

5-Aza-2'-deoxycytidine induces cytotoxicity in BGC-823 cells via DNA methyltransferase 1 and 3a independent of p53 status

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Abstract. The DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-Aza-CdR) has therapeutic value for the treatment of cancer. However, the mechanism by which 5-Aza-CdR induces antineoplastic activity is not clear. The efficacy of 5-Aza-CdR on the contribution of gene reactivation by demethylation and enzyme-DNA adduct formation is an important unresolved question. The aim of this study was to explore the mechanism of the effect of 5-Aza-CdR on human gastric cancer growth. Human BGC-823 cells were treated with different concentrations of 5-Aza-CdR for different durations. Cell viability, DNA damage and gene expression were determined. The results showed that 5-Aza-CdR at low concentrations induced inhibition of gastric cancer BGC-823 cell proliferation as well as increased apoptosis caused by DNA damage. For the first time, we demonstrated that 5-Aza-CdR-induced cytotoxicity against BGC-823 cells was predominantly regulated via upregulation of DNA methyltransferase 1, 3a and partially via reactivation of RUNX3, which was independent of p53 status and its ability to activate p21^{Waf1/Cip1} expression. To our knowledge, this is the first demonstration of p53-independent 5-Aza-CdR action on DNA methyltransferases and demethylation. These results strongly provide the preclinical rationale for the clinical evaluation of 5-Aza-CdR to improve patient outcome in gastric cancer.

Introduction

DNA methylation and related modulation of gene expression contribute to the development of malignancies (1,2).

Specifically, methylation of CpG dinucleotides in promoter regions has been associated with transcriptional silencing of tumor suppressor genes, suggesting DNA methylation as a target for novel therapeutics (3). 5-Azacytidine and 5-Aza-2'-deoxycytidine (5-Aza-CdR) belong to a class of cytosine analogues, which are developed as inhibitors of DNA methylation. Both analogues have been shown to have significant cytotoxic and antineoplastic activities in many experimental tumors (3,4). 5-Aza-CdR, however, is reported to be non-carcinogenic. It incorporates into only DNA but not RNA or proteins (5). Additionally, considerable evidence shows that 5-Aza-CdR has more potent therapeutic effects than 5-Azacytidine in cell culture and animal models of human cancers.

5-Azacytidine is the only DNA methyltransferase (DNMT) inhibitor (MTI) approved by the Food and Drug Administration for treating myelodysplastic syndromes and chronic myelomonocytic leukemia (6). Recently, several clinical trials of 5-Aza-CdR have been reported, including a phase II study of 5-Aza-CdR in patients with metastatic prostate cancer and a phase III study of 5-Aza-CdR (Decitabine) in patients with myelodysplasia (7). Clinical trials evaluating 5-Aza-CdR as a cancer chemotherapeutic have shown promise for the treatment of leukemia, but less effective against solid tumors.

5-Aza-CdR binds DNMT in an irreversible, covalent manner, thus sequestering DNMT enzyme activity and preventing maintenance of the DNA methylation state (8). Consequently, silenced genes are demethylated and are re-expressed. Two major non-mutually exclusive mechanisms of its tumor cytotoxicity have been proposed. One states that 5-Aza-CdR demethylates cellular DNA, with reactivation of silenced genes; and the other states that 5-Aza-CdR induces DNA damage due to the formation of irreversible, covalent enzyme-DNA adducts (9). However, the relative contribution of gene reactivation and enzyme-DNA adduct formation to the efficacy and toxicity of 5-Aza-CdR *in vivo* remains unresolved.

As one of the major causes of cancer death, gastric cancer remains threatening around the world, and most patients in advanced stages need chemotherapy. To date, however, the effects of 5-Aza-CdR and mechanisms against gastric cancer have not been completely characterized. Here, we show that 5-Aza-CdR is cytotoxic against human gastric cancer

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BGC-823 cell growth. Mechanistic studies demonstrated that 5-Aza-CdR induces DNA damage and causes apoptosis of gastric cancer cells. These responses were mediated mainly through upregulation of DNMT and partially through demethylation of runt-related transcription factor 3 (RUNX3), which was independent of p53 status and its ability to activate p21^{Waf1/Cip1} expression. Taken together, these studies provide the preclinical rationale for the clinical evaluation of 5-Aza-CdR to improve patient outcome in gastric cancer.

