

Oxymatrine inhibits proliferation of human bladder cancer T24 cells by inducing apoptosis and cell cycle arrest

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Abstract. Oxymatrine has been shown to exert an antitumor effect on several types of cancer cells. However, the role of oxymatrine in bladder cancer has not yet been evaluated. The present study was designed to investigate the potential anti-proliferative effect of oxymatrine on bladder cancer T24 cells and the possible mechanisms involved. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine cell growth, and the cell morphology was examined using hematoxylin and eosin staining, wrights' staining and electron microscopy. The caspase-3 and survivin mRNA and protein levels were assessed using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The expression of tumor protein p53 (p53), Bcl-2-associated X protein (Bax) and B-cell lymphoma 2 (Bcl-2) were analyzed using immunohistochemistry. Oxymatrine inhibited the proliferation of the T24 cells in a dose- and time-dependent manner. Oxymatrine also induced apoptosis and cell cycle arrest in the cells, in association with the upregulation of caspase-3 and Bax, and the downregulation of survivin, Bcl-2 and p53 expression. Overall, oxymatrine inhibits the proliferation of human bladder cancer cells by inducing apoptosis and cell cycle arrest via mechanisms that involve p53-Bax signaling and the downregulation of survivin expression.

Introduction

Bladder cancer is one of the most common malignant tumors of the urinary system worldwide, affecting 16.6 per 100,000 males and 3.6 per 100,000 females (1). Although surgery is the usual and effective way to treat bladder cancer,

the 5-year recurrence rate is ~50% following surgical treatment (2,3). At present, intravesical chemotherapy is the most frequently used method to prevent the recurrence of bladder cancer, subsequent to surgery (4). However, chemotherapy is often associated with multidrug resistance and toxic side effects, with little specificity to cancer cells, which elucidates the challenges of applying chemotherapy to bladder cancers. Natural compounds have previously emerged as a potential treatment for cancer therapy; therefore, natural compounds may be a promising target for bladder cancer therapy.

Matrine, the active compound that may be extracted from the Chinese herb *Sophora flavescens*, has previously been used to treat chronic hepatitis B, allergic dermatitis and hypoleukocytosis, in China (5,6). Matrine may transform into oxymatrine, which may also be extracted from *Sophora flavescens*, and the nitrogen-oxygen bond may split under certain conditions transforming oxymatrine back to matrine (7). Several studies have demonstrated the multiple beneficial effects of matrine, including anti-arrhythmia (8), anti-inflammation (9,10) and anti-fibrosis (11,12) effects, with minimal side effects reported. In addition, previous studies have shown that matrine induces apoptosis in several cancer cell lines, including breast cancer MCF-7 cells (13) and pancreatic cancer PANC1 cells (14), using various mechanisms, which potentiate the role of matrine in cancer therapy. However, it remains unclear whether matrine is able to inhibit the growth of bladder cancer cells. Therefore, the present study was designed to investigate whether matrine exerts an antitumor effect on the bladder cancer T24 cell line. The results revealed that matrine exhibits an anti-proliferative effect on the T24 cells, by inducing apoptosis and cell cycle arrest.

Materials and methods

Cell culture. The T24 cell line was obtained from the Department of Laboratory Diagnosis, Chongqing Medical University (Chongqing, China). The cells were incubated in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (Sijiqing, Hangzhou, China), at 37°C in a humidified atmosphere with 5% CO₂.

