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Possible involvement of activation of P53/P21 and demethylation of RUNX 3 in the cytotoxicity against Lovo cells induced by 5-Aza-2′-deoxycytidine

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ABSTRACT

Aims: In our model, we aimed to explore the cytotoxicity of 5-Aza-2′-deoxycytidine (5-Aza-CdR) against the colorectal cell line, Lovo, and further characterize the possible mechanisms.
Main methods: After Lovo cells were treated with 5-Aza-CdR at different concentrations for different periods of time, the cell viability was examined using an MTT assay and apoptosis was examined using both flow cytometry and DNA laddering. To examine the mechanisms by which Lovo cells respond to 5-Aza-CdR, we measured both caspase 3 activity as well as DNA damage. Western blotting and RT-PCR assays were used to assess the changes in the expression levels of P53, $P21^{Waf1/Cip1}$, runt-related transcription factor 3 (RUNX 3), DNA methyltransferases (DNMTs) and matrix metalloproteinases (MMPs). Additionally, we performed gelatin zymography to examine the effects of 5-Aza-CdR on metastasis.
Key findings: We observed that the growth and survival advantages of Lovo cells were overcome with 5-Aza-CdR treatment at limited concentrations. Mechanistic exploration demonstrated that 5-Aza-CdR was incorporated into the DNA to induce DNA damage in Lovo cells, which was evidenced by activation of P53, $P21^{Waf1/Cip1}$ and a caspase-independent cell apoptosis pathway. Also, further experiments preliminarily suggested that 5-Aza-CdR results in the deletion of DNMT 3a and DNMT 3b, but not DNMT 1, which reactivates the expression of RUNX 3. Finally, our data revealed that 5-Aza-CdR potentially reduces the activity and expression of MMP 2.
Significance: These data greatly enhance our understanding of how human cancer cells respond to 5-Aza-CdR and also reveal a new role for 5-Aza-CdR in improving patient outcome in human colorectal cancer.

Introduction

The abnormal methylation of the promoters of tumor-associated genes is increasingly being recognized as a mechanism by which their transcription is silenced during human cancer development (Laird, 2005; Robertson, 2005). In fact, a significant proportion of tumor suppressor genes have been demonstrated to be silenced by methylation in cancers, which has led to the realization that abnormal methylation could act as a useful therapeutic target in cancers (Esteller et al., 2001). 5-Aza-cytidine and 5-Aza-2′-deoxycytidine (5-Aza-CdR) both belong to a class of cytosine analogues that have been developed as inhibitors of DNA methylation and have been shown to have significant cytotoxic and anti-neoplastic activities in many experimental tumor models (Natsume et al., 2008; Zorn et al., 2007). However, 5-Aza-CdR has been reported to be non-carcinogenic and it has been found to incorporate into DNA, but not RNA or protein. Additionally, 5-Aza-CdR has empirically been found to have more potent therapeutic effects than 5-aza-cytidine in cell lines and animal models of human cancers (Kuendgen and Lübbert, 2008).

Although there is considerable literature about the possible anti-tumor action of 5-Aza-CdR, its exact in vivo mechanisms remain unclear. One suggested model for its effects is associated with its incorporation into DNA where it binds to DNA methyltransferases (DNMTs) in an irreversible, covalent manner, thus sequestering the enzyme and preventing the maintenance of the methylation state. As a consequence, silenced genes that are induced by hypermethylation are re-expressed by depleting the cells of DNMTs activity. The second model involving its anti-tumor activity is related to the formation of covalent DNMT–DNA adducts in Aza-containing DNA, which lead to DNA damage and cytotoxicity (Kiziltepe et al., 2007). However, the relative contribution of gene reactivation and enzyme–DNA adduct formation to the efficacy as well as the toxicity of 5-Aza-CdR in vivo is an important unresolved question.

Clinical trials evaluating 5-Aza-CdR as a cancer chemotherapeutic candidate have shown promise in the treatment of hematopoietic malignancies, such as acute myelogenous leukemia, chronic myelogenous leukemia, and myelodysplasia. Additionally, growing evidence suggests that 5-Aza-CdR shows potential in treating solid tumors, including renal and lung carcinoma (Momparler et al., 1997; Gollob et al., 2006), but the efficiency is unsatisfactory. Therefore, investigations of the underlying mechanisms regarding the effects of 5-Aza-CdR on solid tumors are needed.

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Colorectal cancer, one of the most commonly occurring tumors, is a leading cause of cancer death worldwide. Here, we delineated that 5-Aza-CdR acts as a promising anti-cancer agent because it overcomes the growth and survival advantages that are found in the human colorectal cancer cell line, Lovo. Mechanistic exploration demonstrated that 5-Aza-CdR incorporates into DNA to induce DNA damage and caspase-independent cell apoptosis. These findings provide the preclinical rationale for the clinical evaluation of 5-Aza-CdR to improve patient outcome in colorectal cancer.

Materials and methods

Cells and treatments

The human colorectal cancer cell line, Lovo, was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 μmol/L (μM) glutamine and 100 units/mL penicillin/streptomycin in a humidified 37 °C incubator with a 5% CO_2 atmosphere. For treatment with 5-Aza-CdR (Sigma), the cells were exposed to a single pulse of 0.01–100 μmol/L 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 1×10^3 cells per well in a 96 well plate, cultured as described above and treated in the presence of increasing concentrations of 5-Aza-CdR (or vehicle control) for 72 h for different lengths of time (24, 48, 72 and 96 h). Twenty microliters of 5 mg/mL of 3-(4,5-Dimethy-lthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Amresco) was then added into each well and the cells were cultured at 37 °C for an additional 4 to 6 h. After culture, the supernatant was discarded, and the resulting formazan crystals were solubilized by adding 150 μL of DMSO to each well. The optical density level under 570 nm was measured and the percentage of cell viability was calculated using the following formula: percentage of cell viability = (absorbance of experimental well − absorbance of blank)/(absorbance of untreated control well − absorbance of blank) × 100%.

Flow cytometric analysis of DNA content

Cells were seeded into a 6-well plate at a density of $4–5\times10^5$ cells per well. They were incubated and allowed to grow to 70 to 80% confluence and then the cells were treated with 0.5, 1 or 5 μmol/L 5-Aza-CdR and incubated for a further 72 h. After being harvested by trypsin release, the cells were washed twice with PBS, and permeabilized with 70% ethanol overnight. On the next day, the ethanol was discarded and the cells were incubated for 15–20 min at 37 °C with 1 mL of a PI solution (0.1% trixton-100, 50 μg PI and 200 μg RNase A). The distribution of the cell cycle phases was determined with a flow cytometer (Beckman, USA).

Annexin V staining

The cells ($5–7\times10^5$) were seeded into 6-well plates and treated with 0.5, 1, and 5 μmol/L 5-Aza-CdR and/or z-VAD-fmk (Beyotime Institute of Biotechnology). After 48 h, they were immediately trypsinized, washed in PBS, and then early apoptosis was detected with and Annexin V Staining Kit (MultiSciences Biotech Co. Ltd) used according to the manufacturer's instructions.

Measurement of caspase 3 activity

Caspase 3 activity was measured using a colorimetric kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology). This kit detected the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of p-nitroanilide (pNA). The absorbance was determined at 405 nm and the activity of caspase 3 was assessed by calculating the ratio of the OD 405 nm of the drug treated cells to the untreated cells.

DNA ladder assay

Briefly, for the DNA ladder assay, cells (5×10^6) were collected by centrifugation and washed twice with PBS. Then the cell pellets were resuspended in lysis buffer (100 mmol/L NaCl, 10 mmol/L Tris–HCl, 25 mmol/L ethylene diamine tetraacetate [EDTA], and 0.5% SDS) containing 0.1 mg/mL proteinase K and then incubated overnight at 55 °C. DNA was cleared from the lysates by centrifugation at 12,000 and then extracted with an equal volume of phenol/chloroform/isoamylol. Following that, the DNA pellet was washed with 75% ethanol and resuspended in TE buffer containing 1 μg/mL RNase A at 37 °C for 1 h. DNA fragments were separated by 2.0% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light.

