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The DNA methyltransferase inhibitor zebularine induces mitochondria-mediated apoptosis in gastric cancer cells in vitro and in vivo

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ABSTRACT

DNA methyltransferase (DNMT) inhibitor zebularine has been reported to potentiate the anti-tumor effect by reactivating the expression of tumor suppressor genes and apoptosis-related genes in various malignant cells. However, the apoptotic signaling pathway in gastric cancer cells induced by zebularine is not well understood. In the study, the effects of zebularine on the growth and apoptosis of gastric cancer cells were investigated by MTT assay, Hoechst assay, Western blot analysis, flow cytometric analysis of annexin V-FITC/PI staining, and TUNEL assay. Zebularine was an effective inhibitor of human gastric cancer cells proliferation in vitro and in vivo. The effects were dose dependent. A zebularine concentration of 50 μM accounted for the inhibition of cell proliferation of 67% at 48 h. The treatment with zebularine upregulated Bax, and decreased Bcl-2 protein. Caspase-3 was activated, suggesting that the apoptosis is mediated by mitochondrial pathways. Moreover, zebularine injection successfully inhibited the tumor growth via apoptosis induction which was demonstrated by TUNEL assay in xenograft tumor mouse model. These results demonstrated that zebularine induced apoptosis in gastric cancer cells via mitochondrial pathways, and zebularine might become a therapeutic approach for the treatment of gastric cancer.

1. Introduction

Gastric cancer results from an accumulation of genetic and epigenetic events [1]. Multiple mutations have been identified in primary gastric cancer [2], but such changes are difficult to overcome. In contrast, most epigenetic modifications are post-transcriptional [3], and inhibition of these mechanisms could be advantageous in the treatment of gastric cancer. As a consequence the role of epigenetic regulators like DNA methyltransferase (DNMT) inhibitors as treatment for gastric cancer is under evaluation.

Zebularine [1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one], a novel inhibitor of DNA methylation with oral bioavailability, is a cytidine analog containing a 2-(1H)-pyrimidinone ring that was originally developed as a cytidine deaminase inhibitor because it lacks an amino group on C-4 of the pyrimidine ring [4,5]. Different from other DNMT inhibitors, zebularine is stable and has low toxicity in most cell lines tested both in vitro and in vivo [6]. Several preclinical studies have evaluated zebularine as a possible therapeutic in cancer cell lines, which preferentially incorporates into DNA, leading to cell growth inhibition and increased expression of cell cycle regulatory genes [7]. In several studies to date, tumor cells exposure to demethylating agents (i.e., zebularine) cause the upregulation of tumor suppressor genes, such as p16, that are silenced by hypermethylation [8]. Once tumor suppressor genes are frequently silenced by methylation in numerous tumor types, the subsequent interruption of proapoptotic pathways is thought to contribute to increased proliferation and/or drug resistance [9]. Zebularine could reverse methylation-induced silencing of tumor suppressors, and then induces apoptosis pathway activation in tumor cells [10].

So far, there is no available information about the anti-tumor effects of zebularine on human gastric cancer cells. In the study, we addressed the hypothesis that zebularine plays an important role in mitochondria-mediated apoptosis in gastric cancer cells. The purpose of this study is to test our hypothesis whether zebularine is capable of inhibiting proliferation and inducing mitochondria-mediated apoptosis in gastric cancer cells in vitro and in vivo.

2. Materials and methods

2.1. Cell culture

BGC823, SGC7901, MGC803 and GES-1 cells were purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All the cell lines were

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cultured in RPMI 1640 medium (GIBCO BRL, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 10 U/mL penicillin, and 10 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

2.2. Antibodies and reagents

Zebularine was purchased from Calbiochem (Darmstadt, Germany). The primary antibodies against human DNMT1, DNMT3a, DNMT3b, p16, Bax, and Bcl-2 (Santa Cruz Biotechnology, CA, USA) were used in Western blot analysis.