Materials and methods

Cells and treatments. Human lung cancer cell line A549 and the gastric cancer cell line BGC-823 were obtained from the China Center for Type Culture Collection (CCTCC). Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂. For treatment with 5-Aza-CdR (Sigma), cells were exposed to a single dose of 0.01-100 μM of drug. 5-Aza-CdR was dissolved in phosphate-buffered saline (PBS) and fresh medium containing 5-Aza-CdR was added every 24 h.

MTT assay. Cell proliferation was measured using the MTT assay. Cells were plated in triplicate at 1x10³ cells/well in 96-well plates, cultured as previously described, and treated with different concentrations of 5-Aza-CdR or z-VAD-fmk (Beyotime Institute of Biotechnology) for indicated times respectively. Twenty microliters of 5 mg/ml of MTT (Amresco) was then added into each well and the cells were cultured at 37°C for an additional 4-6 h. The resulting formazan crystals were solubilized by the addition of 150 μl of DMSO to each well. The optical density (OD) level under 570 nm was measured and the percentage of cell viability was calculated using the following formula: percentage of cell viability = (absorbance_{experimental well} - absorbance_{blank}) / (absorbance_{untreated control well} - absorbance_{blank}) x 100%.

Flow cytometric analysis of DNA content. Cells were seeded into 6-well plates at a density of 4 to 5x10⁵ cells/well. After 72 h of treatment with 0.5, 1 and 5 μM 5-Aza-CdR, cells were washed with PBS, permeabilized with 70% ethanol overnight. The next day, ethanol was removed and cells were incubated for 15-20 min at 37°C with 1 ml PI solution (0.1% Triton X-100, 50 μg PI and 200 μg RNase A). Distribution of cell cycle phases with different DNA contents was determined using a flow cytometer (Beckman Coulter, Miami, FL, USA).

Annexin V staining. Cells (5 to 7x10⁵) were seeded into 6-well plates and treated with 0.5, 1 and 5 μM of 5-Aza-CdR for 48 h. The cells were then trypsinized and washed in PBS. Annexin V staining was then performed following the manufacturer's instructions of the Annexin V Staining kit (Multi Sciences Biotech Co., Ltd).

Measurement of caspase-3 activity. Caspase-3 activity in the cell lysate was measured using a colorimetric kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology). The absorbance was determined at 405 nm

and activity of caspase-3 was determined by calculating the ratio of OD at 405 nm of drug treated cells to untreated cells.

Comet assay for detecting DNA strand breaks. The Comet assay was performed as previously described (10). In brief, slides were cleaned with acid and scrapped with 40 μl of 0.6% agarose. Cell suspension (20 μl) and 80 μl of 1.1% low-melting agarose were mixed before adding to the first gel layer. The gel layer was covered with a coverslip immediately, and then was kept at 4°C for 15 min to allow it to solidify. After gently removing the coverslip, the slides were immersed in fresh prepared cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10.0) with 1% Triton X-100 and 10% DMSO for at least 1 h at 4°C. After electrophoresis in fresh solution (1 mM Na₂EDTA, 300 mM NaOH, pH 13.0) for 30 min, the slides were placed in Tris buffer (0.4 M Tris, pH 7.5) for 15 min twice. The slides were then stained with 40 μl of 0.1 mg/ml propidium iodide (PI). One hundred randomly selected cells were counted per slide. The images were captured and scored for each sample using an Image Analysis software system (IMI version 1.0). The standard of assessing DNA single strand breaks was based on the percentage of cells with tail and tail length (a distance from DNA head to the end of DNA tail) by visual estimation.

RNA preparation and RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) and 1 μg of RNA was used as a template for the synthesis of cDNA using the RevertAidTM first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. Polymerase chain reaction (PCR) analysis was performed in a final volume of 25 μl using the PCR Master Mix (Fermentas). Primer sequences and annealing temperatures are indicated in Table I. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and photographed.

Western blotting. After cells were cultured with the indicated concentrations of 5-Aza-CdR for the specified times, cells were lysed using RIPA buffer containing protease inhibitors. After normalization for total protein content (50 μg/lane), samples were subjected to 15% SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad Laboratories). After blocking with 5% non-fat dry milk and 0.1% Tween-20 in Tris-buffered saline, membranes were incubated with mouse anti-p21^{Waf1/Cip1}, p53 (Santa Cruz Biotechnology), goat anti-RUNX3 (R&D Systems), rabbit anti-caspase-3, β-tubulin (Santa Cruz Biotechnology). After extensive rinsing with TBST buffer, blots were incubated with HRP-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibodies (Pierce) and developed with the use of an enhanced chemiluminescence system (Millipore) and captured on light-sensitive imaging film (Kodak, Tokyo, Japan).