Comet assay for detecting DNA strand breaks

The comet assay, also called the single-cell gel electrophoresis, was performed as described previously (Wang et al., 2006). In brief, slides were scraped with 40 μL 0.6% agarose and then allowed to stand for about 5 min at room temperature. Twenty microliters of the cell suspension and 80 μL of a 1.1% low-melting agarose were mixed together and added to the first gel layer. Immediately after, a coverslip was overlaid and then the slides were kept at 4 °C for 15 min to allow for solidification. After the coverslips were gently removed, the slides were immersed in freshly prepared cold lysis solution (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA and 10 mmol/L Tris at pH 10.0) with 1% Triton X-100 and 10% DMSO for at least 1 h at 4 °C. After the samples were electrophoresed in fresh solution (1 mmol/L Na_2EDTA, 300 mmol/L NaOH at pH 13.0) for 30 min, the slides were then placed in Tris buffer (0.4 mol/L Tris at pH 7.5) for 15 min twice. The slides were then stained with 40 μL of 0.1 mg/mL propidium iodide (PI) and 100 randomly selected cells were counted per slide. The images were captured and scored for each sample using an image analysis software system (IMI ver. 1.0). The standard for assessing DNA damage was based on the percentage of cells with a tail as well as the tail length (a distance from the DNA head to the end of DNA tail) by visual estimation.

Gelatin zymography

After the Lovo cells were incubated with 0.5, 1 or 5 μmol/L 5-Aza-CdR for 72 h, the supernatant samples were collected. Then each sample was subjected to SDS-PAGE and electrophoresed on a 10% polyacrylamide gel containing 1 mg/mL gelatin. Following electrophoresis, the gels were washed for 30 min in a buffer containing 50 mmol/L Tris–HCl (pH 7.5), 2.5% (w/v) Triton X-100, and 5 mmol/L $CaCl_2$ and incubated overnight (16 h) in the incubation buffer at 37 °C [1% (w/v) Triton X-100, 50 mmol/L Tris- HCl, 5 mmol/L $CaCl_2$, 0.02% (w/v) NaN_3, 1 μmol/L $ZnCl_2$ at pH 7.6]. The gels were stained with 0.1% Coomassie blue R 250 and destained with acetic acid in methanol and H_2O (1:3:6), both for 60 min, to visualize the bands with gelatinolytic activity.

RNA preparation and RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) and 1 μg of RNA was used as a template for the synthesis of cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instruction. Polymerase chain reaction (PCR) analysis was performed at a final volume of 25 μL using a PCR Master Mix (Fermentas). Primer sequences and annealing temperatures are shown in Table 1. The amplification was comprised of an initial denaturation step at 94 °C for 5 min, followed by 35 amplification cycles (94 °C for 30 s, annealing temperature for 30 s, elongation at 72 °C

Table 1
Primers and conditions used for RT-PCR

Primers	Sequences	Temperatures	Product length
GAPDH	F: 5′-ACGGATTTGGTCGTATTGGG-3′ R: 5′-TCCTGGAAGATGGTGATGGG-3′	56	211 bp
RUNX 3	F: 5′-GAAAAGGCGTAAGGGAACTC-3′ R: ACCTGGGACCAGCTATAACC-3′	56	395 bp
$P21^{Waf1/Cip1}$	F: 5′-GTGAGC GAT GGA ACT TCGACT-3′ R: 5′-CGA GGC ACA AGG GTA CAA GAC-3′	56	229 bp
P53	F: 5′-GTCTACCTCCCGCCATAA-3′ R: 5′-CATCTCCCAAACATCCCT-3′	55	316 bp
DNMT 1	F: 5′-CGCTGTATCTAGCAAGGGTCA-3′ R: 5′-TCGAATCTCGCGTAGTCTTG-3′	56	313 bp
DNMT 3a	F: 5′-ACCACAGAGGCGGAAATACC-3′ R: 5′-GTCTCCCTGCTGCTAACTGG-3′	56	543 bp
DNMT 3b	F: 5′-CAGGAGACCTACCCTCCACA-3′ R: 5′-TTACGTCGTGGCTCCAGTTA-3′	56	436 bp

for 45 s) and a final incubation at 72 °C for 5 min. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and photographed.

Western blotting

Cells were cultured with 5-Aza-CdR at the indicated concentrations for the specified time periods, harvested, washed, and lysed using RIPA buffer containing protease inhibitors. After normalization for total protein content (50 μg/lane), the samples were subjected to 15% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5% nonfat dry milk and 0.1% Tween-20 in Tris buffered saline, the membranes were incubated with mouse anti-$P21^{Waf1/Cip1}$, -P53 and -MMP 2 (Santa Cruz), goat anti-RUNX 3 (R&D) and -MMP 9, rabbit anti-caspase 3 and -β-Tubulin (Santa Cruz). After extensive rinsing with TBST buffer, the blots were incubated with HRP-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibodies (Pierce) and developed with the enhanced chemiluminescence system (Millipore) and captured on light-sensitive imaging film (Kodak, Tokyo, Japan).

Statistical analysis

All experiments were repeated in triplicate. Data are reported as the mean ± SEM. Statistical analyses were performed using Student's *t*-test or one-way ANOVA and a post hoc Tukey's test. Probability (*P*) values of 0.01 were considered to be significant (SPSS 11.5 for Windows).

Results

Dose and duration dependent inhibition of cell proliferation by 5-Aza-CdR

In this study, human colorectal cancer Lovo cells were treated with 5-Aza-CdR at different concentrations for 72 h and the cell viability was determined by an MTT assay. As shown in Fig. 1A, a dose dependent inhibition of cell proliferation was observed in Lovo cells. For example, cell viability was decreased by 85% when Lovo cells were treated with 0.2 μmol/L 5-Aza-CdR and 53% or 36% when cells were treated with 5-Aza-CdR at 1 μmol/L or 50 μmol/L, respectively.

Subsequently, the Lovo cells were treated with 1 μmol/L 5-Aza-CdR for the indicated times (24, 48, 72 and 96 h) to evaluate the duration effects of 5-Aza-CdR on cell viability. This dose was selected because it induces growth inhibition in Lovo cells and is frequently used in other published reports. In this model, we observed that 5-Aza-CdR not only has a dose dependent inhibitory effect on the growth of Lovo cells, but also inhibits the growth of Lovo cells in a time dependent manner (Fig. 1B).

Effects of 5-Aza-CdR on cell cycle status

To determine the mechanism of the anti-proliferative effects of 5-Aza-CdR on Lovo cells, we further clarified whether growth inhibition is closely associated with specific cell cycle arrest. Exponentially growing Lovo cells were treated with 5-Aza-CdR at the indicated concentrations for 72 h and harvested for flow cytometric analysis of the DNA content by PI staining. A cell cycle distribution analysis displayed an increase in the number of cells in the G_1 phase of the cell cycle following treatment with 5-Aza-CdR as compared with the untreated control, which provides evidence that 5-Aza-CdR treatment induces G_1 arrest in Lovo cells. Furthermore, the addition of 5-Aza-CdR for 72 h induced an accumulation of sub-G_0–G_1 (apoptotic) DNA in a dose-dependent manner (Fig. 2A).