2.3. 3-(4,5-Dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

All normal and cancer cell lines were seeded into 96-well plates (6.0×10^3 cells/well) and allowed to attach overnight. After cellular adhesion, freshly prepared zebularine at the appropriate concentration (0–100 μM) was added. The viability of the cells was evaluated using an MTT assay according to the manufacturer's specifications (Roche Applied Science, Indianapolis, IN, USA). Briefly, MTT was added at a concentration of 500 mg/L, and the cells were incubated for 4 h at 37 °C. The absorbance reading of each well was determined using a computer-controlled microtiter plate reader at a wavelength of 570 nM. The cell growth inhibitory rates were defined as the relative absorbance of treated versus untreated cells.

2.4. Hoechst staining assay

BGC823 cells were cultured in 6-well tissue culture plates and treated with 0, 10, 50, and 100 μM zebularine for 48 h. Next, Hoechst 33258 (Sigma–Aldrich, St. Louis, MO, USA) was added to the culture medium of living cells; changes in nuclear morphology were detected by fluorescence microscopy (Nikon, Japan) with a filter for Hoechst 33258 (365 nm). The percentages of Hoechst-positive nuclei per optical field (with a minimum of 50 fields) were counted.

2.5. Apoptosis analysis

BGC823 cells were incubated in the presence of zebularine for an additional 48 h, then harvested and fixed with 2.5% glutaraldehyde for 30 min and washed twice with ice-cold PBS. Apoptotic cells were detected using flow cytometry (Molecular Probes, Invitrogen, USA) with annexin V-FITC and propidium iodide (BioVision, USA).

2.6. Caspase-3 activity

A Caspase-3 Colorimetric Assay Kit (Nanjing Keygen Biotech. Co., Ltd. China) was used to measure the activity of Caspase-3. Following the manufacturer's instruction, the cells were incubated in the presence or absence of zebularine for 48 h. The cells were then harvested, resuspended in 50 μL of lysis buffer and incubated on ice for 30 min. At this point the cellular debris was pelleted, and the lysates (50 μL) were transferred to 96-well plates. The lysates were added to 50 μL of 2.0× reaction buffer along with 5 μl of Caspase-3 Substrate and incubated for 4 h at 37 °C in a 5% CO_2 incubator. The activities were quantified spectrophotometrically at a wavelength of 405 nm.

2.7. Western blot assay

Protein expression levels were analyzed by Western blot. Briefly, the cells were washed with PBS and lysed with lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 50 μg/ml leupeptin, 30 μg/ml aprotinin, and 1 mM PMSF). Protein was loaded at a concentration of 40 μg per lane, separated on a 12.5% sodium dodecyl sulfate polyacrylamide (SDS–PAGE) gel, and then transferred onto a nitrocellulose membrane (Millipore, Bedford, MA) using a wet transfer system (Bio-Rad, Hercules, CA). Next, the membrane was blocked with 10% nonfat dry milk in TBST (Tris buffered saline with Tween-20, pH 8.0) and then incubated with primary antibodies: DNMT1, DNMT3a, DNMT3b, p16, Bax, Bcl-2, and actin overnight at 4 °C. The appropriate horseradish peroxidase (HRP) conjugated secondary antibodies were used at 1:3000 for all antibodies. Positive antibody reactions were detected with the enhanced chemoluminescence system and Hyperfilm X-ray film.

2.8. In vivo assay for tumor growth

All animal procedures were approved by the Committee on Animal Experimentation of Wuhan University, and the procedures complied with the NIH Guide for the Care and Use of Laboratory Animals. BGC823 cells were injected into the flanks of BALB/c nude mice (Nu/Nu, female, 4–6 weeks old) purchased from the Center of Experimental Animals of Wuhan University and maintained under pathogen-free conditions. Nude mice were randomly divided into four groups: control group, low-dose group (10 mg/kg oral), mid-dose group (50 mg/kg oral), and high-dose group (100 mg/kg oral). Each group consisted of five nude mice (at least six tumors per group; one or two nude mice per group were randomly killed at earlier time points to establish a time course of expression). When the tumors were 100–150 mm^3 in size, the nude mice were treated with zebularine via oral gavage in a solution of 0.45% saline every 4 days. And control group treated with 0.45% saline administered by oral gavage every the same 4 days. Tumor growth was monitored by measuring tumor volume (TV), which was calculated by the formula: TV (mm^3) = width^2 (mm^2) × length (mm)/2. The fold differences in tumor growth among the various nude mice groups are calculated using relative tumor volume (RTV), which are calculated as follows: $RTV = TV_n/TV_0$, where TV_n is the tumor volume at a given day n and TV_0 is the tumor volume at day 0 (initial treatment). At the end of the experiment, the tumors were harvested for further analyses, as described below. Differences in tumor growth were tested for statistical significance.

