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Role of 5-Aza-CdR in mitomycin-C chemosensitivity of T24 bladder cancer cells

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Abstract. Chemotherapeutic insensitivity is one of key obstacles to effectively treating muscle invasive bladder cancer. 5-Aza-2'-deoxycytidine (5-Aza-CdR) has been identified as a tumor suppressive agent in various types of cancer. The aim of the present study was to identify the effects of 5-Aza-CdR on the mitomycin-C (MMC) chemosensitivity of T24 bladder cancer cells and investigate the underlying mechanisms. T24 cells were treated with a combination of MMC and 5-Aza-CdR at various concentrations. The rates of proliferation and apoptosis were assessed by an MTT assay and flow cytometry, respectively. The expression of drug resistance-associated proteins, including P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1), and autophagy-associated proteins, including beclin 1, nucleoporin 62 (p62) and autophagy protein 5 (ATG5) were detected with western blotting. Treatment with 5-Aza-CdR significantly promoted the MMC chemosensitivity of T24 cells. The proliferation of T24 cells was significantly inhibited with increasing 5-Aza-CdR concentration, whereas apoptosis was significantly increased. This was associated with the decreased expression of P-gp, MRP1, beclin 1, p62 and ATG5. In conclusion, 5-Aza-CdR enhanced MMC chemosensitivity in bladder cancer T24 cells, which may be caused by the suppression of drug resistance- and autophagy-associated proteins.

Introduction

Bladder cancer ranks 13th in the causes for cancer-associated mortality worldwide and is the most common type of urological cancer in China (1). Muscle-invasive bladder cancer constitutes ~30% of newly diagnosed cases of bladder cancer (2). Approximately 10% of non-invasive bladder cancer cases eventually progress to invasive cancer following the transurethral resection of the bladder tumor (3). Compared with non-invasive bladder cancer, patients with invasive disease have a poor prognosis, with a 5-year survival rate of 50% (4). Systemic chemotherapy remains the major therapeutic option for muscle-invasive bladder cancer in neoadjuvant and adjuvant settings, as well as for metastatic disease. Although it often leads to an initial therapeutic success in patients with metastatic bladder cancer, 60-70% of responding patients relapse within the first year of treatment, with a median survival time of 12-14 months (5). This limited efficacy appears to be largely associated with drug resistance of the tumor during treatment. Therefore, there is an urgent requirement to develop chemosensitization strategies.

Mitomycin-C (MMC) is widely used as a chemotherapeutic drug in the treatment of bladder cancer. However, only a limited number of patients were microscopically free of tumor cells following MMC treatment. The development of resistance to MCC is a major concern in bladder cancer therapy, and the mechanism remains largely unclear. Several methods have been demonstrated to increase the anticancer efficacy of MMC therapy in bladder cancer (6,7). In our previous study, it was revealed that as a DNA methyltransferase inhibitor, 5-Aza-2'-deoxycitidine (5-Aza-CdR) could inhibit the proliferation, migration and invasion of the T24 bladder cancer cell line (8). However, whether 5-Aza-CdR could affect the chemosensitivity of T24 cells was not studied. Therefore, the present study aimed to investigate the effects of 5-Aza-CdR on the MMC chemosensitivity of bladder cancer T24 cells. The underlying mechanisms were also investigated.

Materials and methods

Cell culture and chemicals. MMC was purchased from the Zhejiang Haizheng Group Co., Ltd. (Taizhou, China). It was dissolved in physiological saline to a concentration of 0.01 mg/ml, and then stored until use at -4˚C. 5-Aza-CdR was purchased from Sigma-Aldrich (Merck KGaA; Darmstadt, Germany). It was dissolved in physiological saline to a concentration of 0.25, 1 and 10 μmol/l, and then stored until use at -20˚C, protected from light.

T24 human bladder cancer cells were obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). The cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with high glucose (4,500 mg/l), supplemented with 10% fetal

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bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell culture was conducted with 5% CO_2 at 37˚C.