Effects of 5-Aza-CdR on the induction of apoptosis

To further confirm the nature of the cell death, we used the Annexin V flow cytometric assay and DNA laddering methods. First, Lovo cells were treated with 0.5, 1, or 5 μmol/L 5-Aza-CdR for 48 h and

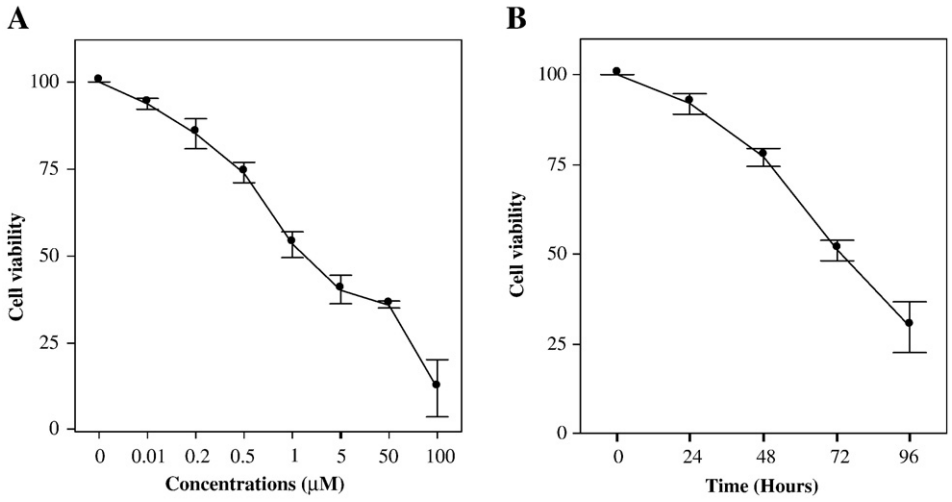

Fig. 1. 5-Aza-CdR-induced cell proliferation inhibition in colorectal cancer Lovo cells. Cell viability was measured with an MTT after Lovo cells were treated with 5-Aza-CdR at 0–100 μmol/L (μM) for 72 h (A) or at 1 μmol/L (μM) for different intervals (B). Results are presented as the average of triplicate measurements and the bar indicates the standard deviation.

detected for early apoptosis using the Annexin V flow cytometry assay. Our data revealed that 5-Aza-CdR treatment increased the proportion of positive cells with Annexin V staining from 4.6% in untreated cells to 16.6% in cells treated with 0.5 μmol/L 5-Aza-CdR, which peaked at 5 μmol/L (52.4%), presenting a dose dependent trend. The results paralleled the data from the cell cycle analysis and strongly suggest that apoptosis rather than necrosis is the mechanism by which cytotoxicity in Lovo cells is induced by 5-Aza-CdR (Fig. 2B).

In the DNA fragmentation assays, Lovo cells also displayed a characteristic "DNA laddering" pattern of apoptosis in the course of treatment with 1 and 5 μmol/L 5-Aza-CdR as compared with the untreated cells, which provides further evidence that suggests that 5-Aza-CdR triggers apoptosis in a dose dependent manner (Fig. 2C).

Treatment with 5-Aza-CdR causes DNA damage

Since 5-Aza-CdR has been reported to incorporate into DNA, but not RNA (Kuendgen and Lübbert, 2008), we explored the effects of 5-Aza-CdR on DNA damage using a comet assay. Lovo cells were treated with 5-Aza-CdR at 0.5, 1, or 5 μmol/L for 72 h and then submitted to

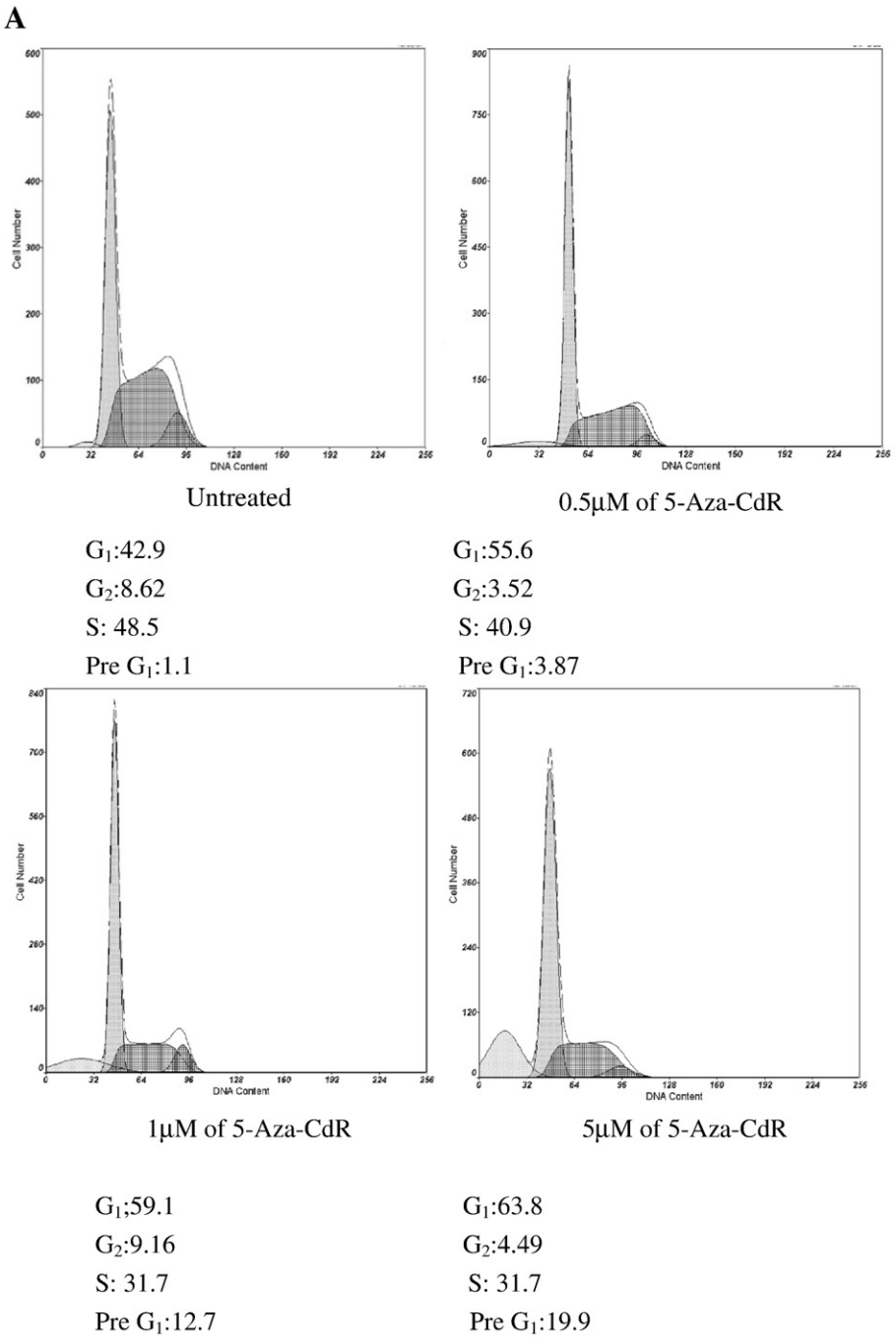

Fig. 2. The apoptotic effects of 5-Aza-CdR on Lovo cells. (A) Effects on cell cycle profiles of Lovo cells after treatment with 5-Aza-CdR for 72 h at the indicated concentrations. DNA content was determined by flow cytometric analysis using PI staining. (B) Percentage of apoptotic cells were scored after the cells were exposed to 5-Aza-CdR and/or z-VAD-fmk at the indicated concentration for 48 h by Annexin V flow cytometric assay, which can detect cells at an earlier stage of apoptosis and distinguish between apoptotic and necrotic cells. (C) Lovo cells treated with 5-Aza-CdR at the higher concentrations (1 and 5 μmol/L) were observed to have marked nucleosomal DNA fragmentation. Lane 1: marker; Lane 2: untreated; Lane 3: 0.2 μmol/L; Lane 4: 0.5 μmol/L; Lane 5: 1 μmol/L; Lane 6: 5 μmol/L (5-Aza-CdR).