2.9. TUNEL assay

Each group of nude mice was weighed at the end of the experiment. Next, to detect apoptotic cells in tumor tissue sections, an in situ apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA) was used. Tumor sections were dewaxed with dimethylbenzene, rehydrated with gradient ethanol, incubated with proteinase K, and rinsed with ddH_2O. A 3% H_2O_2 solution was used to block endogenous peroxidase. After incubation with equilibration buffer and terminal deoxynucleotidyl transferase enzyme, the sections were incubated with antidigoxigenin–peroxidase conjugate. The peroxidase activity in each section was detected using diaminobenzidine. Finally, the sections were counterstained with hematoxylin. Positive cells were identified and counted (three random fields per slide) under a light microscope (Carl Zeiss, NY, USA).

2.10. Statistical analysis

All experimental data were shown as the mean ± S.E.M. The means of the different groups were compared using one-way ANOVA. All statistical analyses were performed with the SPSS 13.0 software (SPSS Inc., IL, USA). Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Zebularine inhibits human gastric cancer cells growth in a dose- and time-dependent manner

The three human gastric cancer cell lines were treated with 10 μM zebularine, and the percentage of surviving cells was assessed by MTT assay from 24 to 72 h. After treatment, the growth rates for these cell lines were significantly inhibited, especially in BGC823 cells (Fig. 1A). Furthermore, the growth rate of BGC823 cells was greatly decreased by incubation 50 and 100 μM zebularine (Fig. 1B).

Then, we determine the general cytotoxicity of zebularine in normal human gastric mucosa epithelial (GES-1) cells. As these cells divide more slowly than gastric cancer cell lines, we treated GES-1 cells with increasing doses of zebularine for 96 h rather than for 48 h. As shown in Fig. 1B, GES-1 cells growth rates were reduced by only 37% at 100 μM zebularine, even after 96 h. In contrast, about 78% inhibition of the three cancer cell lines after 48 h of treatment at 100 μM zebularine. Moreover, zebularine exhibited an IC_{50} of 214 μM following 96-h treatment in GES-1 cells, 4.7- to 5.4-fold higher than the 50% effective doses for the 48-h-treated gastric cancer cell lines. These data indicated that zebularine not only has a potential to prevent gastric cancer cell growth, but also has minimally toxic to normal human gastric mucosa epithelial cells.