Statistical analysis. All experiments were repeated in triplicate. Data are presented as means ± SD. Levels of significance for comparisons between samples were determined using the Student's t-test distribution. Statistical analyses among more than three samples were performed by ANOVA with Tukey's honest significant difference post hoc test applied to significant main effects or interactions (SPSS 11.5 for Windows).

Table I. Primers and conditions used for RT-PCR.

Primers	Sequences	Temperature (°C)	Product length (bp)
GAPDH	F: 5'-ACGGATTTGGTCGTATTGGG-3' R: 5'-TCCTGGAAGATGGTGATGGG-3'	56	211
RUNX3	F: 5'-GAAAAGGCGTAAGGGAAGCTC-3' R: 5'-ACCTGGGACCAGCTATAACC-3'	56	395
p21 ^{Waf1/Cip1}	F: 5'-GTGAGCGATGGAAGCTTCGACT-3' R: 5'-CGAGGCACAAGG GTACAAGAC-3'	56	229
p53	F: 5'-GTCTACCTCCCGCCATAA-3' R: 5'-CATCTCCCAAACATCCCT-3'	55	316
DNMT1	F: 5'-CGCTGTATCTAGCAAGGGTCA-3' R: 5'-TCGAATCTCGCGTAGTCTTG-3'	56	313
DNMT3a	F: 5'-ACCACAGAGGCGGAAATACC-3' R: 5'-GTCTCCCTGCTGCTAACTGG-3'	56	543
DNMT3b	F: 5'-CAGGAGACCTACCCTCCACA-3' R: 5'-TTACGTCGTGGCTCCAGTTA-3'	56	436

Results

5-Aza-CdR inhibits BGC-823 cell growth by induction of apoptosis. Human lung cancer A549 cells were used as a control since it is well-established that 5-Aza-CdR potentially overcomes growth advantages (11). Human BGC-823 cells and A549 cells were exposed to 5-Aza-CdR at different concentrations for 72 h. The cell viability was determined by the MTT assay. A dose-dependent inhibition of cell proliferation was observed in both BGC-823 and A549 cells. As shown in Fig. 1A, BGC cell viability was 76.23% after 1 μ M 5-Aza-CdR treatment, while its viability was only about 25% after 100 μ M 5-Aza-CdR treatment.

Thus, 1 μ M 5-Aza-CdR treatment resulted in cell growth inhibition in both cell lines. Therefore, to determine the duration effect of 5-Aza-CdR on cell viability, BGC-823 cells were treated with 1 μ M of 5-Aza-CdR for different durations. As expected, we observed not only a dose-dependent growth suppression, but also a duration-dependent growth suppression in BGC-823 cells (Fig. 1B). BGC-823 cell viability was 90, 82, 70 and 38% after 24, 48, 72 and 96 h of treatment, respectively.

To dissect the mechanism of the anti-proliferative effects of 5-Aza-CdR in BGC-823 cells, we determined whether inhibition in cell viability was associated with specific cell cycle arrest. Exponentially growing BGC-823 cells were treated with 0.5, 1 or 5 μ M 5-Aza-CdR for 72 h. The cells were harvested for flow cytometric analysis of DNA content by PI staining as described in Materials and methods. The results showed that BGC-823 cell cycles were not affected by the presence of 5-Aza-CdR (Fig. 1C).

Next, we aimed to examine whether 5-Aza-CdR could efficiently trigger apoptosis, which led to cytotoxicity against BGC-823 cells. BGC-823 cells were treated with 0.5, 1 and 5 μ M of 5-Aza-CdR for 48 h. The cells were subjected to apoptosis analysis using Annexin V staining flow cytometry

assay as described in Materials and methods. As shown in Fig. 1D, the data revealed that 5-Aza-CdR treatment increased the proportion of cells positive for Annexin V from 0.7% in pretreated cells to 15.5, 32.7 and 49.6% after 5-Aza-CdR treatment at 0.5, 1 and 5 μ M, respectively. The results strongly suggested that apoptosis rather than necrosis is the mechanism of 5-Aza-CdR induced growth inhibition in BGC-823 cells.