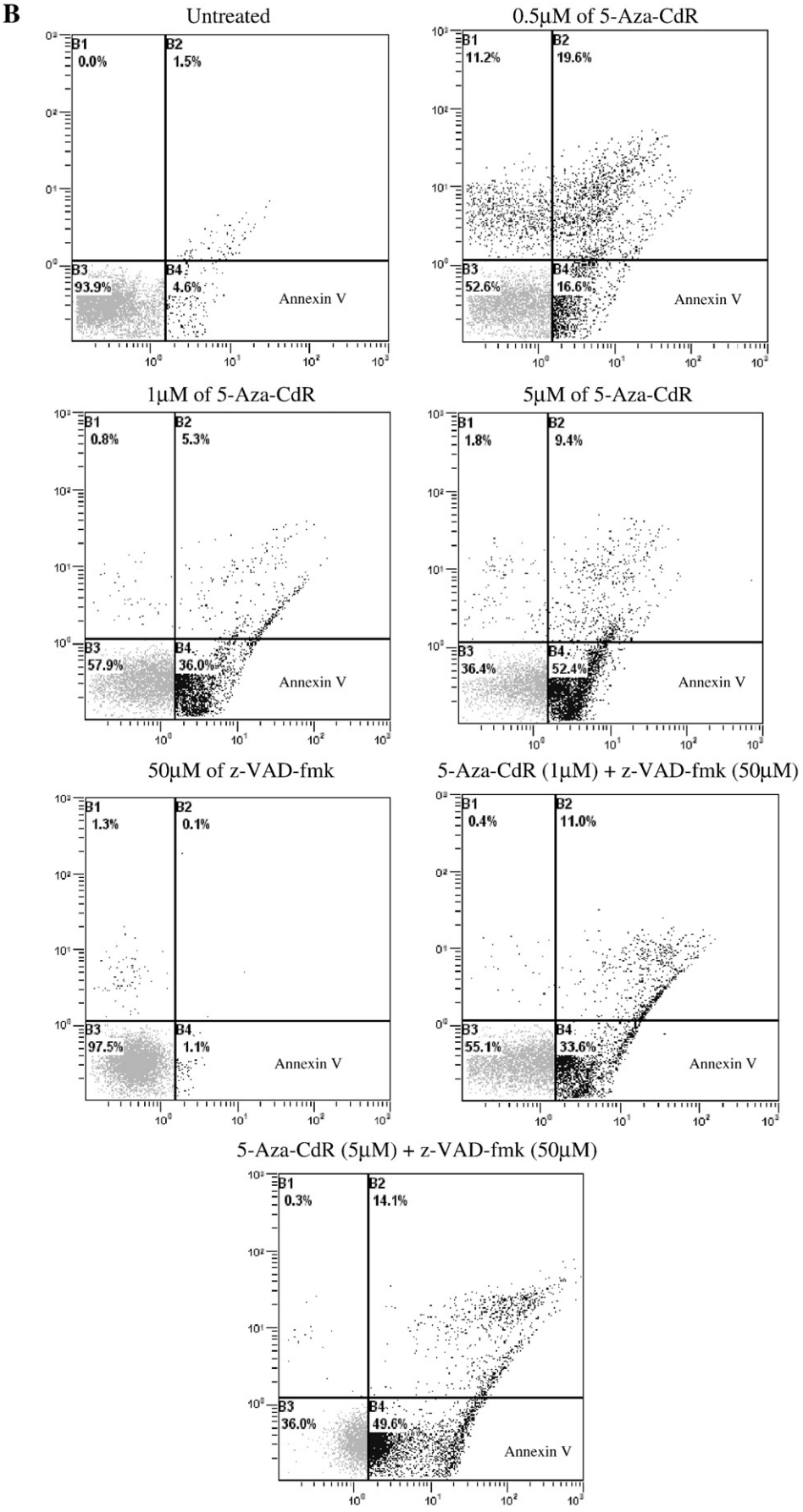

Fig. 2 (*continued*).

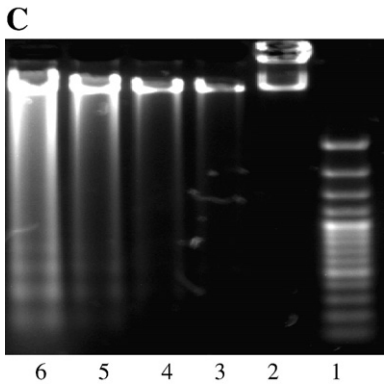

Fig. 2 (*continued*).

this assay. As shown in Fig. 3A, dose dependent DNA damage was observed following 72 h of treatment in Lovo cells. Compared with the untreated cells, 5-Aza-CdR treatment, even at a low concentration (0.5 μmol/L), induces DNA damage, as indicated by the presence of cells with a comet tail (14.7%). More comet cells and longer DNA tail length indicate that there is more extensive DNA damage. These features of DNA damage that are induced by 5-Aza-CdR were much more obvious in the Lovo cells treated with a higher concentration of 5-Aza-CdR than in those treated with a lower concentration (Fig. 3A).

Activation of caspase 3 in Lovo cells exposed to 5-Aza-CdR

To analyze whether caspase is responsible for the recruitment of the apoptotic pathways that are induced by 5-Aza-CdR, we performed a colorimetric enzyme assay to examine the caspase 3 activity and Western blot to detect procaspase 3 expression. Here, caspase 3 enzymatic activity was detectable in Lovo cells after exposure to 5 μmol/L 5-Aza-CdR. However, no obvious alteration was observed among the cells treated with 0, 0.5 or 1 μmol/L 5-Aza-CdR (Fig. 3B). Similar results also were found using Western blot analysis in which 5 μmol/L 5-Aza-CdR presumably decreases the expression level of caspase 3, since we examined the level of procaspase 3 (Fig. 3C). Next, we further demonstrated the role played by caspases in 5-Aza-CdR induced apoptosis. Lovo cells were pretreated with the pan-caspase inhibitor, z-VAD-fmk (50 μmol/L), and then treated with 5-Aza-CdR for 48 h. z-VAD-fmk slightly inhibited cell apoptosis triggered by 5-Aza-CdR (Fig. 2B), suggesting that the apoptotic effect is independent of the caspase pathway.

Dose response of 5-Aza-CdR on P53, P21$^{Waf1/Cip1}$

Because it has been proposed that P53 status stands as a vital point in growth arrest and apoptosis in response to DNA damage, we first analyzed the induction of P53 after 5-Aza-CdR treatment for 72 h in Lovo cells. PCR analysis showed no increase or decrease in the P53 mRNA level in the presence of 5-Aza-CdR (Fig. 4A). To identify whether P53 accumulation after DNA damage is a result of posttranslational modifications rather than transcriptional activation, we detected the protein expression of P53 in Lovo cells using Western blot analysis. As expected, compared to untreated Lovo cells, 5-Aza-CdR treatment gave rise to a dose-dependent upregulation in the expression of the P53 protein (Fig. 4B).

As P21$^{Waf1/Cip1}$ is a target regulator of the P53 transcription factor and it is attributed to G_1 arrest (Jiemjit et al., 2008), we assessed the expression of P21$^{Waf1/Cip1}$ in Lovo cells using Western blot analysis and RT-PCR assays. As presented in Fig. 4B, 5-Aza-CdR increased the protein expression of P21$^{Waf1/Cip1}$ along with the elevation of P53 expression and G_1 cell cycle arrest in Lovo cells, but the mRNA level of P21$^{Waf1/Cip1}$ was unaffected even though the Lovo cells were exposed to 5-Aza-CdR at different concentrations (Fig. 4A).

Effects of 5-Aza-CdR on the expression of DNA methyltransferases and RUNX 3

Since 5-Aza-CdR has been shown to act as an inhibitor of DNA methyltransferases (DNMTs), it is essential to evaluate the association between levels of human DNMTs, including DNMT 1, DNMT 3a, and DNMT 3b, and the cytotoxicity of 5-Aza-CdR against Lovo cells. As shown in Fig. 4C, treatment of cells with an increasing concentration of 5-Aza-CdR (0.5 to 5 μmol/L) caused dose-dependent depletion of DNMT 3a and DNMT 3b. DNMT 3b was affected to a greater extent than DNMT 3a, which showed a decrease of mRNA only at the higher dose of 5-Aza-CdR (1 μmol/L). However, the level of DNMT 1 remained unchanged regardless of whether the cells received 5-Aza-CdR treatment or not (Fig. 4C).