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3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. T24 cells in the exponential growth phase

(24–96 h after plating) were seeded into 96-well plates at $\sim 4 \times 10^4$ cells/well, and then treated with oxymatrine (0.625, 1.25, 2.5, 5.0 and 10 mg/ml; Ningxia Qiyuan Pharmaceutical Co., Ltd., Yinchuan, Ningxia, China) for 24, 48 and 72 h. Subsequent to incubation for various times, 20 μ l MTT reagent (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for an additional 4 h. Dimethyl sulfoxide was used to dissolve the MTT formazan. Mitomycin C (MMC) was used as a positive control. The absorbance values at 600 nm were obtained using a spectrometer (Infinite® 200; Tecan Group Ltd., Männedorf, Switzerland).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA of the treated cells was isolated using the Invitrogen TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocols. The ReverTra Ace® qPCR RT kit (Toyobo Co., Ltd., Shanghai, China) was used to synthesize cDNA from the total RNA, producing a 0.5 μ g sample per assay. A SYBR® Premix Ex Taq II kit (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) was used to perform the qPCR on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and the sequences were as follows: i) Survivin forward, 5'-GCCAGTGTCTTCTCTGCTT-3' and reverse, 5'-CCGGACGAATGCTTTTATG-3'; ii) caspase-3 forward, 5'-GAGTGCTCGCAGCTCATACCT-3' and reverse, 5'-CCTCACGGCCTGGGATTT-3'; iii) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-AGCCACATCGCTCAGACAC-3' and reverse, 5'-GCCCAATACGACCAATCC-3'. The PCR protocol was as follows: Survivin: 94°C for 2 min, 94°C for 30 sec 54°C for 30 sec (30 cycles) and 72°C for 5 min; and caspase-3: 94°C for 5 min, 95°C for 30 sec, 52°C for 30 sec (35 cycles) and 72°C for 5 min. The gene expression was normalized to GAPDH, using the $2^{-\Delta\Delta C_q}$ method (15) and the experiments were performed in triplicate.

Western blot analysis. The treated T24 cells were digested using 1% trypsin for 3–10 min at 37°C. The cells were then treated with 100 μ l lysis buffer for each sample. The cells were subsequently placed on ice for 30 min. Each sample was centrifuged at 14,000 $\times g$ for 10 min and the supernatants were collected. The protein concentrations of the supernatants were determined using a bicinchoninic acid kit (Beyotime Institute of Biotechnology, Haimen, China). For the western blot analysis, 30 μ g denatured total protein for each sample was separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked in 5% skimmed milk for 2 h and were incubated with primary antibodies [rabbit anti-human survivin monoclonal antibody (catalog no., BA14055; dilution, 1:1,000); and rabbit anti-human caspase-3 monoclonal antibody (catalog no., BA3592; dilution, 1:1,000) purchased from Wuhan Boster Biological Technology, Ltd., Wuhan, China] overnight at 4°C. The horseradish peroxidase-conjugated secondary antibodies [goat anti-rabbit IgG (catalog no., BA1055; dilution, 1:5,000) purchased from Wuhan Boster Biological Technology, Ltd.] were used to detect the primary antibodies on the membrane

and the bands were visualized using 3,3-diaminobenzidine (DAB). Each experiment was performed in triplicate.

Immunohistochemistry (IHC). The cells were seeded onto coverslips and examined using IHC staining. The HRP Conjugated anti-Mouse/Rabbit IgG SABC kit (catalog no., SA1020) was purchased from Wuhan Boster Biological Technology, Ltd., and the staining procedure was performed according to the manufacturer's protocols. DAB was used to develop the color, and the cells were counterstained with hematoxylin and eosin (H&E). The rate of expression was calculated manually.

H&E and Wright's staining. The T24 cells were cultured in RPMI-1640 medium containing 10% bovine serum (Gibco; Thermo Fisher Scientific) at 37°C with 5% CO₂ and placed on coverslips where they were treated with 1.25 mg/ml oxymatrine. The staining was performed using the Hematoxylin-Eosin Staining kit (Wuhan Boster Biological Technology, Ltd.), according to the manufacturer's protocols.