Treatment with 5-Aza-CdR causes DNA damage independent of the activation of the caspase pathway in BGC-823 cells. Since 5-Aza-CdR has been reported to incorporate into DNA and not RNA (5), we evaluated the effect of 5-Aza-CdR on DNA damage. BGC-823 cells were treated with 5-Aza-CdR at 0.5, 1 and 5 μ M for 72 h. DNA damage was determined using the Comet assay. As shown in Fig. 2A, dose-dependent DNA damage was observed upon 5-Aza-CdR treatment in both BGC-823 cells. Not only more comet cells but also longer DNA tail lengths were observed with increased dosage treatment, indicating more extensive DNA damage at higher concentrations. As described in Materials and methods, the image was scored for the percentage of cells with tail and for DNA tail length. As shown in Fig. 2A, the percentage of cells with tail increased from 5.85% in untreated cells to 15.68, 48.20 and 60.97% in 0.5, 1 and 5 μ M treated cells, respectively; while the length of DNA tail increased from 12.10 in untreated cells to 36.33, 57.87, and 71.33 in 0.5, 1 and 5 μ M treated cells, respectively.

To analyze whether caspase-3 was responsible for the recruitment of apoptotic pathways by 5-Aza-CdR, we performed a colorimetric enzyme assay to examine the activity of caspase-3 and western blotting to detect the protein level of procaspase-3. The results showed that caspase-3 enzymatic activity was not affected in the 5-Aza-CdR-treated cells (Fig. 2B). The protein level of procaspase-3 was not affected by 5-Aza-CdR treatment either, as monitored by western blot analysis (Fig. 2C).