It has been well established that 5-Aza-CdR exerts its cytotoxicity through the depletion of DNA methyltransferase, by which DNA hypomethylation occurs. To address this issue, we selected the 5-Aza-CdR inducible, methylation-regulated target gene (RUNX 3), which is highly methylated in colorectal cancer cells and may explain the cytotoxicity of 5-Aza-CdR against colorectal cancer. As indicated in Fig. 4D, we observed that the RUNX 3 mRNA level was higher following the addition of 5-Aza-CdR at 0.5 μmol/L than in untreated cells. Treatment of the cells with 1 μmol/L 5-Aza-CdR caused further increase in RUNX 3 expression and peaked at 5 μmol/L, suggesting a dose dependent trend. Similarly, Western blot analysis demonstrated that 5-Aza-CdR treatment induced a dose dependent increase in the protein expression of RUNX 3.

5-Aza-CdR inhibited the activity and expression of MMP 2

To evaluate the inhibitory effect of 5-Aza-CdR on matrix metalloproteinase (MMP) production in Lovo cells, we performed an assay involving gelatin zymography. Following gelatin zymography, four main lysis bands corresponding to the gelatinases were observed in untreated Lovo cells: 92 kDa latent MMP 9, 84 kDa active MMP 9, 72 kDa latent MMP 2 and 66 kDa active MMP 2. When the cells were incubated with 5-Aza-CdR for 72 h, the gelatinolytic activity of MMP 2 was observed to be reduced in a dose dependent manner, but the activity of MMP 9 was not altered significantly even with higher doses (Fig. 5A). At the same time, after addition of 5-Aza-CdR for 72 h, Western blot analysis also displayed a dose dependent decrease in the expression of MMP 2, but not in MMP 9 (Fig. 5B).

Discussion

The field of epigenetic therapy for cancer has raised considerable interest over the last few years. A number of studies have reported that 5-Aza-CdR treatment inhibits cell proliferation and induces apoptosis in various cancers, but the mechanisms remain unclear. In our investigation, we found that 5-Aza-CdR significantly abrogates Lovo colorectal cancer cell growth advantages in a dose- and duration-dependent manner, which is in agreement with previous studies that reported that 5-Aza-CdR acts as a chemotherapeutic agent against tumor cells (Fan et al., 2007; Tang et al., 2004). Regarding the mechanism underlying the anti-cancer actions of 5-Aza-CdR, we detected that apoptosis is induced as a result of DNA damage, while no activation of caspase 3 is apparent. This result is inconsistent with Gomyo et al.'s report that 5-Aza-CdR triggers a caspase dependent apoptosis (Gomyo et al., 2004), implying that a different caspase-independent apoptotic pathway may also be recruited by 5-Aza-CdR (Carter et al., 2003; Shang et al., 2008). However, the mechanism by which apoptosis is induced warrants further exploration.

P53 is a tumor suppressor that stands at the crossroads of cellular responses to various stresses. In response to DNA damage, both the quantity and activity of P53 are greatly augmented (Römer et al., 2006). In this system, we observed that the DNA damage induced by

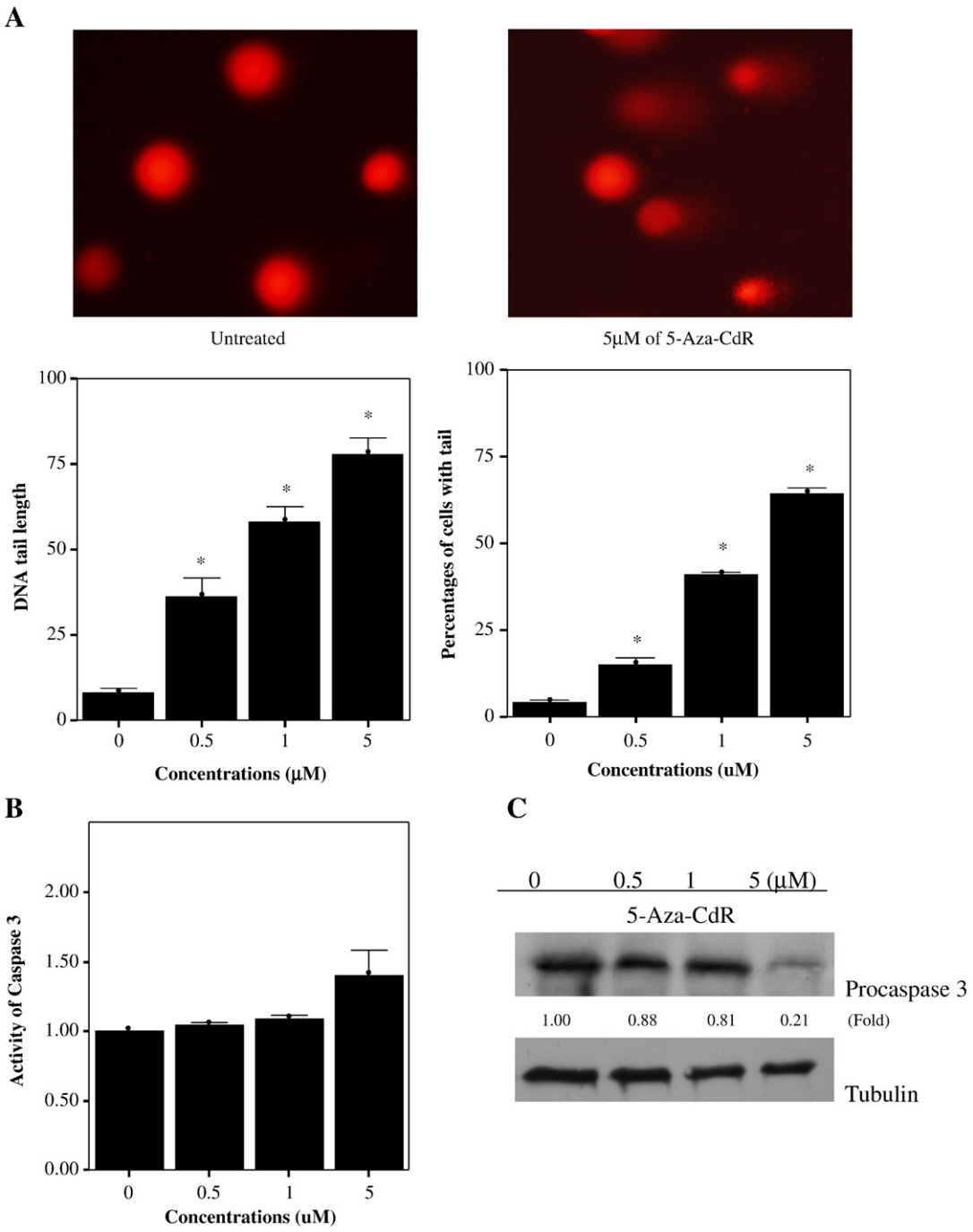

Fig. 3. Change in caspase 3 and DNA damage caused by 5-Aza-CdR in Lovo cells. (A) To detect DNA damage the comet assay was used. DNA damage was characterized by the percentage of cells with a comet tail/100 cells (%) and the comet tail length (from the center of the DNA head to the end of the DNA tail). Lovo cells with more significant damage show larger percentages of comet tails (%) or longer the DNA tail length. Representative pictures are shown. (B) Activation of caspase 3 activity in Lovo cells was determined using a colorimetric method after treatment with 5-Aza-CdR for 72 h as indicated. (C) Lovo cells were exposed to 0.5, 1 or 5 μmol/L 5-Aza-CdR for 72 h. Western blot analysis was used to examine the expression of procaspase 3 between treated and untreated cells. Blots were reprobed with an anti-β-Tubulin antibody as a loading control. Results are represented as the average of three independent experiments. *$P<0.01$, as compared with the untreated cells. Columns, mean of triplicate cultures; bars, SD.

5-Aza-CdR in Lovo cells does lead to a dose dependent increase in the expression of P53. Of interest, we observed no alteration in the transcriptional level of P53 in these cells, supporting the model that the cytotoxicity that is caused by 5-Aza-CdR is part of a classical response to DNA damage via inducing the expression of P53 post-translationally rather than through the hypomethylation of the P53 promoter (Wang et al., 2008).