T24 cells were harvested in the exponential phase. Cells were seeded in 96-well plates at a density of $2x10^4$ cells per well. Following incubation overnight in the previously specified conditions, the cells were treated with 0.01 mg/ml MMC and 5-Aza-CdR at different concentrations (including 0, 0.25, 1 and 10 μmol/l). T24 cells without MMC and 5-Aza-CdR served as a control. At 48 h, the cells were obtained for subsequent experiments.

MTT assay. Cells were treated with 20 μl MTT dye (Sigma-Aldrich; Merck KGaA) and incubated at 37˚C for 4 h. The supernatant was then removed, and 100 μl of dimethylsulfoxide was added to every well to dissolve the formazan product. The absorbance was recorded at a wavelength of 570 nm using a microplate reader (Bio-Rad Laboratories, Inc.). The following calculation was used: Cellular inhibition rate, % = (1-treated group absorbance/control group absorbance) x100.

Flow cytometric analysis. The cells were washed twice with PBS, harvested with pancreatin and centrifugated (164.3 x g, 5 min) at room temperature, and resuspended. The samples were incubated with 5 μl Annexin V-fluorescein isothiocyanate and 5 μl propidium iodide for exactly 5 min at room temperature in the dark and then measured on a FACS Vantage Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting. The cells were lysed with lysis buffer (ProMab Biotechnologies, Inc., Richmond, CA, USA). The total protein concentration was quantified by a bicinchoninic acid protein assay. Appropriate amounts of protein (20-40 μg per well) were separated with 10% SDS-PAGE. The proteins were then transferred to a polyvinylidene difluoride membrane. The membranes were blocked at 37˚C for 3 h in a blocking solution consisting of 5% nonfat milk and TBS with 0.1% Tween-20. The primary antibodies, including mouse anti-P-gp (cat. no. ab80594; dilution, 1:1,000), mouse anti-MRP1 (cat. no. ab24102; dilution, 1:1,000), rabbit anti-beclin 1 (cat. no. ab62557; dilution, 1:1,000), mouse anti-p62 (cat. no. ab56416; dilution, 1:1,000), rabbit anti-ATG5 (cat. no. ab108327; dilution, 1:1,000) and mouse anti-GAPDH (cat. no. ab8245; dilution, 1:1,000) (all from Abcam, Cambridge, UK) were incubated overnight with the membrane at 4˚C, followed by corresponding horseradish peroxidase-conjugated secondary antibodies goat anti-rabbit immunoglobulin (Ig) G/horse radish peroxidase (HRP) (cat. no. ab6721; dilution, 1:2,000) and goat anti-mouse IgG/HRP (cat. no. ab6789; dilution, 1:2,000) (all from Abcam) at room temperature for 1 h. Immunoreactive bands were detected using an Enhanced Chemiluminescence-plus kit (GE Healthcare, Chicago, IL, USA) according to the manufacturer's protocol. The chemiluminescence was analyzed using ChemiDoc XRS system with Image Lab Software version 6.0 (Bio-Rad Laboratories, Inc., Hercules, CA).The levels of the target protein were presented as the relative density vs. GAPDH.

Statistical analysis. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data are presented as the mean ± standard deviation. The significance of the data was determined by Student's t-test and one-way analysis of variance. The post hoc test was performed using Student-Newman-Keuls method. P<0.05 was considered to indicate a statistically significant difference.