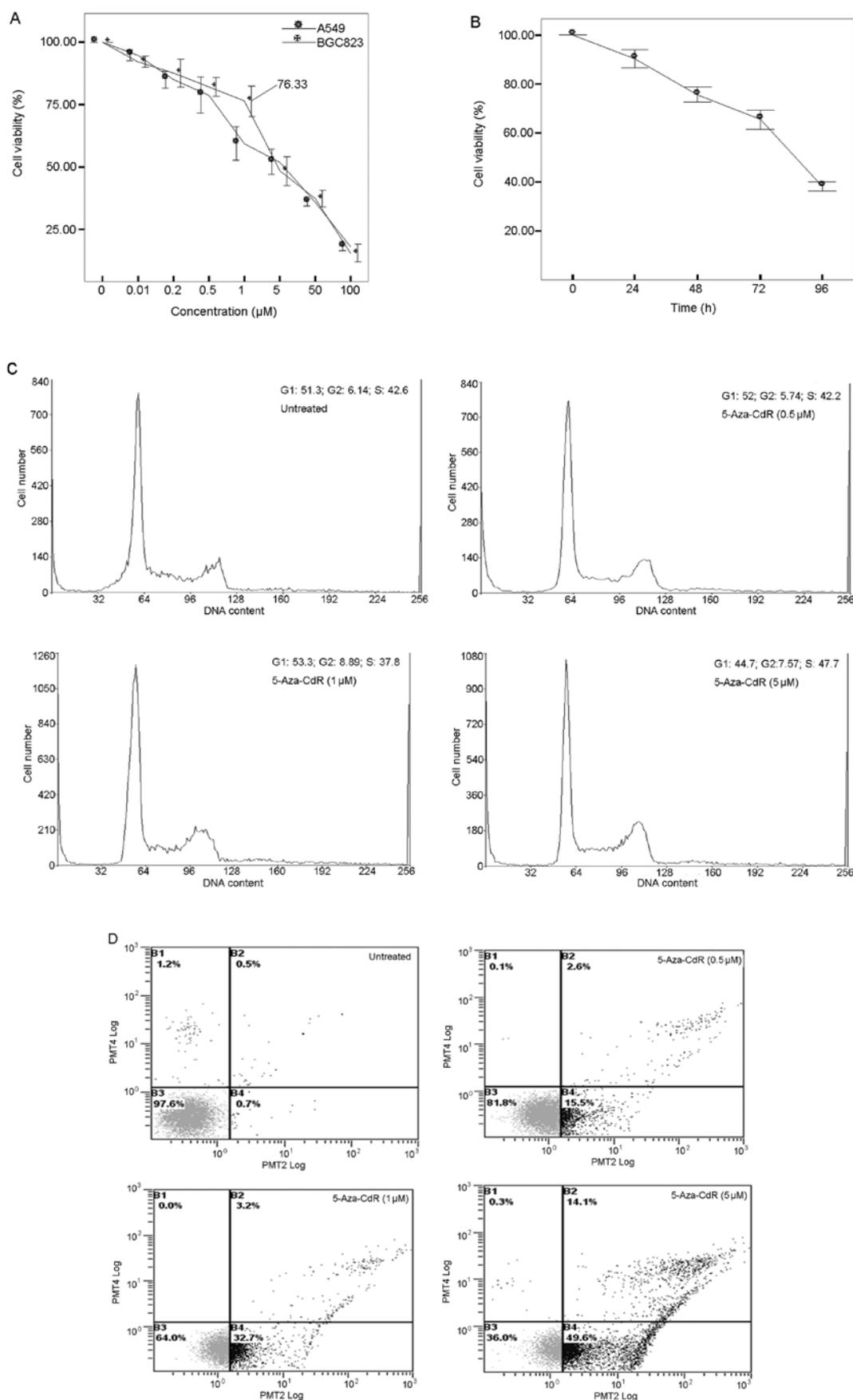


Figure 1. 5-Aza-CdR-induced cell proliferation inhibition and apoptosis in A549 and BGC-823 cells. Cell viability was measured with MTT when A549, BGC-823 cells were treated with 5-Aza-CdR at 0-100 μM for 72 h (A) and at 1 μM for different intervals (B). Results are presented as the average of quadruplicate measurements, and the bar is the standard deviation. (C) Effects on cell cycle profiles of BGC-823 cells after treatment with 5-Aza-CdR for 72 h at indicated concentrations. DNA content was determined by flow cytometric analysis by PI staining. (D) Percentage of apoptotic cells were scored after cells exposure to 5-Aza-CdR at 0, 0.5, 1 and 5 μM for 48 h using the Annexin V flow cytometric assay method.