$P21^{Waf1/Cip1}$, a major transcriptional target of the tumor-suppressor P53, plays an important role in cell cycle control by interacting with the cyclin-dependent kinase complexes, resulting in cell cycle arrest in G_1 or G_2 after DNA damage (Jiemjit et al., 2008). While analyzing the DNA fragmentation in 5-Aza-CdR treated Lovo cells, we detected that the cell cycle arrest in G_1 phase is accompanied by an increase in the protein expression of $P21^{Waf1/Cip1}$ after DNA damage; nevertheless, the transcriptional level of $P21^{Waf1/Cip1}$ was no different. It is reasonable to assume that the $P21^{Waf1/Cip1}$ promoter is unmethylated in Lovo cells, since $P21^{Waf1/Cip1}$ was readily reactivated at the transcriptional level upon exposure of the cells to 5-Aza-CdR if the promoter of $P21^{Waf1/Cip1}$ is hypermethylated. These data parallel a previous report that showed that DNA demethylation is not required for $P21^{Waf1/Cip1}$ induction

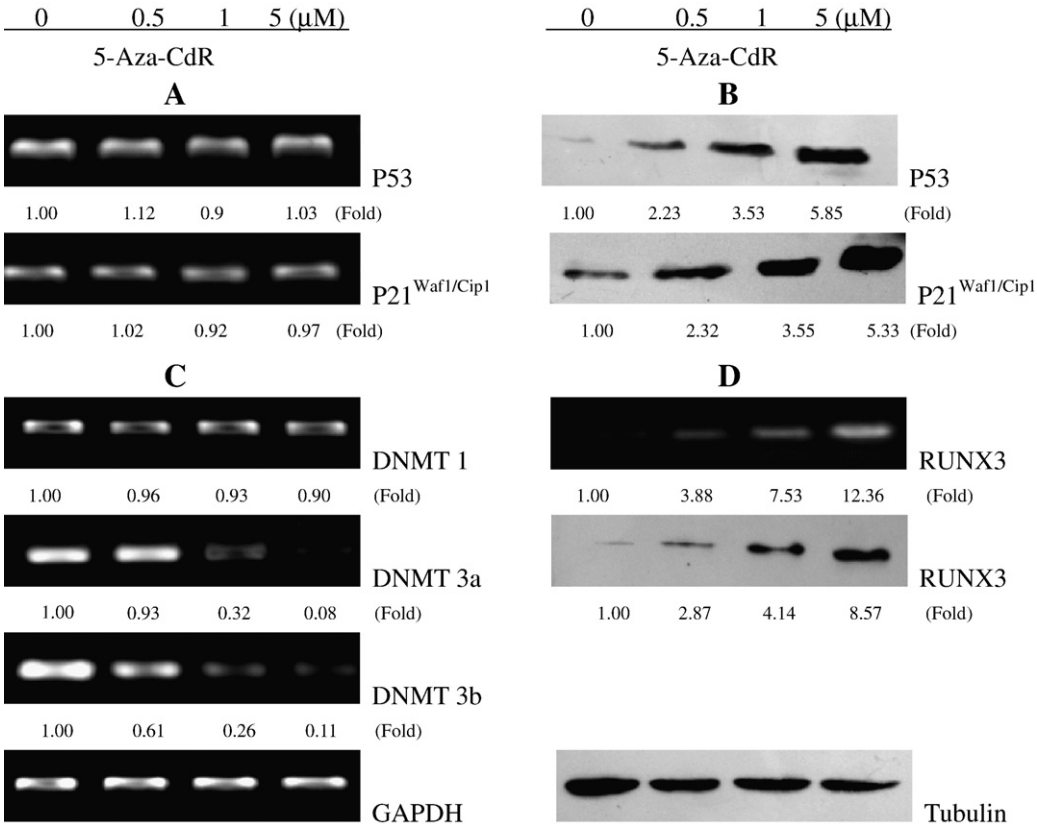

Fig. 4. Dose response of 5-Aza-CdR on the expressions of P53, $P21^{Waf1/Cip1}$, RUNX 3 and DNA methyltransferases (DNMTs). After exposure to 5-Aza-CdR at the indicated concentrations for 72 h, the transcriptional levels of P53 and $P21^{Waf1/Cip1}$ (A) and protein expression (B) in Lovo cells were detected using RT-PCR and Western blot analysis. (C) Lovo cells were observed to develop a dose dependent decrease in the levels of DNMT 3a and DNMT 3b, whereas there was no change in the level of DNMT 1. (D) The expression of RUNX 3 was reactivated following addition of 0.5 μmol/L 5-Aza-CdR, which peaked at 5 μmol/L. GAPDH and β-Tubulin were used as internal controls.

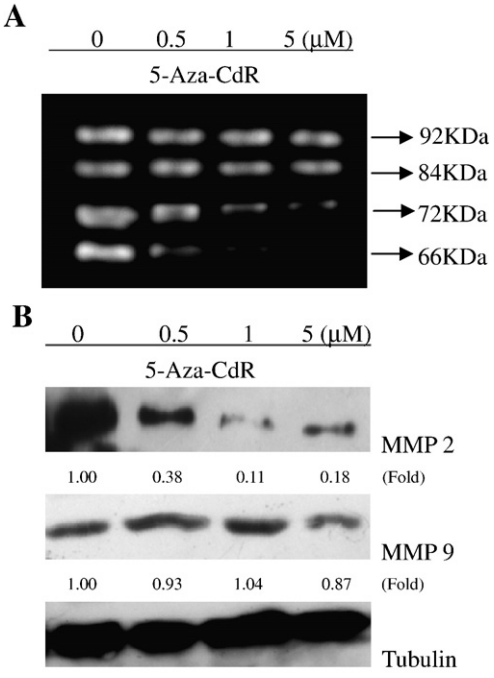

Fig. 5. Suppressive effects of 5-Aza-CdR on MMP production. 5-Aza-CdR at a concentration of 0.5, 1 or 5 μmol/L was applied to Lovo cells for 72 h. After incubation, supernatant samples were collected and gelatin zymography was performed. ProMMP 9 (92 kD), proMMP 2 (72 kD), and active MMP 2 and MMP 9 (66 kD and 84 kD) were present. There is no detectable difference in the MMP 9 level between the control and drug treated cells. However, MMP 2 secretion is reduced by 5-Aza-CdR (A). Similar results are observed using Western blot analysis (B). β-Tubulin was used as an internal control.

(Schmelz et al., 2005), implying that the anti-neoplastic activity that operates by damaging DNA is independent of the demethylation of P53 and $P21^{Waf1/Cip1}$. In this regard, many more studies related to the evaluation of the DNA methylation status, such as a methylation specific PCR assay, are needed to show whether the observed effect is mediated through this proposed mechanism in Lovo cells.

In mammals, global DNA methylation is catalyzed mainly by three DNA methyltransferases: DNMT 1, DNMT 3a, and DNMT 3b. DNMT 1 has a high preference for hemimethylated DNA and is essential for maintaining methylation patterns during DNA replication, and thus is called the maintenance DNA methyltransferase. DNMT 3a and DNMT 3b, on the other hand, are responsible for the wave of de novo methylation. It has been well known that DNMT 1 is highly expressed in various cancer cells (Melki et al., 1998; Zhu et al., 2007), whereas there is increasing evidence that de novo DNA methyltransferases are also closely related to cancer development (Park et al., 2006; Roll et al., 2008). As a result, the abnormal expression of the de novo DNA methyltransferases serves as a marker for cancers, as well as a potential target for future cancer therapies.