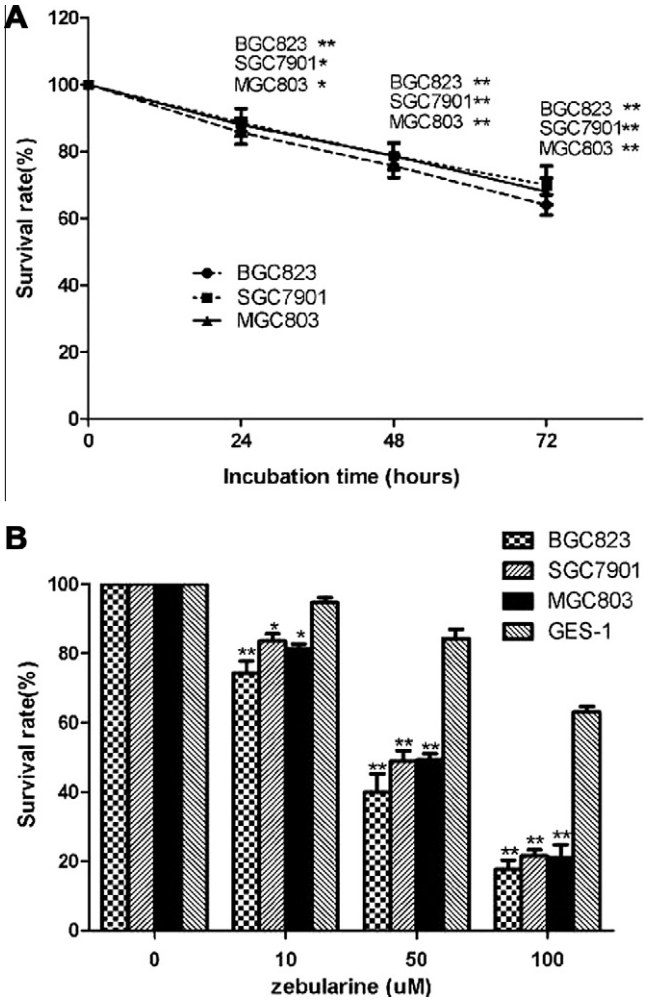

Fig. 1. Effect of zebularine on growth of gastric cancer cells. An MTT assay was used to detect the growth of gastric cancer cells. (A) Growth rate of three gastric cancer cell lines BGC823, SGC7901 and MGC803 treated with 10 μM zebularine for 0, 24, 48, and 72 h. (B) Growth rate of gastric cancer cell lines and normal gastric mucosa epithelial (GES-1) cells treated with various concentrations of zebularine for 48 h. *P < 0.05, **P < 0.01 versus 0.

3.2. Zebularine induces apoptotic cell death in gastric cancer cells

Apoptosis is one of the main types of programmed cell death which involves a series of biochemical events leading to specific cell morphology characteristics such as cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation [11]. The morphology characteristics of the apoptotic BGC823 cells were detected by Hoechst 33258 staining. As shown in Fig. 2A, in the control group, the nuclear were stained a weak homogeneous blue, while in zebularine treatment groups, bright chromatin condensation and nuclear fragmentation could be found.

Zebularine has been reported to have an anti-tumor effect by apoptosis induction in various solid tumors [12,13]. Thus, we analyzed the induction of apoptosis in gastric cancer cell lines BGC823 upon treatment with zebularine. BGC823 cells were treated with zebularine (10–100 μM) for 48 h followed by annexin V-FITC/PI staining to examine the proportion of apoptotic cells. As shown in Fig. 2B, it revealed that zebularine caused cell apoptosis in a dose-dependent manner.

3.3. Zebularine induces p16 reexpression and activates the mitochondrial apoptotic pathway in gastric cancer cells

Zebularine is a DNMT inhibitor in other model systems. Cheng et al. [7] had previously shown the effects of continuous treatment with zebularine on the protein levels of DNMTs in T24 bladder cancer cells using Western blot analysis. In our study, zebularine treatment was associated with a statistically significant dose-dependent depletion of DNMT1, DNMT3a, and DNMT3b proteins in BGC823 cells (Fig. 3A). It is now confirmed that the antiproliferative effects of DNA methylation inhibitors are largely due to the reexpression of tumor suppressor genes such as p16 [8]. p16 is a tumor suppressor protein, which is lost expression in gastric cancer because of hypermethylation [14]. Untreated control cells showed no p16 expression, whereas cells treated with zebularine showed robust expression of p16 (Fig. 3B). Zebularine successfully induced p16 expression in a dose-dependent manner. To examine the mechanism of zebularine-induced apoptosis in gastric cancer cells, we analyzed mitochondrial features of the intrinsic apoptotic pathway. The proapoptotic Bcl-2 family members, such as Bax and Bcl-2, are essential for the initiation of mitochondrial dysfunction during apoptosis. The key apoptosis-associated proteins were explored by Western blot analysis. Results showed that in BGC823 cells, treatment with increased doses of zebularine was associated with enhanced expression of Bax and reduced expression of antiapoptotic Bcl-2 (Fig. 3B). Additionally, the activity of caspase-3 were upregulated (Fig. 3C). It suggested that Bcl-2 inhibited Bax activity, which reduced mitochondrial membrane potential, leading to caspase-3 upregulation and cell apoptosis [15]. Together these findings suggested that zebularine treatment of gastric cancer cells induces apoptosis through the mitochondrial apoptosis pathway.