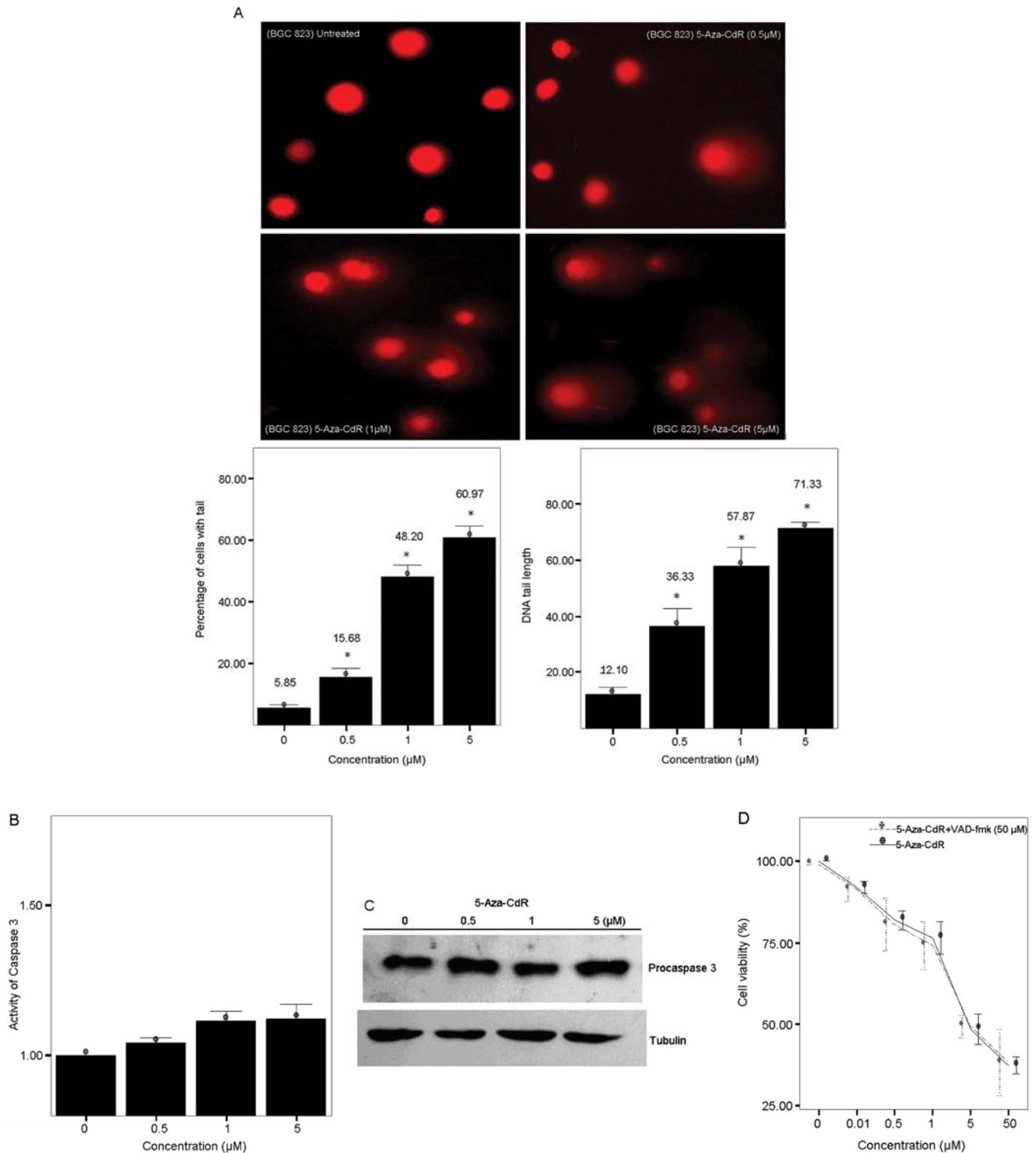


Figure 2. DNA damage and change of caspase-3 caused by 5-Aza-CdR in BGC-823 cells. (A) After 5-Aza-CdR treatment (0, 0.5, 1 and 5 μM) for 72 h, DNA damage in BGC 823-cells were detected using the Comet assay. DNA damage was characterized by the percentage of cells with a Comet tail/100 cells (%) and the comet tail length (from the center of DNA head to the end of the DNA tail) in BGC-823 cells. Representative pictures of BGC-823 cells are shown. * $P < 0.01$, as compared with respective untreated cells. Bars, mean of triplicate experiments; error bars, SD. (B) Induction of caspase-3 activity level. (C) Expression of procaspase-3 in BGC-823 cells was determined using colorimetric and western blotting methods respectively. (D) The pan-caspase inhibitor, z-VAD-fmk did not significantly alter the cytotoxicity of 5-Aza-CdR-treated BGC-823 cells, as examined by the MTT assay. Results represent the average of 3 independent experiments.

Furthermore, BGC-823 cells were pretreated with the pan-caspase inhibitor, z-VAD-fmk (5 μM), and then treated with various concentrations of 5-Aza-CdR for 72 h. Treatment with

z-VAD-fmk did not alter the cytotoxic effect of 5-Aza-CdR, implying that the 5-Aza-CdR-induced apoptosis was independent of the caspase pathway (Fig. 2D).

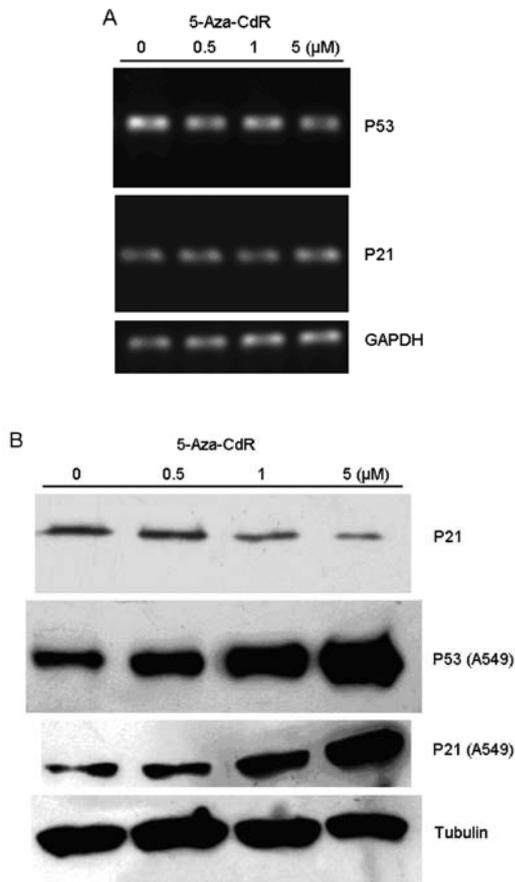


Figure 3. Effects of treatment with 5-Aza-CdR for 72 h on the expression of p53 and p21^{Waf1/Cip1}. (A) p53 or p21^{Waf1/Cip1} mRNA levels measured by RT-PCR after BGC-823 cells were treated with 5-Aza-CdR at indicated doses. GAPDH was used as an internal control. (B) The protein level of p21^{Waf1/Cip1} in BGC-823 cells and p53 or p21^{Waf1/Cip1} protein levels in A549 cells measured by western blotting. β -tubulin was used as an internal control.

5-Aza-CdR has no effect on p53 and p21^{Waf1/Cip1} expression. Because it has been proposed that p53 has a vital role in growth arrest and apoptosis in response to DNA damage, we explored the p53 expression in response to 5-Aza-CdR treatment in BGC-823 cells. p53 mRNA levels were determined by RT-PCR using total-RNA isolated from BGC-823 cells treated with 0.5, 1 and 5 μ M 5-Aza-CdR for 72 h. The results revealed that p53 mRNA levels were not changed in the presence of 5-Aza-CdR (Fig. 3A). Of interest, the protein expression of p53 was undetectable (data not shown).