Electron microscopy. The treated cells were fixed in 1% osmic acid and subsequently subjected to gradient dehydration in ethanol. The cells were then embedded in epoxy resin, sectioned (100 μ m thick) and stained with lead citrate. Electron microscopic images were captured using the JEM-100CXII transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Flow cytometric analysis of DNA content. The T24 cells were synchronized for 24 h and were treated with 1.25 and 2.50 mg/ml oxymatrine for 72 h. Following treatment, the cells were collected using trypsin and resuspended in pre-cooled ethanol. The cell suspensions were mixed with an equal volume of propidium iodide (PI) staining buffer for 30 min, and then passed through a 40- μ m strainer. The PI stain was excited at a wavelength of 488 nm. The results were analyzed using ModFit LT 2.0 software (Verity Software House, Inc., Topsham, ME, USA).

Statistical analysis. The data are presented as the mean \pm standard error. Student's t-test was used to compare the differences between two groups and the differences among three or more groups were compared using a one-way analysis of variance, followed by the Bonferroni post hoc test. A two-tailed P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Oxymatrine inhibits the proliferation of T24 cells. Previous studies have shown that matrine exerts a growth inhibition effect on breast cancer cells (13); however, whether matrine has a similar effect on bladder cancer cells has not been elucidated. In order to address this issue, an MTT assay was performed to examine the role of oxymatrine in cell growth. As shown in Fig. 1A, the exponential growth phase of the T24 cells was between 24–96 h after plating. The cells were treated with various doses of oxymatrine for 24, 48 and 72 h. Low doses (0.625 mg/ml) of oxymatrine showed no significant effect on the cell inhibition ratio, whereas a medium to high dose (1.25–10.00 mg/ml) of oxymatrine significantly inhibited

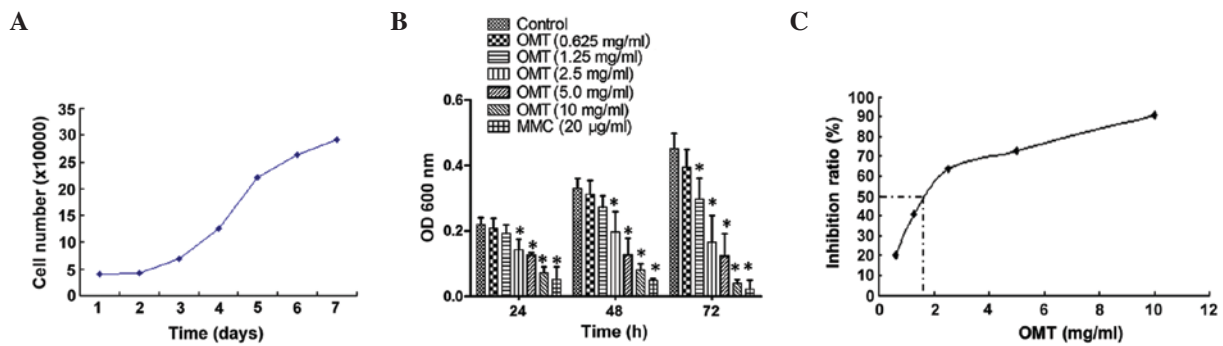


Figure 1. Effect of oxymatrine on cell proliferation. (A) Growth curve of the T24 cells. Cells were plated onto 96-well plates and incubated for 7 days, and the cell count was then determined. (B) Effect of various doses of OMT on T24 cell proliferation at 24, 48 and 72 h, detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (n=3; *P<0.05 vs. control). MMC at 20 µg/µl was used as a positive control. (C) Regression analysis of the effect of oxymatrine on the inhibition ratio at 72 h, showing the half maximal inhibitory concentration. OMT, oxymatrine; MMC, mitomycin C.

