenetic silencing of gene expression in the KAS-6/1 multiple myeloma cell line. Cancer Res 64:3465–3473
17. Beurel E, Kornprobst M, Blivet-Van Eggelpoel MJ, Ruiz-Ruiz C, Cadoret A, Capeau J, Desbois-Mouthon C (2004) GSK-3β inhibition by lithium confers resistance to chemotherapy-induced apoptosis through the repression of CD95 (Fas/APO-1) expression. Exp Cell Res 300:354–364
18. Grivicich I, Regner A, Zanoni C, Correa LP, Jotz GP, Henriques JA, Schwartsmann G, Da Rocha AB (2007) Hsp70 response to 5-fluorouracil treatment in human colon cancer cell lines. Int J Colorectal Dis 22:1201–1208
19. Boissel JP, Bros M, Schröck A, Gödtel-Armbrust U, Förstermann U (2004) Cyclic AMP-mediated upregulation of the expression of neuronal NO synthase in human A673 neuroepithelioma cells results in a decrease in the level of bioactive NO production: analysis of the signaling mechanisms that are involved. Biochemistry 43:7197–7206
20. Li X, Friedman AB, Zhu W, Wang L, Boswell S, May RS, Davis LL, Jope RS (2007) Lithium regulates glycogen synthase kinase-3β in human peripheral blood mononuclear cells: implication in the treatment of bipolar disorder. Biol Psychiatry 61:216–222
21. Aggarwal S, Kim SW, Ryu SH, Chung WC, Koo JS (2008) Growth suppression of lung cancer cells by targeting cyclic AMP response element-binding protein. Cancer Res 68:981–988
22. Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M (2005) Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci USA 102:4459–4464
23. Sugino Y, Misawa A, Inoue J, Kitagawa M, Hosoi H, Sugimoto T, Imoto I, Inazawa J (2007) Epigenetic silencing of prostaglandin E receptor 2 (PTGER2) is associated with progression of neuroblastomas. Oncogene 26:7401–7413
24. Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T (2003) Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. J Biol Chem 278:12752–12758
25. Leone V, Di Palma A, Ricchi P, Acquaviva F, Giannouli M, Di Prisco AM, Iuliano F, Acquaviva AM (2007) PGE2 inhibits apoptosis in human adenocarcinoma Caco-2 cell line through Ras-PI3 K association and cAMP-dependent kinase A activation. Am J Physiol Gastrointest Liver Physiol 293:G673–G681
26. Rowe MK, Chuang DM (2004) Lithium neuroprotection: molecular mechanisms and clinical implications. Expert Rev Mol Med 6:1–18
27. Yazlovitskaya EM, Edwards E, Thotala D, Fu A, Osusky KL, Whetsell WO Jr, Boone B, Shinohara ET, Hallahan DE (2006) Lithium treatment prevents neurocognitive deficit resulting from cranial irradiation. Cancer Res 66:11179–11186
28. Shintani Y, Ohta M, Hirabayashi H, Tanaka H, Iuchi K, Nakagawa K, Maeda H, Kido T, Miyoshi S, Matsuda H (2003) New prognostic indicator for non-small-cell lung cancer, quantitation of thymidylate synthase by realtime reverse transcription polymerase chain reaction. Int J Cancer 104:790–795
29. Pearl LH, Prodromou C, Workman P (2008) The Hsp90 molecular chaperone: an open and shut case for treatment. Biochem J 410:439–453
30. Banerji U, Walton M, Raynaud F, Grimshaw R, Kelland L, Valenti M, Judson I, Workman P (2005) Pharmacokinetic-pharmacodynamic relationships for the heat shock protein 90 molecular chaperone inhibitor 17-allylamino, 17-demethoxygeldanamycin in human ovarian cancer xenograft models. Clin Cancer Res 11:7023–7032
31. Hellebrekers DM, Griffioen AW, Van Engeland M (2007) Dual targeting of epigenetic therapy in cancer. Biochim Biophys Acta 1775:76–91
32. Viswanathan M, Tsuchida N, Shanmugam G (2003) Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. Int J Cancer 105:41–46
33. Shi H, Wei SH, Leu YW, Rahmatpanah F, Liu JC, Yan PS, Nephew KP, Huang TH (2003) Triple analysis of the cancer epigenome: an integrated microarray system for assessing gene expression, DNA methylation, and histone acetylation. Cancer Res 63:2164–2171
34. Cheng JC, Yoo CB, Weisenberger DJ, Chuang J, Wozniak C, Liang G, Marquez VE, Greer S, Orntoft TF, Thykjaer T, Jones PA (2004) Preferential response of cancer cells to zebularine. Cancer Cell 6:151–158
35. Shintani S, Nakahara Y, Mihara M, Ueyama Y, Matsumura T (2001) Inactivation of the p14(ARF), p15(INK4B) and p16(INK4A) genes is a frequent event in human oral squamous cell carcinomas. Oral Oncol 37:498–504
36. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R (2000) Reversal of drug resistance in human tumor xenografts by 2′-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res 60:6039–6044
37. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395:89–93