3.4. Anti-tumor effect of zebularine on human gastric cancer cells in vivo

Because zebularine inhibited growth and induced apoptosis in BGC823 in vitro, we next examined the anti-tumor effect of zebularine in vivo. BGC823 cells were inoculated subcutaneously into the right and left flanks of female BALB/c nude mice at 4–6 weeks of age. When the tumor xenografts reached a mean size of 100 mm^3 (approximately 6–10 days later), the nude mice were

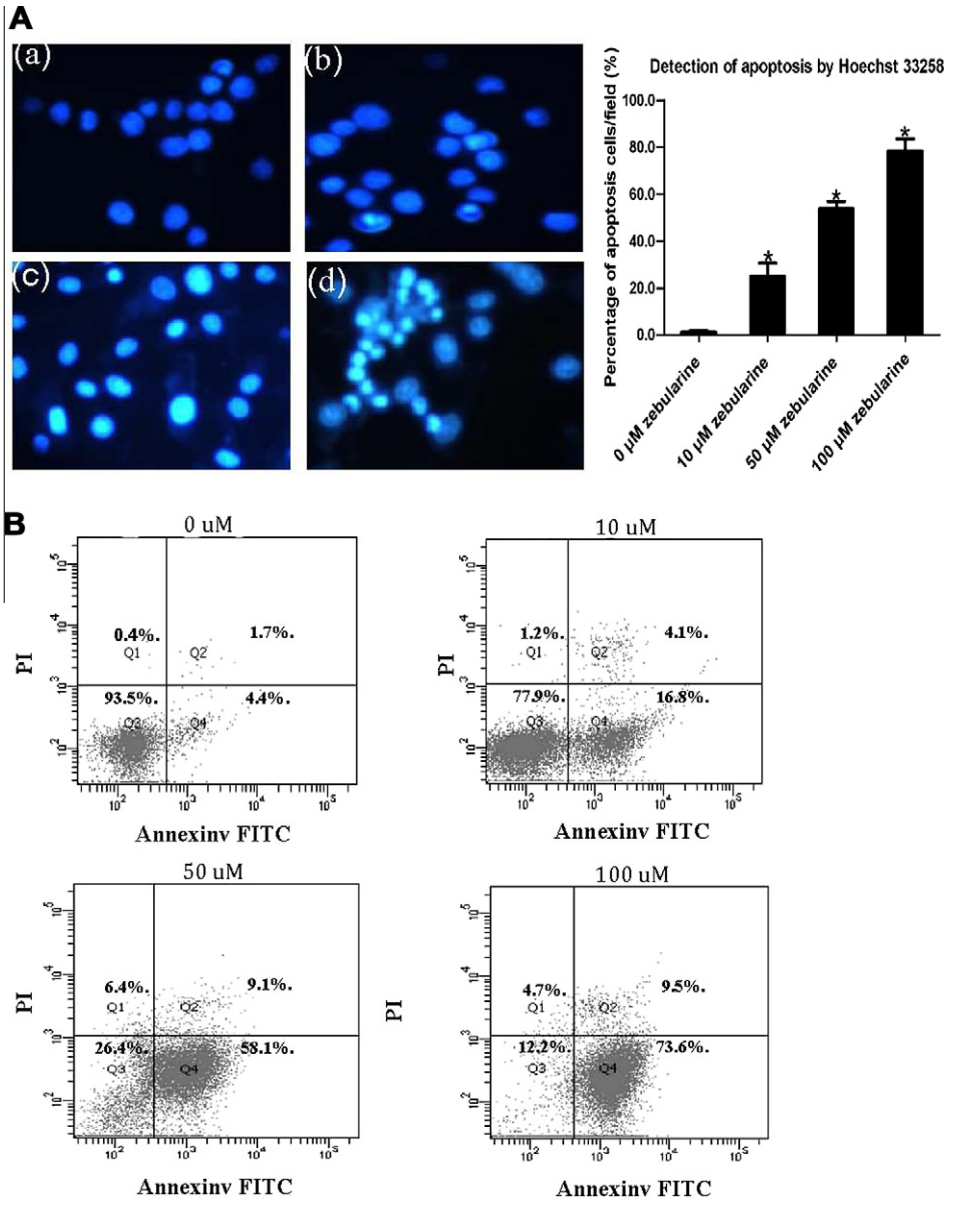

Fig. 2. Effect of zebularine on cell apoptosis. (A) Detection of apoptosis by Hoechst 33258. BGC823 cells were incubated with 0, 10, 50, and 100 μM of zebularine for 48 h (a–d). Original magnification: 400×. *P < 0.05 versus 0 μM. (B) Flow cytometry analysis of apoptotic cells. BGC823 cell was treated with various concentrations (0–100 μM) of zebularine for 48 h. Percentage value indicates the proportion of apoptotic cells.

treated with different concentrations of zebularine via oral gavage every 4 days. As shown in Fig. 4A, Tumors from control nude mice had a high ratio of tumor cells to stroma, whereas tumors from nude mice treated with zebularine (10, 50, and 100 mg/kg oral) had a much lower ratio of tumor cells to stroma. Treatment with zebularine reduced tumor growth in a dose-dependent manner. At the end of 20 days, compare with oral control group, tumor weight was only 0.212 g in high-dose group (Fig. 4B).