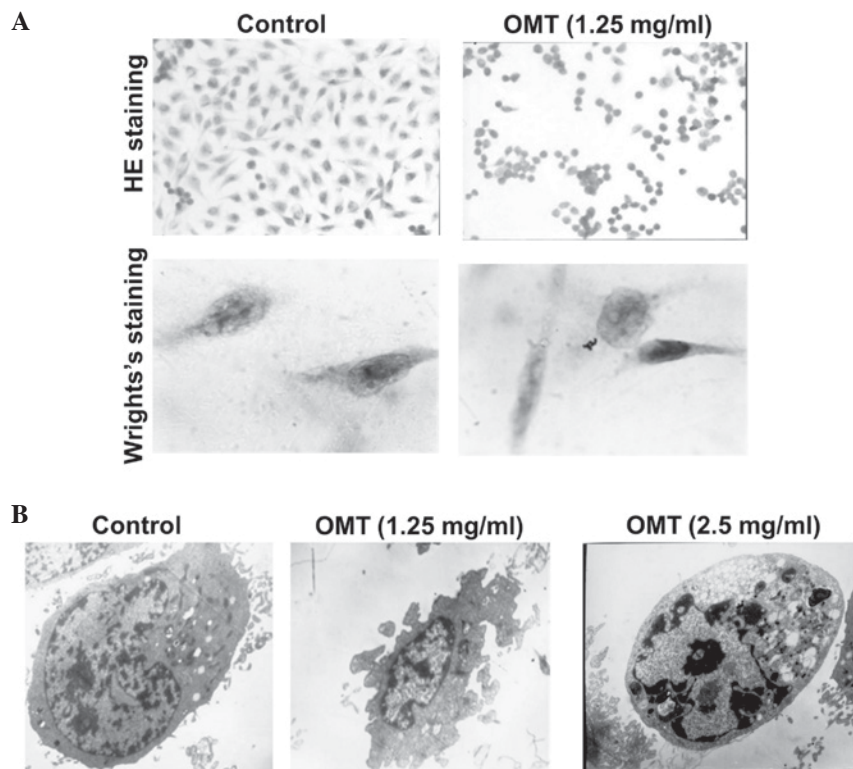


Figure 2. Effect of oxymatrine on cell morphology. (A) Representative low-power microscopic views of H&E stained cells (magnification, x200), and representative high power microscopic views of Wright's stained cells (magnification, x1,000). (B) Representative electron microscopy image showing apoptotic bodies in oxymatrine-treated cells (magnification, x8,000). H&E, hematoxylin and eosin.

the growth of the T24 cells in a time- and dose-dependent manner (Fig. 1B). In order to better understand the effect of oxymatrine on T24 cells, the data was analyzed using a regression equation, which indicated that the 50% inhibition concentration of oxymatrine on the T24 cells was 1.82 mg/ml (Fig. 1C).

Oxymatrine induces apoptosis in T24 cells. Multiple causes may account for the growth inhibition effect of oxymatrine, including apoptosis and necrosis. In order to examine whether the growth inhibition was caused by apoptosis, the T24 cells were subjected to 1.25 mg/ml oxymatrine for 72 h. H&E staining revealed that the cells in the control group were spindle-shaped, the nuclei were hyperchromatic and the nucleoli

were presented clearly. By contrast, the oxymatrine-treated cells exhibited an irregular cell shape and shrinkage of the cytoplasm (Fig. 2A). Wright's staining clearly showed that the nuclei were condensed and that the nuclear membranes were crescent-shaped, an indication of apoptotic bodies (Fig. 2A). In addition, the cells displayed the typical features of apoptosis, including a decreased nuclear-cytoplasmic ratio, a decreased nucleolus size, condensed chromatin and the invagination of cell membranes, following treatment with oxymatrine (Fig. 2B). These results suggest that oxymatrine induces apoptosis in T24 cells.

Oxymatrine induces cell cycle arrest in T24 cells. In order to evaluate the effect of oxymatrine on cell cycle progression, the

Table I. Cell cycle distribution and apoptotic ratio following treatment with oxymatrine (%).