It has been well established that the expression of p21^{Waf1/Cip1} is regulated by p53 (12); therefore, we examined the expression of p21^{Waf1/Cip1} in BGC-823 cells. p21^{Waf1/Cip1} gene expression was altered in BGC-823 cells exposed to 5-Aza-CdR (Fig. 3A). As a control, 5-Aza-CdR treatment increased the protein level of p21^{Waf1/Cip1} along with elevation of p53 protein levels in a dose-dependent manner in A549 cells (Fig. 3B).

5-Aza-CdR induces RUNX3, DNMT1 and DNMT3a gene transcription. 5-Aza-CdR has been reported to exert its cytotoxicity through depleting DNA methyltransferase levels, resulting in DNA hypomethylation (8). We thus studied the expression of RUNX3, a 5-Aza-CdR inducible, methylation-regulated target gene. RUNX3 promoter is highly methylated

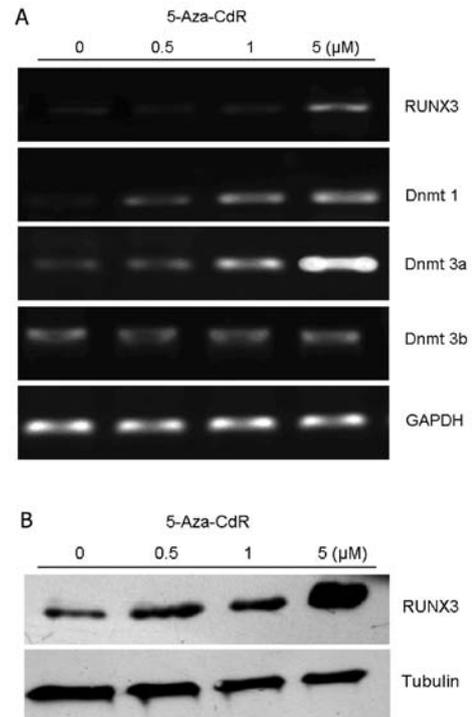


Figure 4. Change of RUNX3 and DNA methyltransferases induced by 5-Aza-CdR. After BGC-823 cells were exposed to 5-Aza-CdR for 72 h, the reactivation of RUNX3 was determined by RT-PCR (A) and western blotting (B). GAPDH and β -tubulin were used as an internal control.

in gastric cancer cells (13). The RUNX3 protein and mRNA level were determined in BGC-823 cells treated with 0.5, 1 and 5 μ M 5-Aza-CdR for 72 h. As shown in Fig. 4A, it was indicated that RUNX3 mRNA levels were increased in the presence of 5-Aza-CdR at 5 μ M compared to untreated cells. However, 0.5 and 1 μ M of 5-Aza-CdR exposure did not affect the levels of RUNX3, consistent with the finding from western blot analysis (Fig. 4B).

Since 5-Aza-CdR is an inhibitor of DNMTs, we analyzed the association between 5-Aza-CdR and DNMT1, DNMT3a, and DNMT3b in BGC-823 cells with RT-PCR analysis. Upon exposure of BGC-823 cells to 0.5 μ M of 5-Aza-CdR for 72 h, the mRNA levels of DNMT1 and DNMT3a were upregulated in a dose-dependent fashion. The mRNA levels of DNMT3b, however, remained unchanged between drug-treated cells and untreated cells (Fig. 4A).

Discussion

A number of studies have reported that 5-Aza-CdR treatment decreases cell proliferation and induces apoptosis in tumors, but the mechanisms remain unclear (14,15). In our investigation, we observed that 5-Aza-CdR increased DNA methyltransferase expression and demethylation of RUNX3, independent of the activation of the caspase pathway and the p53/p21 pathway.

From our results, we found that 5-Aza-CdR significantly inhibited BGC-823 cell growth in a dose- and duration-dependent manner. Regarding the mechanism underlying the 5-Aza-CdR-induced inhibition of cell growth, we demonstrated that induction of apoptosis resulted from DNA damage, while

cell cycle arrest and activation of the caspase pathway were not apparent. This finding is not in line with a previous report that 5-Aza-CdR led to a caspase-dependent induction of apoptosis (16), suggesting different apoptotic pathways rather than caspase may be activated by 5-Aza-CdR (17,18,25).

p53, a tumor suppressor, stands at the crossroads of cellular responses to various stresses. Both the quantity and activity of p53 are greatly increased in response to DNA damage. Data from PCR and western blotting in BGC-823 cells support the model that 5-Aza-CdR-induced cytotoxicity is part of a classical response to DNA damage through induction of the expression of p53 post-translationally rather than hypomethylation at the p53 promoter (19). However, we did not detect any expression changes of p53 mRNA levels in 5-Aza-CdR-treated BGC-823 cells. This may be due to gene mutation (20), as p53 protein levels were undetectable in untreated and treated BGC-823 cells.