In vitro studies on the mechanisms of action of 5-Aza-CdR have indicated that the interaction of the cytosine methyltransferases with 5-Aza-CdR substituted DNA in the presence of S-adenosyl-methionine results in the irreversible binding of the cysteine in the catalytic center of the enzyme to the 6-position of the cytidine ring. Consequently, 5-Aza-CdR-treated cells are depleted of active DNA MTase through the sequestration of the enzyme by the azacytosine residues in DNA, resulting in genome-wide demethylation. Among the DNMTs, it is reasonable to suspect that DNMT 3a and DNMT 3b respond to a much greater extent to the inhibitory effects of 5-Aza-CdR residues on the incorporation into DNA, since they are randomly incorporated in place of cytidine, and, unlike DNMT 1, DNMT 3a and DNMT 3b are capable of methylating

cytidine residues that are not in CpG islands (Aoki et al., 2001). Our findings further support this speculation, since we observed that 5-Aza-CdR causes a marked down-regulation of DNMT 3b and DNMT 3a mRNA levels, in contrast to a null effect on DNMT 1, which was inconsistent with Palii's report that DNMT 1-deficient cells demonstrated profound defects in their response to 5-Aza-CdR induced DNA damage (Palii et al., 2008). However, this study alone is not enough to establish the above hypothesis given that the mRNA level, rather than the protein level and activity, was measured. 5-Aza-CdR functions to covalently trap the enzyme, not to degrade it. To our knowledge, the relationship between the cytotoxic effects of 5-Aza-CdR against cancer cells and DNMTs is contradictory, since Oka et al. reported that DNMT 3a and DNMT 3b null ES cells are highly resistant to 5-Aza-CdR when compared to wild type cells (Oka et al., 2005). Later, another report extended upon these findings and further set up a theory in which the deletion of DNMTs failed to abolish growth advantages in lung cancer cell lines (Chai et al., 2008). Therefore, more studies involving the correlation between DNMTs and 5-Aza-CdR are warranted.

The human RUNX 3 belongs to the runt-domain family of transcription factors, which are master regulators of gene expression in major developmental pathways. More recently, RUNX 3 was shown to function as a candidate tumor suppressor gene for cancer, because the gastric mucosa in RUNX $3^{-/-}$ mice undergoes hyperplasia due to a reduced sensitivity of the gastric epithelial cells to growth inhibitory activity transforming growth factor (TGF)-β and TGF-β-mediated apoptosis (Li et al., 2002). In colorectal cancer, Tan et al. reported that down-regulation or silencing of RUNX 3 gene expression was mainly due to promoter hypermethylation rather than gene mutation or anything else (Tan et al., 2007). From our findings, it is clear that 5-Aza-CdR is a potent demethylating agent, in that 5-Aza-CdR is readily incorporated into DNA, and it is able to confer the ability to trap DNMT 3a and 3b. In theory, inhibition of DNA methylation will increase the expression of genes if they are methylated in their promoter regions, because methyl-binding proteins specifically bind to methylated DNA. In fact, the effectiveness of 5-Aza-CdR in the treatment of cancer is the subject of a number of human clinical trials by several different groups. Also, there are reports indicating that methylation in the promoter region of caspase 8 or caspase 1 is one reason that cells develop resistance to chemotherapy (Fulda et al., 2001; Harada et al., 2002), and treatment with 5-Aza-CdR could help abolish this resistance and restore cell sensitivity to chemotherapy (Eggert et al., 2001; Fulda et al., 2001). In line with previous experiments, we observed that 5-Aza-CdR induces a dose-dependent increase in the expression of RUNX 3. Importantly, reactivation of RUNX 3 coincides with the cell growth that is overcome and the apoptosis that is induced by 5-Aza-CdR. These findings echo those reported in Nagahama's work, that enhancement of RUNX 3 expression by transfection results in growth inhibition and cell death (Nagahama et al., 2008). Re-expression of RUNX 3 occurs as a consequence of promoter demethylation, since it is clearly associated with a re-induction of RUNX 3 as measured with RT-PCR, which might be attributed to a depletion of DNMT 3b and DNMT 3a by 5-Aza-CdR treatment. This result provides us with enough evidence to support the notion that RUNX 3 is a relevant target for the methylation-dependent efficacy of 5-Aza-CdR in Lovo colorectal cancer cells.

Increasingly, the importance of epigenetic changes involving global DNA methylation to the metastatic process is being realized (Rodenhiser, 2008; Long et al., 2008). Thus, investigation of the matrix metalloproteinases activity in this study was designed mainly to assess this association between 5-Aza-CdR and cancer metastasis. It has been established that MMP 2 and 9 can degrade the extracellular matrix and seem to play multiple other roles in colorectal cancer progression. Based on our data, we suspect that 5-Aza-CdR may contribute to the inhibition of Lovo cell invasion, since it efficiently suppresses the activity of MMP 2. Conversely, a recent investigation indicates that 5-Aza-CdR promotes cancer cell progression, since the investigators found that 5-Aza-CdR not only restores the genes related to apoptosis, but also restores prometastatic genes (Ateeq et al., 2008). Therefore, it is necessary to determine the exact effects and mechanisms of 5-Aza-CdR on cancer progression as quickly as possible using many more techniques, including the wounding assay and matrigel invasion chambers, which would benefit the pharmacological evaluation of 5-Aza-CdR as it applies to the clinic.

Conclusion

Our results show that 5-Aza-CdR potently inhibits Lovo colorectal cancer cell growth and induces apoptosis with the activation of P53 and $P21^{Waf1/Cip1}$. Further studies indicate that reactivation of RUNX 3 expression, which is the result of the deletion of DNMT 3a and DNMT 3b, plays an active role in the cytotoxicity of 5-Aza-CdR against Lovo cells. To our knowledge, this is the first demonstration that 5-Aza-CdR may be involved in the suppression of invasion through the reduction of the activity of MMP 2. We believe that this novel finding will be helpful in formulating more effective strategies to clinically treat colorectal cancer.

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References

Aoki, A., Suetake, I., Miyagawa, J., Fujio, T., Chijiwa, T., Sasaki, H., Tajima, S., 2001. Enzymatic properties of de novo-type mouse DNA (cytosine-5)-methyltransferases. Nucleic Acids Research 29 (17), 3506–3512.

Ateeq, B., Unterberger, A., Szyf, M., Rabbani, S.A., 2008. Pharmacological inhibition of DNA methylation induces proinvasive and prometastatic genes in vitro and in vivo. Neoplasia 10 (3), 266–278.

Carter, B.Z., Kornblau, S.M., Tsao, T., Wang, R.Y., Schober, W.D., Milella, M., Sung, H.G., Reed, J.C., Andreeff, M., 2003. Caspase-independent cell death in AML: caspase inhibition in vitro with pan-caspase inhibitors or in vivo by XIAP or Survivin does not affect cell survival or prognosis. Blood 102 (12), 4179–4186.

Chai, G., Li, L., Zhou, W., Wu, L., Zhao, Y., Wang, D., Lu, S., Yu, Y., Wang, H., McNutt, M.A., Hu, Y.G., Chen, Y., Yang, Y., Wu, X., Otterson, G.A., Zhu, W.G., 2008. HDAC inhibitors act with 5-aza-2′-deoxycytidine to inhibit cell proliferation by suppressing removal of incorporated abases in lung cancer cells. PLoS ONE 3 (6), e2445.

Eggert, A., Grotzer, M.A., Zuzak, T.J., Wiewrodt, B.R., Ho, R., Ikegaki, N., Brodeur, G.M., 2001. Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. Cancer Research 61 (4), 1314–1319.

Esteller, M., Corn, P.G., Baylin, S.B., Herman, J.G., 2001. A gene hypermethylation profile of human cancer. Cancer Research 61 (8), 3225–3229.

Fan, H., Zhao, Z.J., Cheng, Y.C., Shan, Y.F., Lu, Z.H., Zhang, J.Q., Xie, W., 2007. Gene induction and apoptosis in human hepatocellular carci-noma cells SMMC-7721 exposed to 5-aza-2′-deoxycytidine. Chinese Medical Journal (Engl) 120 (18), 1626–1631.

Fulda, S., Küfer, M.U., Meyer, E., van Valen, F., Dockhorn-Dworniczak, B., Debatin, K.M., 2001. Sensitization for death receptor- or drug-induced apoptosis by reexpression of caspase-8 through demethylation or gene transfer. Oncogene 20 (41), 5865–5877.