Groups	G ₀ /G ₁	S	G ₂ /M	Apoptotic ratio
Control	43.31	54.10	2.59	0.00
1.25 mg/ml oxymatrine	54.52	44.42	1.06	5.37
2.50 mg/ml oxymatrine	52.41	40.59	6.99	- ^a

^aApoptotic ratio could not be analyzed for 2.50 mg/ml oxymatrine as necrosis occurred in a high number of cells and was therefore difficult to clearly determine.

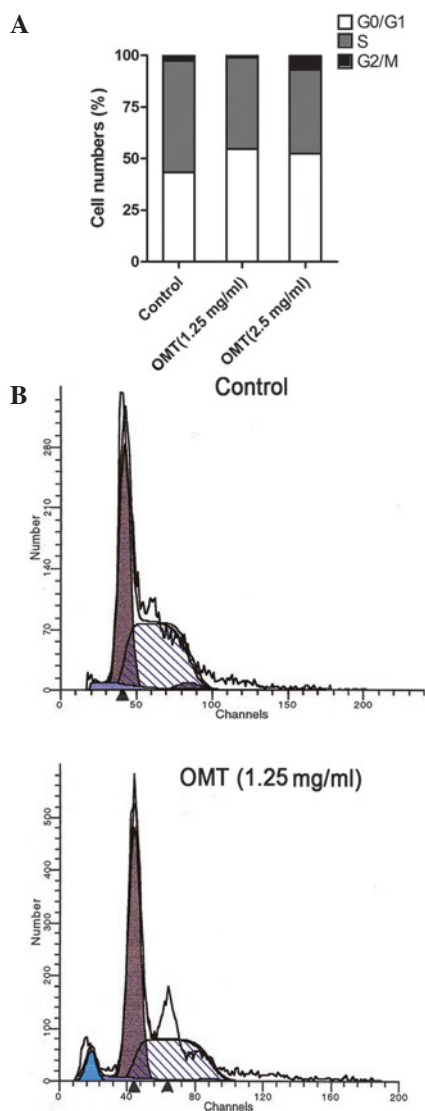


Figure 3. Effect of oxymatrine on cell cycle distribution. (A) Statistical analysis of cell cycle distribution following treatment with 1.25 and 2.50 mg/ml oxymatrine for 72 h. (B) A typical apoptotic peak was observed in the cells treated with 1.25 mg/ml oxymatrine.

T24 cells were treated with oxymatrine at the doses of 1.25 and 2.50 mg/ml. The DNA content was determined using flow cytometry. As shown in Fig. 3A and Table I, the cell number in the S phase was significantly decreased for each dose (% cell population in phase, 40.59 and 44.42% for 1.25 and 2.5 mg/ml, respectively, vs. 54.10% for control; $P < 0.05$) in the oxymatrine-treated groups compared with the control group.

Cell cycle arrest at the G₀/G₁ phase was also observed in the treated groups (% cell population in phase, 54.52 and 52.41% for 1.25 and 2.5 mg/ml, respectively, vs. 43.31% for control; $P < 0.05$). Consistent with the findings of the morphological studies, the apoptotic sub-G₁ peak was detected, and the apoptotic ratio of the cells that were treated with 1.25 mg/ml oxymatrine was 5.37% (Fig. 3A and B).

Oxymatrine affects the expression of apoptosis regulator proteins. In order to investigate the mechanism that mediates the pro-apoptotic effect of oxymatrine on T24 cells, the cells were firstly treated with oxymatrine. The levels of caspase-3 and survivin were evaluated, and the results from the RT-qPCR and western blot analysis revealed that the mRNA and protein expression levels of the apoptosis effector, caspase-3, increased in a dose-dependent manner (Fig. 4A and B). By contrast, the mRNA and protein expression levels of the anti-apoptotic gene, survivin, were decreased (Fig. 4A and B). In addition, the expression of tumor protein p53 (p53), B-cell lymphoma 2 (Bcl-2) and BCL2-associated X protein (Bax) was examined using IHC. As shown in Fig. 4C, p53 expression was significantly decreased (25.75 vs. 76.25%; $P < 0.05$), Bax expression was markedly increased (58.25 vs. 23.38%; $P < 0.05$) and Bcl-2 expression was notably increased (27.13 vs. 64.38%; $P < 0.05$) in the T24 cells of the oxymatrine-treated group compared with the control group.