Actually, some investigators have indicated that the cell lines with wild-type p53 are more sensitive to 5-Aza-CdR than that of mutant p53 (11,21). However, Nieto *et al* (22) have shown that p53 null mouse embryonic fibroblast (MEF) cells are more sensitive to 5-Aza-CdR than p53-positive cells. In line with this report, when the null expression of p53 was examined in BGC-823 cells, the 5-Aza-CdR-induced inhibition of cell survival was as sensitive as that in A549 cells and triggered apoptosis in a dose-dependent manner. Our data further showed that the cytotoxicity of 5-Aza-CdR was independent of the p53 status in BGC-823 cells. In addition, we observed upregulation of p21^{Waf1/Cip1} with increased p53 expression in A549 cells (12), in keeping with a previous observation that p21^{Waf1/Cip1} functions as a downstream target of p53. Our data suggest that the absence of cell cycle arrest and p21^{Waf1/Cip1} gene expression differences between 5-Aza-CdR-treated and untreated cells is due to the p53 mutation in BGC-823 cells.

RUNX3 is part of the runt-domain family of transcription factors that act as master regulators of gene expression in major developmental pathways. A more recent study shows that RUNX3 functions as a candidate gastric cancer suppressor gene with hypermethylation at the promoter region (13). Enhancing RUNX3 expression by transfection or other strategies completely abrogated growth advantages and activated apoptosis in gastric cancer (23). In line with previous reports, we observed that 5 μ M of 5-Aza-CdR increased RUNX3 expression both at the mRNA and protein levels (24). It is also noteworthy that the expression of RUNX3 was only enhanced at 5 μ M, even though 0.5 μ M of 5-Aza-CdR treatment had triggered cell apoptosis. Therefore, 5-Aza-CdR induced apoptosis is, at least in part, due to demethylation of silenced RUNX3. In mammals, global DNA methylation is catalyzed mainly by three DNMTs: DNMT1, DNMT3a and DNMT3b. Recently, increased expression of DNA methyltransferases were shown in various cancer cells (26-28). *In vitro* studies indicated that 5-Aza-CdR-treated cells were depleted of active DNA methyltransferases through sequestration of the enzyme to azacytosine residues in DNA, resulting in genome-wide demethylation (8). According to our data, although 5-Aza-CdR treatment induced demethylation and expression of RUNX3 the demethylation activity alone could not explain the observed cytotoxicity against BGC-823 cells. We assumed that the cellular effects of 5-Aza-CdR were a

direct result of the formation of stable adducts between DNA methyltransferases and 5-Aza-CdR-substituted genomic DNA rather than the DNA hypomethylation. A previous study demonstrated that DNMT1 knockout cells were more resistant to 5-Aza-CdR treatment than that of wild-type cells (29). Also, Oka *et al* (30) reported that 5-Aza-CdR may mediate DNMT3a and DNMT3b *de novo* DNA methyltransferases in that DNMT3a and DNMT3b null embryonic stem cells were highly resistant to 5-Aza-CdR when compared to that of wild-type cells. However, our data demonstrate that the levels of DNMT1 and DNMT3a were substantially increased rather than decreased after 5-Aza-CdR treatment (31,32). Our data support the hypothesis, that, cytotoxicity of 5-Aza-CdR results from higher DNA methyltransferase levels. Higher DNA methyltransferase levels were expected to produce more DNA methyltransferase DNA adducts, resulting in an increased response of BGC-823 cells to 5-Aza-CdR. Alternatively, the elevation of DNMT1 and DNMT3a may be not a causative effect but just a side effect of the treatment of the cells with 5-Aza-CdR. We are planning to address this issue in a future study.

In conclusion, our results show that 5-Aza-CdR, a cytosine analogue designed to inhibit DNMTs, inhibited gastric cancer BGC-823 cell growth via cell apoptosis induced by DNA damage. The upregulation of DNMT1, DNMT3a, and RUNX3 contributed to the cytotoxicity. To our knowledge, this is the first demonstration of p53-independent 5-Aza-CdR action on DNA methyltransferases and demethylation.

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