In Fig. 4C, the results of TUNEL assay of the subcutaneous tumor sections demonstrated that zebularine caused obvious cell death in tumor mass via apoptosis, whereas less apoptosis was found in control group ($P < 0.05$). These data proved that zebularine has significant anti-tumoral potential in vivo.

4. Discussion

Gastric cancer is the second cause of cancer related mortality rate worldwide after lung cancer and the fourth most common cancer, with approximately 900,000 new cases and 700,000 deaths per year [16]. In East Asia especially, like China and Japan, more than one million new cases are diagnosed with gastric cancer each year [17]. To increase the survival of gastric cancer and develop medicines with high efficacy, low toxicity and new mechanisms of action is required. Tumorigenesis and progression are relevant to genetic and epigenetic changes, and one of the epigenetic changes is DNA methylation [3]. Accumulated evidence demonstrates that the DNA of tumor suppressor genes is hypermethylated in varied cancers, which is considered to be an important mechanism for inactivating tumor suppression genes during tumorigenesis [18]. Recently, DNA methylation inhibitor agents [19,20] have been made to reverse the hypermethylation status of tumor-suppressor genes for treating cancers.

Zebularine is considered as a novel mechanism-based inhibitor of DNA methylation, which was demonstrated anti-tumor activity both in vitro and in vivo in various cancer cells that are resistant to the conventional anti-tumor drugs [21,22]. Moreover, although numerous studies have suggested that zebularine may be selective