Discussion

The present study demonstrates that oxymatrine initiates an anti-proliferative effect on the bladder cancer T24 cell line, in addition to inducing apoptosis and cell cycle arrest, which is consistent with previous studies on other types of cancer cells.

Bladder cancer is the most common malignancy of the urinary system (16,17). As recurrent bladder cancers are often characterized by a high occurrence, poor differentiation and a high invasive ability, searching for effective chemotherapy drugs is a priority for improving the prognosis. The natural compound oxymatrine, which is an alkaloid that is extracted from the traditional Chinese herb *Sophora flavescens*, has emerged as an important chemical compound in the treatment of cancer.

Consistent with several previous studies that have reported the antitumor effects of matrine on cancer cells (18-20), the present study reinforced the findings of the anti-proliferative effects of oxymatrine in bladder cancer cells. The MTT assay indicated that oxymatrine decreased the cell survival rate in a dose- and time-dependent manner, which indicates

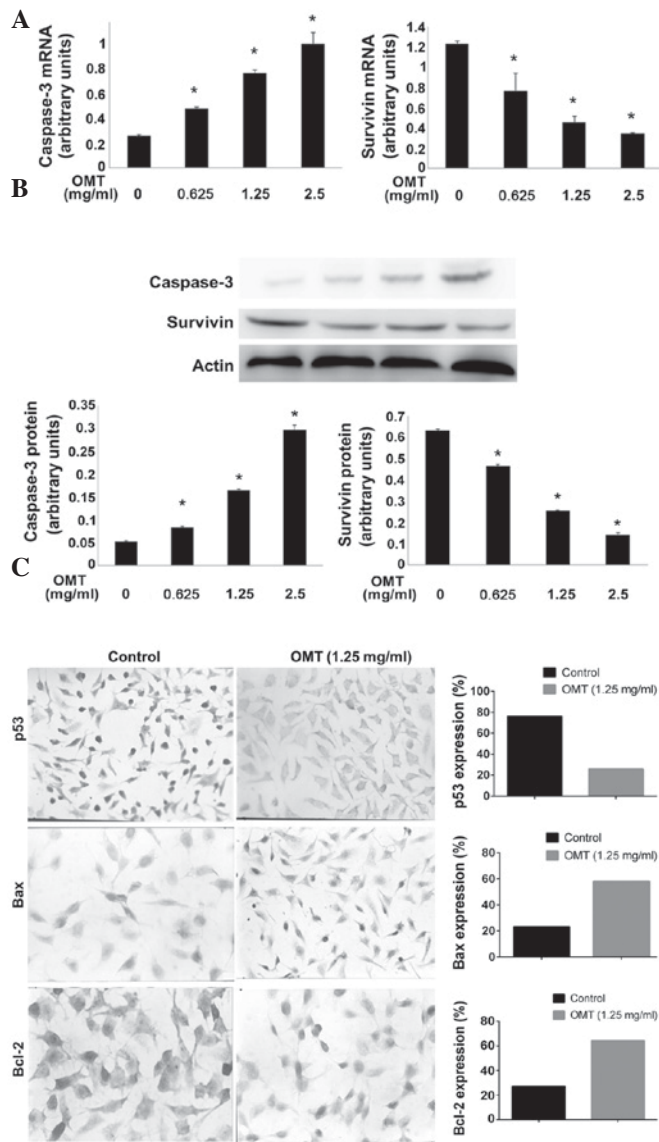


Figure 4. Effect of OMT on the expression of apoptosis-associated mRNAs and proteins. Effect of various doses of OMT on: (A) Caspase-3 and survivin mRNA levels, determined using reverse transcription-quantitative polymerase chain reaction; and (B) caspase-3 and survivin protein levels, detected by western blot analysis. The bottom panel shows the statistical band density analysis ($n=3$; $P<0.05$ vs. control). (C) Representative images of the immunohistochemistry of p53, Bax and Bcl-2. The cells were treated with 1.25 mg/ml OMT for 72 h. Left, control group; right, OMT (1.25 mg/ml)-treated group. p53 is present in the nucleus, while Bax and Bcl-2 are present in the cytoplasm. OMT, oxymatrine; p53, tumor protein p53; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2.