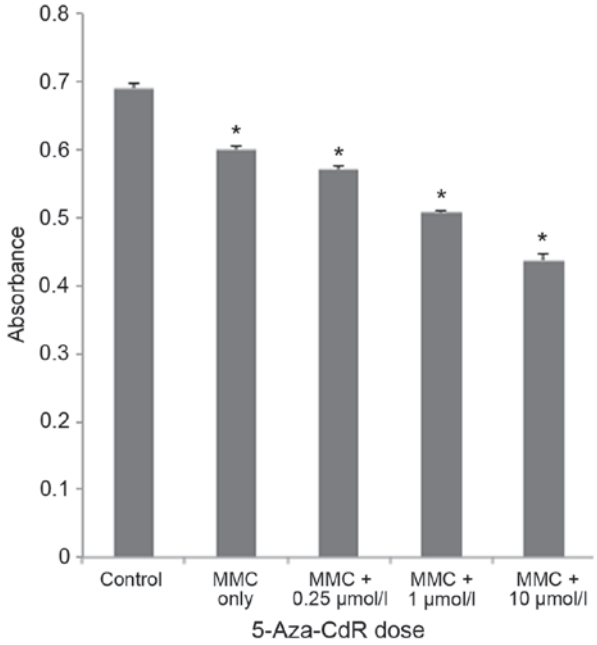


Figure 1. Effects of 5-Aza-CdR on MMC-induced growth inhibition in T24 cells. A reduced extent of cell proliferation was observed with increasing 5-Aza-CdR treatment. The data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control. MMC, mitomycin-C; 5-Aza-CdR, 5-Aza-2'-deoxycitidine.

Results

Effect of 5-Aza-CdR on MMC-induced proliferation inhibition in T24 cells. To investigate the effects of 5-Aza-CdR treatment, an MTT assay was performed subsequent to treating T24 cells with different concentrations of 5-Aza-CdR (0, 0.25, 1 and 10 μmol/l) and MMC (0.01 mg/ml). 5-Aza-CdR enhanced MMC-induced proliferation inhibition in T24 cells (Fig. 1). The rate of T24 cell growth inhibition was 1.63±0.01 with MMC only, and 5.0±0.04, 15.50±0.06 and 27.16±0.11%, with 0.025, 1 or 10 μmol/l 5-Aza-CdR, respectively; growth inhibition was thus induced in a dose-dependent manner by 5-Aza-CdR (P<0.05).

Effects of 5-Aza-CdR on MMC-induced apoptosis in T24 cells. To determine whether 5-Aza-CdR could enhance MMC-induced apoptosis in T24 cells, flow cytometry was performed to identify the apoptotic rate of cells. The apoptotic rate increased in a 5-Aza-CdR-dose-dependent manner, including early and late apoptotic cell death (P<0.05; Fig. 2). In the control group, 3.23±0.08% of cells underwent apoptosis, whereas 4.85±0.04, 8.81±0.06, 21.14±0.05 and 38.17±0.07% of cells underwent apoptosis in the 0, 0.25, 1 and 10 μmol/l 5-Aza-CdR groups, respectively.

Effects of 5-Aza-CdR on the expression of chemoresistance-associated proteins in T24 cells. To elucidate the