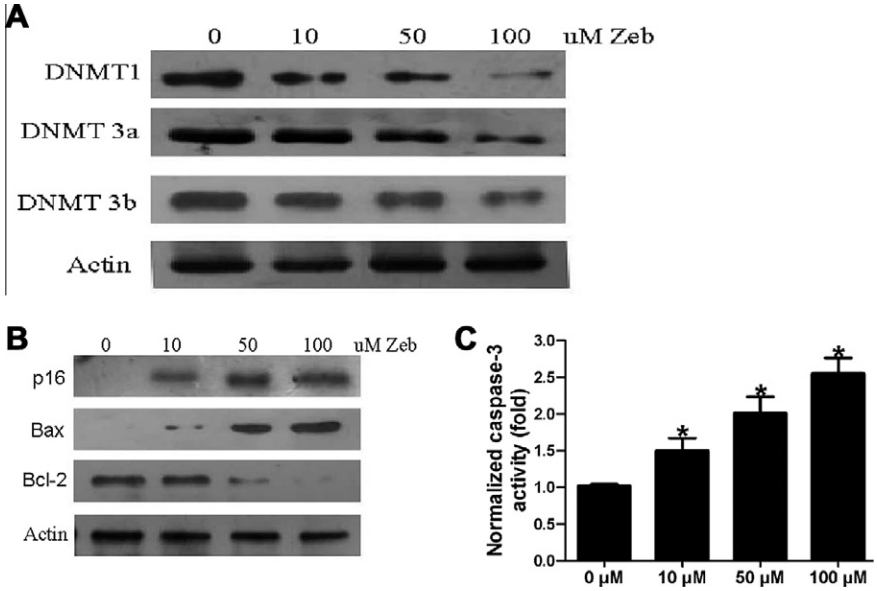

Fig. 3. Effects of zebularine treatment on DNMT protein levels, proapoptotic and antiapoptotic elements in BGC823 cells. BGC823 cells were treated with 0, 10, 50, and 100 μM zebularine for 48 h. (A) The protein levels of DNMT1, −3a, and −3b were determined by Western blot. (B) p16, Bax and Bcl-2 expression were determined by Western blot. Zebularine increased the expressions of p16, and Bax, but decreased Bcl-2 expression. Actin expression was used as an internal control. (C) Analysis of relative caspase-3 activity in cells. Zebularine increased the activity of caspase-3. $^{*}P < 0.05$ versus 0 μM.

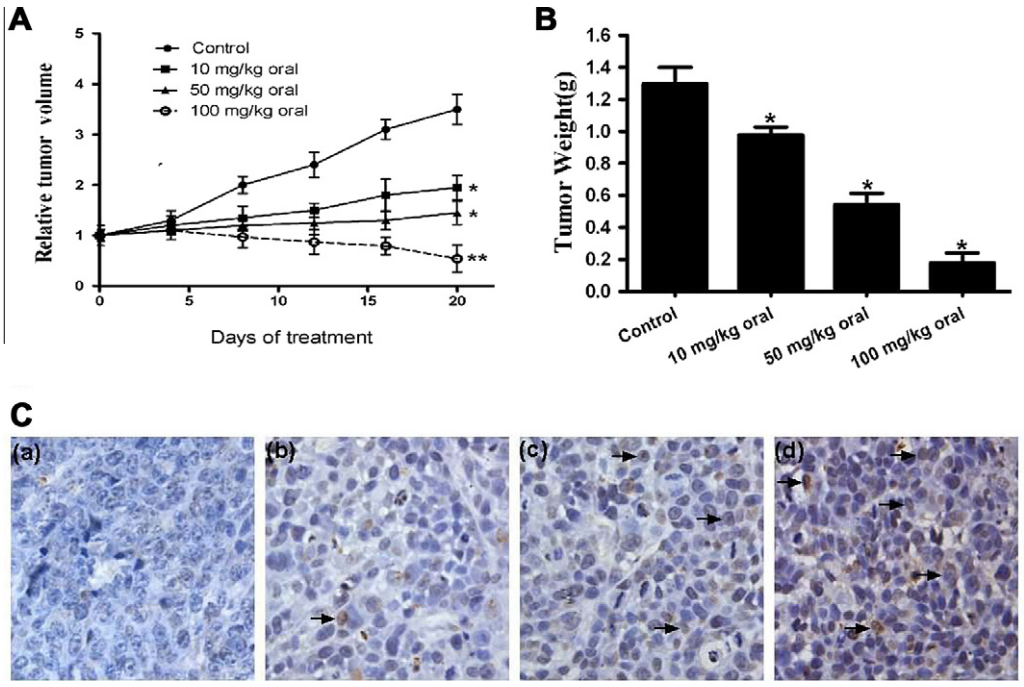

Fig. 4. Zebularine suppresses of tumor growth in vivo. (A) Tumor volume was measured for all nude mice groups at the indicated time points, averaged within each group, and plotted as the mean relative tumor volume (RTV) versus days of treatment for each group. Data represent the relative tumor volume (RTV) ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. (B) Tumor weight was obtained at the end of the experiment. Data represent the mean body weight ± S.E.M. $^{*}P < 0.05$ versus control. (C) Detection of apoptotic cells in tumor tissues was performed using the TUNEL assay. Tumors from BGC823. (a) Tumor with untreated group, (b) tumor with low-dose zebularine (10 mg/kg oral), (c) tumor with mid-dose noscapine (50 mg/kg oral), and (d) tumor with high-dose noscapine (100 mg/kg oral). The brown color of apoptotic signals is shown by the arrows. Original magnification: 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