the significant inhibitory effect of oxymatrine on T24 cell proliferation. In addition, the morphologies of the cells were evidently altered to an apoptotic phenotype by oxymatrine. Oxymatrine induced the shrinkage of T24 cell cytoplasm and the condensation of the nucleus. In particular, crescent-shaped apoptotic bodies were also present in the cells. Normal cell cycle progression is important for the proliferation and division of cancer cells. In the present study, the findings of the flow cytometry analysis showed that oxymatrine induced T24 cell cycle arrest at the G_0/G_1 phase and therefore decreased the cell number in the S phase. The mechanisms underlying the anti-proliferative effect of oxymatrine were investigated in

order to strengthen the current understanding of the therapeutic value of oxymatrine in prostate cancer.

One of the major features of oxymatrine in the present study is the induction of apoptosis, a self-killing process that relies on the stepwise activation of caspases and substrates. Among the caspase family, caspase-3 acts as the rate-limiting enzyme that determines the extent of apoptosis. Survivin is another important regulator of cell survival that has been demonstrated to inhibit apoptotic signals by inhibiting the activation of endogenous caspase-9 (21). Survivin has also demonstrated the ability to exert an anti-apoptotic effect through competitively binding with the p21-cdk4 complex, thereby promoting the release of p21. p21 and caspase-3 form a complex that may inhibit caspase-3 activity (22). The results of the present study showed the increased expression of caspase-3 mRNA and the decreased expression of survivin mRNA caused by oxymatrine. In addition, similar results were observed for the protein expression of caspase-3 and survivin. The present study indicates that the transcriptional regulation of apoptosis via caspase-3 and survivin regulation may be one of the mechanisms underlying the effect of oxymatrine on cell proliferation. Another important molecule that regulates apoptosis is p53. p53 is important for G_1/S cell cycle regulation, and cells that express wild-type p53 are arrested in the G_1 phase, initiating apoptosis as a response to DNA damage. However, cells that express mutated forms of p53 may exhibit a cancerous aneuploidy phenotype, and are unable to initiate apoptosis (23). p53 mutations have been identified in the majority of cancer cell types (24). As the half-life of wild-type p53 is extremely short, IHC is commonly used to detect the expression of mutant p53. The results of the present study indicated that oxymatrine significantly suppressed the nuclear expression of mutant p53. Proteins located in the mitochondrial membrane are also important for the regulation of apoptosis. In the present study, Bcl-2 and Bax demonstrated anti-apoptotic and pro-apoptotic effects, respectively. Additionally, p53 has been previously indicated to transcriptionally activate Bax expression (25). The abnormal expression of the Bcl-2 and Bax proteins in bladder cancer has been previously illustrated (26,27). The results of the present study indicated an increased Bax/Bcl-2 ratio due to oxymatrine, which indicates that a p53-Bax dependent mechanism may be involved in this process.

Certain limitations were evident in the present study. For example, the results were all obtained from *in vitro* studies; therefore, careful assessment of the effect of oxymatrine on bladder cancer cell apoptosis *in vivo* is recommended in future studies.

In summary, the present study elucidated the anti-proliferative effect of the natural compound oxymatrine on bladder cancer cells. Oxymatrine was indicated to induce apoptosis and cell cycle arrest in T24 cells via the regulation of survivin and p53-Bax signaling. The findings may have clinical implications for future chemotherapy strategies.

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