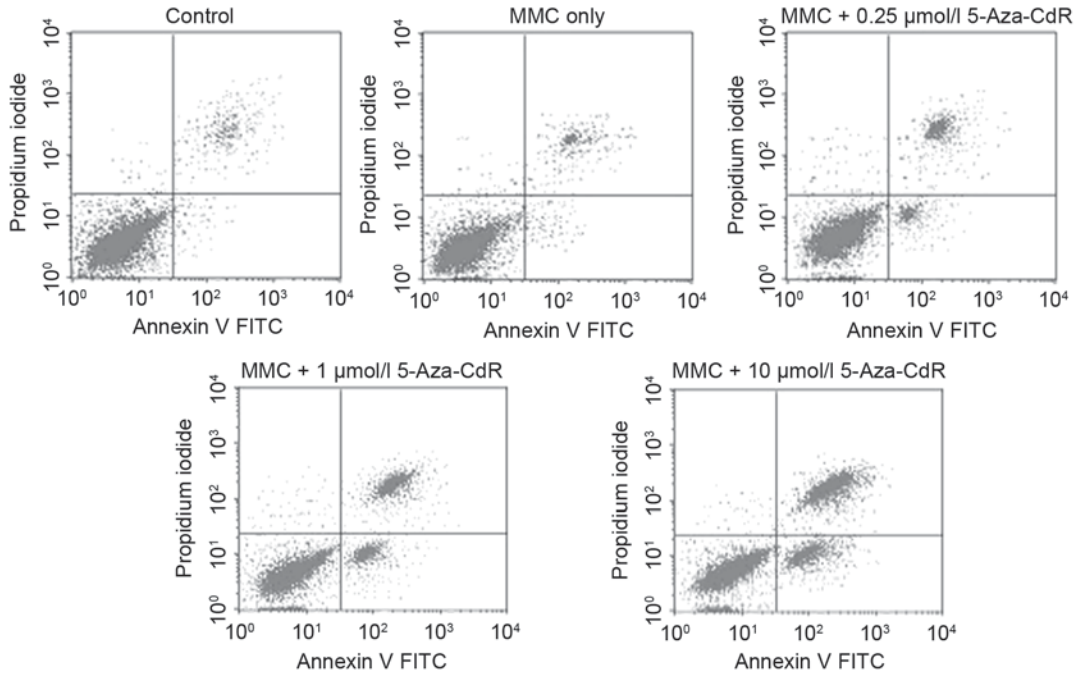


Figure 2. Effects of 5-Aza-CdR on MMC-induced apoptosis in T24 cells. An increased rate of apoptosis was observed with increasing 5-Aza-CdR treatment. The experiments were conducted in triplicate. MMC, mitomycin-C; 5-Aza-CdR, 5-Aza-2'-deoxycitidine; FITC, fluorescein isothiocyanate.

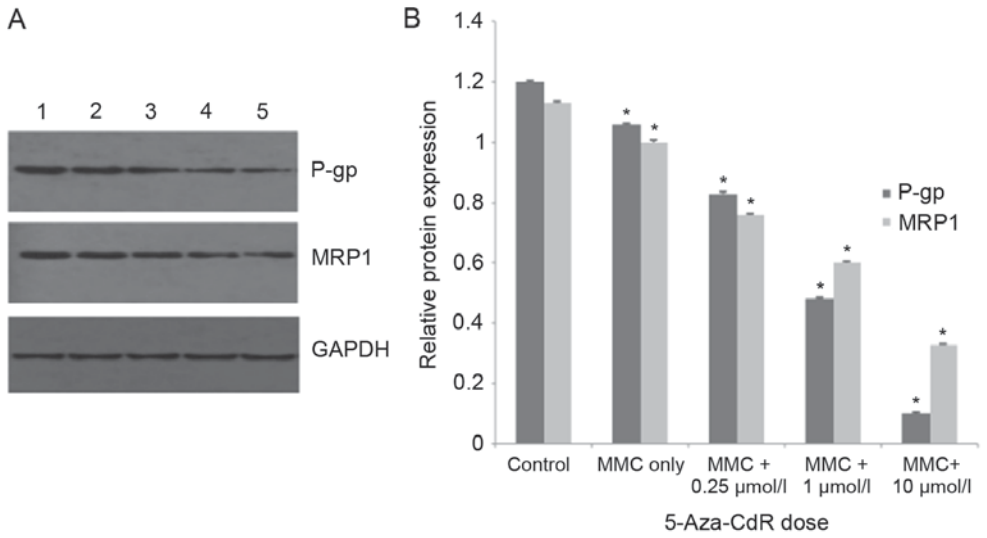


Figure 3. Western blot analysis of P-gp and MRP1 protein in T24 cells. (A) P-gp and MRP1 in T24 cells was measured by western blotting. 1 is the control, 2 MMC only, 3 MMC + 0.25 μmol/l, 4 MMC + 1 μmol/l and 5 MMC + 10 μmol/l 5-Aza-2' deoxycitidine. GAPDH was used as an internal control. (B) The relative protein levels, normalized to GAPDH. P-gp and MRP1 expression were decreased with increasing 5-Aza-CdR treatment. The data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control. P-gp, P-glycoprotein; MRP1, multidrug resistance-associated protein 1; MMC, mitomycin-C; 5-Aza-CdR, 5-Aza-2'-deoxycitidine.

potential mechanism for 5-Aza-CdR-mediated chemosensitivity alterations, western blot analysis was performed to examine the effects on P-gp and MRP1 expression. The results demonstrated that with increasing 5-Aza-CdR, the expression levels of P-gp and MRP1 were significantly decreased (P<0.05; Fig. 3).