toward cancer cells and have potential as an anticancer therapy [23], its effect on gastric cancer has not yet been addressed. In the study, our results are the first to show zebularine effectively inhibited BGC823, SGC7901, and MGC803 cells proliferation by inducing the cell death, especially in BGC823 cells.

The effect of zebularine on cell proliferation was assayed by MTT. Although minimal effects were seen after 24 h of 10 μM zebularine exposure, a significant dose- and time-dependent inhibition of cell growth was observed thereafter. Additionally, the IC50s of zebularine were approximate 42, 33 and 27 μM in BGC823 cells on exposure for 48, 72 and 96 h, respectively. To compare growth inhibition by cancer cell lines versus normal human gastric mucosa epithelial (GES-1) cells, we examined the effect of zebularine on proliferation of the gastric cancer cell lines

BGC823, SGC7901, MGC803 and GES-1 cells. Zebularine, at 100 μM, elicited >75% growth inhibition of all three cancer lines but only 37% inhibition of normal human gastric mucosal cells (Fig. 1B), suggesting minimal toxicity. Based on our MTT assay, it is unlikely that zebularine induces substantial cell cycle arrest or apoptosis in normal gastric mucosal cells.

It also revealed that zebularine resulted in apoptosis of treated cells in a dose-dependent manner (Fig. 2). Zebularine is a DNMT inhibitor and its ability to induce epigenetic alterations has already been reported [24]. In the present report, zebularine depleted expression of DNMTs protein (Fig. 3A). This reduction of DNMT activity is associated with re-expression of epigenetically silenced genes [25]. Here we observed also that DNMT inhibition with zebularine was able to reactivate the expression of p16 (Fig. 3B), which was a tumor suppressor gene, but hypermethylation in gastric cancer [14]. This was likely responsible for enhancing the expression of Bax, and inhibiting the expression of Bcl-2 associated with the increasing of caspases-3 activity (Fig. 3B and C). The results were consistent with the results of Kim et al. [26], which reported that zebularine could regulate caspase-3 activity and induce apoptosis of lung cancer cell line (A549). Moerover, these are interesting results as there are few previous reports describing the signaling pathway of zebularine-induced apoptosis. In cells, apoptosis appears to be triggered by both extrinsic and intrinsic pathways through inhibition of antiapoptotic Bcl-2 and activation of Bax. It has been reported that the ratio of Bax and Bcl-2 determines the response to a death signal via modulating membrane permeability transition (MPT) pore opening [27]. The result was cooperative activation of the executioner caspase-3 activity by both caspase pathways in the end. In agreement with the hypothesis, activated caspase-3 was detected in BGC823 cells treated with zebularine in each group (Fig. 3C). The data indicated that zebularine could induce apoptosis by reactivating p16 gene and activating mitochondria apoptosis pathway.

In the xenograft tumor-bearing nude mouse model, zebularine was revealed to have significant anti-cancer effect (Fig. 4A and B). The therapeutic effect has been occurred, at least in part, through apoptosis induction, as determined by TUNEL staining of tumor sections (Fig. 4C). what is unique and exciting about zebularine is that this is the first time a methylation inhibitor zebularine has been shown to exhibit an in vivo antitumor effect in BGC823 cell line-bearing mouse models. These findings raise the possibility that zebularine may be clinically useful to treat certain cancers.

In conclusion, our results strongly suggest that zebularine is not only an effective demethylating agent, but also a high-efficacy therapeutic drug for gastric cancer. We demonstrated the mechanism of action in inducing apoptosis in gastric cancer cell lines by zebularine, in which mitochondrial dysfunction was involved. These results provide a rationale to continue research with zebularine, and may lead to the development of novel DNMT inhibitors that can be used in combination for gastric cancer treatment.

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