Gollob, J.A., Sciambi, C.J., Peterson, B.L., Richmond, T., Thoreson, M., Moran, K., Dressman, H.K., Jelinek, J., Issa, J.P., 2006. Phase I trial of sequential low-dose 5-aza-2′-deoxycytidine plus high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. Clin Cancer Research 12 (15), 4619–4627.

Gomyo, Y., Sasaki, J., Branch, C., Roth, J.A., Mukhopadhyay, T., 2004. 5-Aza-2′-deoxycytidine upregulates caspase-9 expression cooperating with p53-induced apoptosis in human lung cancer cells. Oncogene 23 (40), 6779–6787.

Harada, K., Toyooka, S., Shivapurkar, N., Maitra, A., Reddy, J.L., Matta, H., Miyajima, K., Timmons, C.F., Tomlinson, G.E., Mastrangelo, D., Hay, R.J., Chaudhary, P.M., Gazdar, A.F., 2002. Deregulation of caspase 8 and 10 expression in pediatric tumors and cell lines. Cancer Research 62 (20), 5897–5901.

Jiemjit, A., Fandy, T.E., Carraway, H., Bailey, K.A., Baylin, S., Herman, J.G., Gore, S.D., 2008. $P21^{WAF1}$ induction by 5-azacytosine nucleosides requires DNA damage. Oncogene 27 (25), 3615–3623.

Kiziltepe, T., Hideshima, T., Catley, L., Raje, N., Yasui, H., Shiraishi, N., Okawa, Y., Ikeda, H., Vallet, S., Pozzi, S., Ishitsuka, K., Ocio, E.M., Chauhan, D., Anderson, K.C., 2007. 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. Molecular Cancer Therapeutics 6 (6), 1718–1727.

Kuendgen, A., Lübbert, M., 2008. Current status of epigenetic treatment in myelodysplastic syndromes. Annals of Hematology 87 (8), 601–611.

Laird, P.W., 2005. Cancer epigenetics. Human Molecular Genetics 14 (1), 65–76.

Li, Q.L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X.Z., Lee, K.Y., Nomura, S., Lee, C.W., Han, S.B., Kim, H.M., Kim, W.J., Yamamoto, H., Yamashita, N., Yano, T., Ikeda, T., Itohara,

S., Inazawa, J., Abe, T., Hagiwara, A., Yamagishi, H., Ooe, A., Kaneda, A., Sugimura, T., Ushijima, T., Bae, S.C., Ito, Y., 2002. Causal relationship between the loss of RUNX 3 expression and gastric cancer. Cell 109 (1), 113–124.

Long, N.K., Kato, K., Yamashita, T., Makita, H., Toida, M., Hatakeyama, D., Hara, A., Mori, H., Shibata, T., May 14, 2008. Hypermethylation of the RECK gene predicts poor prognosis in oral squamous cell carcinomas. Oral Oncology.

Melki, J.R., Warnecke, P., Vincent, P.C., Clark, S.J., 1998. Increased DNA methyltransferase expression in leukaemia. Leukemia 12 (3), 311–316.

Momparler, R.L., Bouffard, D.Y., Momparler, L.F., Dionne, J., Belanger, K., Ayoub, J., 1997. Pilot phase I-II study on 5-aza-2-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs 8 (4), 358–368.

Nagahama, Y., Ishimaru, M., Osaki, M., Inoue, T., Maeda, A., Nakada, C., Moriyama, M., Sato, K., Oshimura, M., Ito, H., 2008. Apoptotic pathway induced by transduction of RUNX3 in the human gastric carcinoma cell line MKN-1. Cancer Science 99 (1), 23–30.

Natsume, A., Wakabayashi, T., Tsujimura, K., Shimato, S., Ito, M., Kuzushima, K., Kondo, Y., Sekido, Y., Kawatsura, H., Narita, Y., Yoshida, J., 2008. The DNA demethylating agent 5-aza-2′-deoxycytidine activates NY-ESO-1 antigenicity in orthotopic human glioma. International Journal of Cancer 122 (11), 2542–2553.

Oka, M., Meacham, A.M., Hamazaki, T., Rodić, N., Chang, L.J., Terada, N., 2005. De novo DNA methyltransferases Dnmt3a and Dnmt3b primarily mediate the cytotoxic effect of 5-aza-2′-deoxycytidine. Oncogene 24 (19), 3091–3099.

Palii, S.S., Van Emburgh, B.O., Sankpal, U.T., Brown, K.D., Robertson, K.D., 2008. DNA methylation inhibitor 5-aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B. Molecular and Cellular Biology 28 (2), 752–771.

Park, H.J., Yu, E., Shim, Y.H., 2006. DNA methyltransferase expression and DNA hypermethylation in human hepatocellular carcinoma. Cancer Letters 233 (2), 271–278.

Robertson K.D. DNA methylation and human disease. Nature Reviews. Genetics 6 (8), 597–610, 2005.

Rodenhiser, D.I., 2008. Epigenetic contributions to cancer metastasis. Clinical & Experimental Metastasis.

Roll, J.D., Rivenbark, A.G., Jones, W.D., Coleman, W.B., 2008. DNMT 3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. Molecular Cancer 7 (15).

Römer, L., Klein, C., Dehner, A., Kessler, H., Buchner, J., 2006. p53—a natural cancer killer: structural insights and therapeutic concepts. Angewandte Chemie (International ed. in English) 45 (39), 6440–6460.

Schmelz, K., Wagner, M., Dörken, B., Tamm, I., 2005. 5-Aza-2′-deoxycytidine induces p21WAF expression by demethylation of p73 leading to p53-independent apoptosis in myeloid leukemia. International Journal of Cancer 114 (5), 683–695.

Shang, D., Liu, Y., Matsui, Y., Ito, N., Nishiyama, H., Kamoto, T., Ogawa, O., 2008. Demethylating agent 5-aza-2′-deoxycytidine enhances susceptibility of bladder transitional cell carcinoma to Cisplatin. Urology 71 (6), 1220–1225.

Tan, S.H., Ida, H., Lau, Q.C., Goh, B.C., Chieng, W.S., Loh, M., Ito, Y., 2007. Detection of promoter hypermethylation in serum samples of cancer patients by methylation-specific polymerase chain reaction for tumour suppressor genes including RUNX3. Oncology Reports 18 (5), 1225–1230.

Tang, Q.B., Sun, H.W., Zou, S.Q., 2004. Inhibitory effect of methylation inhibitor 5-aza-2-deoxycytidine on bile duct cancer cell line in vivo and in vitro. Hepatobiliary & Pancreatic Diseases International: HBPD INT 3 (1), 124–128.

Wang, C., Zhang, Y., Liang, J., Shan, G., Wang, Y., Shi, Q., 2006. Impacts of ascorbic acid and thiamine supplementation at different concentrations on lead toxicity in testis. Clinica Chimica Acta 370 (1–2), 82–88.

Wang, H., Zhao, Y., Li, L., McNutt, M.A., Wu, L., Lu, S., Yu, Y., Zhou, W., Feng, J., Chai, G., Yang, Y., Zhu, W.G., 2008. An ATM- and Rad3-related (ATR) signaling pathway and a phosphorylation–acetylation cascade are involved in activation of p53/p21Waf1/Cip1 in response to 5-aza-2′-deoxycytidine treatment. The Journal of Biological Chemistry 283 (5), 2564–2574.

Zhu, Y.M., Huang, Q., Lin, J., Hu, Y., Chen, J., Lai, M.D., 2007. Expression of human DNA methyltransferase 1 in colorectal cancer tissues and their corresponding distant normal tissues. International Journal of Colorectal Disease 22 (6), 661–666.

Zorn, C.S., Wojno, K.J., McCabe, M.T., Kuefer, R., Gschwend, J.E., Day, M.L., 2007. 5-Aza-2′-deoxycytidine delays androgen-independent disease and improves survival in the transgenic adenocarcinoma of the mouse prostate mouse model of prostate cancer. Clinical Cancer Research 13 (7), 2136–2143.