Effects of 5-Aza-CdR on the expression of autophagy-associated proteins in T24 cells. The expression level of the autophagy-associated proteins beclin 1, p62 and ATG5 was determined with western blot analysis. Data revealed that with increasing 5-Aza-CdR concentration, the expression levels of beclin 1, p62 and ATG5 were all significantly decreased (P<0.05; Fig. 4).

Discussion

Resistance to chemotherapeutic drugs is an important reason for clinical chemotherapy failure in bladder cancer. In the present study, it was identified that 5-Aza-CdR may enhance the MMC sensitivity of T24 bladder cancer cells. The apoptosis of T24 cells was significantly promoted by combined treatment with MMC and 5-Aza-CdR, compared with those treated

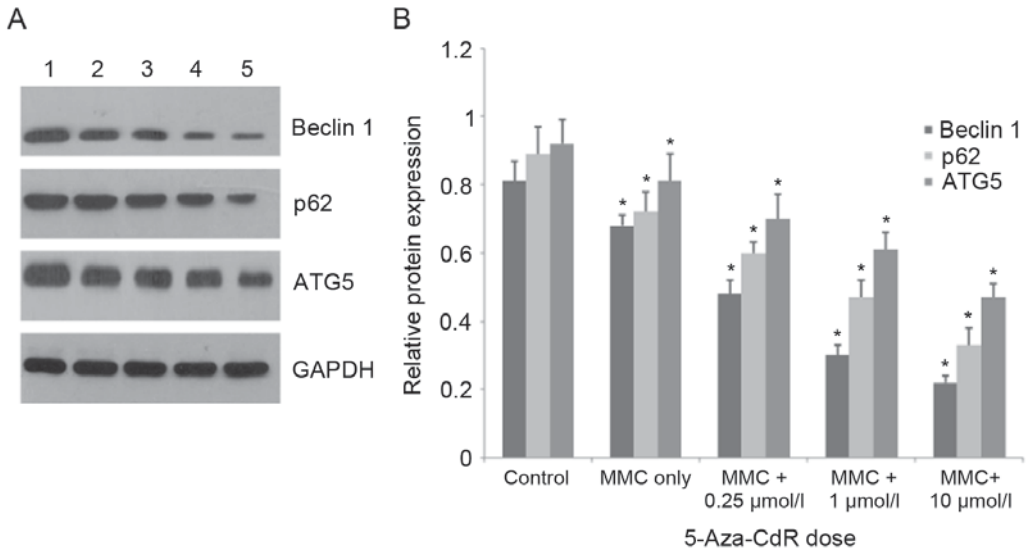


Figure 4. Western blotting analysis of the expression of beclin 1, p62 and ATG5 protein in T24 cells. (A) Beclin 1, p62 and ATG5 expression levels in T24 cells were measured by western blotting. 1 is the control, 2 MMC only, 3 MMC + 0.25 μmol/l, 4 MMC + 1 μmol/l and 5 MMC + 10 μmol/l 5-Aza-2' deoxycitidine. GAPDH was used as an internal control. (B) The relative protein levels, normalized to GAPDH. Beclin 1, p62 and ATG5 expression levels were decreased with increasing 5-Aza-CdR treatment. The data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control. p62, nucleoporin 62; ATG5, autophagy 5; MMC, mitomycin-C; 5-Aza-CdR, 5-Aza-2'-deoxycitidine.

by MMC alone. Furthermore, with increasing 5-Aza-CdR concentrations, the cellular inhibition rates increased in a dose-dependent manner. This data indicated that 5-Aza-CdR serves a role in the enhancement of MMC chemosensitivity of bladder cancer T24 cells.

To investigate the mechanism of 5-Aza-CdR in the chemosensitivity of bladder cancer T24 cells, western blotting was used to detect the expression of P-gp and MRP1 protein, which are associated with chemotherapeutic resistance. The data revealed that the expression levels of P-gp and MRP1 protein in T24 cells following treatment with MMC were significantly decreased in a dose-dependent manner with increasing 5-Aza-CdR concentration. P-gp and MRP1 protein belong to a family of ATP-dependent efflux transporters termed the ATP-binding cassette (ABC) family of membrane transport proteins (9). Members of the ABC transporter family have the capacity to efflux small molecules, causing drug accumulation in the cell (including the accumulation of anticancer drugs) to decrease, and thus contribute to drug resistance (10). The clinical significance of P-gp and MRP1 in drug resistance is supported by evidence that their expression indicates an adverse prognosis in patients with a range of types of cancer (11,12). Yang *et al* (13) demonstrated that Nsc23925 could prevent the development of paclitaxel resistance by inhibiting the expression of P-gp and enhancing apoptosis. Therefore, it was concluded from the data of the present study that 5-Aza-CdR enhances MMC chemosensitivity of T24 cells by suppressing P-gp and MRP1 expression.

Based on this result, studying the mechanism for the effect of 5-Aza-CdR on the chemosensitivity of T24 bladder cancer cells may yield clinical value. The expression levels of beclin 1, p62 and ATG5 protein were then detected, which were associated with autophagy. It was demonstrated that the expression levels of beclin 1, p62 and ATG5 protein in T24 cells were decreased in a dose-dependent manner following treatment with MMC and increasing 5-Aza-CdR concentrations. Beclin 1, p62 and ATG5 are considered as the key regulators of autophagic cell death (14-16). Autophagy is a lysosome-dependent self-digesting system primarily responsible for the removal and recycling of long-lived proteins, and damaged or obsolete intracellular organelles, in order to maintain cell homeostasis (17). The exact role of autophagy in cancer remains controversial. A number of studies provide evidence that autophagy suppresses tumorigenesis (18,19), whereas other studies propose that autophagy is associated with tumor development and protects tumor cells from apoptosis (20,21). In addition, a role for autophagy in the chemosensitivity of cancer cells has been identified; Wu *et al* (22) reported that autophagy may facilitate the resistance of lung adenocarcinoma cells to cisplatin treatment by the activation of the AMP-activated protein kinase/mechanistic target of rapamycin signaling pathway. Yang *et al* (23) demonstrated that the inhibition of autophagy could reduce pancreatic cancer stem cell activity and potentiate the tumoricidal effect of gemcitabine. In the present study, the expression of beclin 1, p62 and ATG5 in T24 cells was decreased in a dose-dependent manner following treatment with MMC and increasing 5-Aza-CdR treatment, indicating the reduced autophagy activity. Based on the regulatory role of autophagy in chemosensitivity, it was speculated that 5-Aza-CdR enhanced MMC chemosensitivity of T24 cells partially by suppression of autophagy. Future studies involving autophagy and chemosensitivity are warranted to confirm the conclusions of the present study.

In our previous study, 5-Aza-CdR was revealed to exhibit an inhibitory effect on the proliferation, migration and invasion of T24 bladder cancer cells (8). In the present study, it was demonstrated that 5-Aza-CdR could enhance the cytotoxicity of MMC in T24 cells. This effect may be partially mediated by the suppression of drug resistance- and autophagy-associated proteins. Although the mechanism remains to be clarified, the conclusions of the present study may provide a new therapeutic option to overcome chemoresistance in bladder cancer.

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