


## Medicarpin induces G1 arrest and mitochondria-mediated intrinsic apoptotic pathway in bladder cancer cells

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### ABSTRACT

Bladder cancer (BC) is the tenth most commonly diagnosed cancer. High recurrence, chemo-resistance, and low response rate hinder the effective treatment of BC. Hence, a novel therapeutic strategy in the clinical management of BC is urgently needed. Medicarpin (MED), an isoflavone from *Dalbergia odorifera*, can promote bone mass gain and kill tumor cells, but its anti-BC effect remains obscure. This study revealed that MED effectively inhibited the proliferation and arrested the cell cycle at the G1 phase of BC cell lines T24 and EJ-1 *in vitro*. In addition, MED could significantly suppress the tumor growth of BC cells *in vivo*. Mechanically, MED induced cell apoptosis by upregulating pro-apoptotic proteins BAK1, Bcl2-L-11, and caspase-3. Our data suggest that MED suppresses BC cell growth *in vitro* and *in vivo* via regulating mitochondria-mediated intrinsic apoptotic pathways, which can serve as a promising candidate for BC therapy.

**Keywords:** bladder cancer, medicarpin, cell cycle arrest, apoptosis, therapy

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Bladder cancer (BC) is the tenth most common neoplasm worldwide, the sixth most common tumor in men, and the most common tumor of the urinary system, with an estimated 573, 278 cases and 212, 536 deaths in 2020 (1). The number of patients diagnosed with this disease has been showing an increasing trend in recent years (1, 2). BC can be classified into two subtypes, non-muscle invasive BC (NMIBC) or muscle-invasive BC (MIBC), depending on the distinct genetic background and clinical prognosis (3), NMIBC and MIBC account for 75 and 25 % of all BC cases, respectively (4).

The treatment of BC mainly depends on surgery, chemotherapy, targeted therapy and immunotherapy. A surgical resection is a dominant approach for BC patients (5). According to the literature, it is predicted that 54 % of patients with BC carcinoma *in situ* (CIS) will progress to MIBC (6), and after radical cystectomy (RC), approximately half of MIBC patients will relapse, and most will develop distant metastases (7). To reduce recurrence rate, neoadjuvant and adjuvant therapies, which consist of immunotherapy, targeted therapy, and chemotherapy have emerged. Cisplatin-based neoadjuvant or adjuvant therapies, a standard first-line treatment for BC, have shown improvement in the 5-year survival or progression-free survival (PFS) of BC patients, but there is a lack of evidence of a survival advantage for overall survival (OS) of BC patients (8–11). However, chemotherapy often led to several adverse side effects and drug-resistance. For cisplatin-ineligible BC patients, compared with first-line gemcitabine-carboplatin, atezolizumab (PD-1 inhibitor) therapy could have prolonged OS of BC patients from 9.3 months to 15.9 months (12, 13). Targeted therapy against cisplatin-resistant BC patients can be performed *via* mutated oncogenes or cell surface antigens. Vascular endothelial growth factor receptor (VEGFR) inhibitors have been shown to possess anti-BC activity when tested in patients with advanced BC. However, drug resistance is an inevitable challenge in targeted therapy and only a small part of patients responded to immunotherapy. Taken together, the current treatment against BC could not meet the clinical requirements and it is imperative to find novel therapeutic approaches in the battle against BC.

Recent studies suggested that traditional Chinese medicine (TCM) could inhibit proliferation and vascularization, and overcome the chemoresistance of different tumor cells (14). In addition, matrine, polyphyllin II and berberine also exhibited anti-BC properties (15–17). Specifically, matrine, a primary active ingredient derived from the dry roots of *Sophora flavescens*, inhibited BC *via* upregulation of apoptosis-related proteins and down-regulation of the VEGF/PI3K/Akt pathway combined with cisplatin (17). Berberine, a bio-active compound derived from the Chinese medicinal plant *Coptis chinensis*, inhibited the BC cells proliferation by perturbing the JAK1-STAT3 pathway (15).

Medicarpin (MED), a flavonoid extracted from *Medicago sativa* L., is found to possess various biological activities that include antibacterial and antifungal activities (18, 19). It promoted bone mass gain (20) and exhibited an inhibitory effect on various tumor cells. For instance, MED induced cell apoptosis of the doxorubicin-resistant P388 leukemia cells, which is a monocyte/macrophage-like cell line derived from a mouse lymphoma (21). Furthermore, MED sensitized myeloid leukemia cells K562 and U937 cells to tumor necrosis factor  $\alpha$ -related apoptosis-inducing ligand (TRAIL)-induced apoptosis (22). However, whether MED has an inhibitory effect on BC remains obscure. This study proved that MED inhibited the proliferation and induced cell apoptosis against BC cells *in vitro* and *in vivo*.

## EXPERIMENTAL

### *Cell culture and experimental reagents*

SV-HUC-1 (RRID:CVCL\_3798), T24 (RRID: CVCL\_0554) and EJ-1 (RRID:CVCL\_2893) cell lines, which were obtained from Procell Life Science & Technology Co., Ltd. (China), were cultured in 1640 medium containing 10 % fetal bovine serum (Gibco, USA) and 1 % penicillin-streptomycin (Gibco) in an incubator containing 5 % CO<sub>2</sub> at 37 °C. MED (C<sub>16</sub>H<sub>14</sub>O<sub>6</sub>) with ≥ 98 % of purity was brought from Desite (CAS NO: 32383-76-9, Chengdu, China), and dissolved in dimethyl sulfoxide (DMSO) to obtain the mother liquor.

### *Cell counting kit-8 assay*

SV-HUC-1, T24 and EJ-1 cells were seeded in 96-well culture plates at a seeding density of 5000 cells per well, as mentioned in a previous study (24). Cells were incubated with MED at concentrations of 0, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0, 175.0 and 200.0 μmol L<sup>-1</sup> for 24 hours (h) and 48 h. At a certain incubated time period (24 h or 48 h), the original complete medium was replaced with fresh complete medium with 10 % CCK-8 reagent (Beyotime Biotechnology Co., Ltd., China) and incubated for 2 hours at 37 °C. Finally, the absorbance values were obtained by Multiskan FC microplate reader (51119080, Thermo Fisher Scientific, USA) at 450 nm. The experimental results were analyzed by GraphPad Prism 8 software to calculate the IC<sub>50</sub> values and cell viability. The cell viability was measured by the formula presented as follows:

$$\text{Cell viability (\%)} = [(A_{\text{experimental well}} - A_{\text{blank well}}) / (A_{\text{control well}} - A_{\text{blank well}})] \times 100 \%$$

### *Cell cycle assay*

T24 and EJ-1 cells were seeded in 6 well culture plates at a seeding density of  $2 \times 10^5$  cells/well, as mentioned in a previous study (25). After adherence to the well, cells were treated with FBS-free 1640 medium for 24 h to ensure the consistency of the cell cycles. Subsequently, T24 cells were treated with 65.9 μmol L<sup>-1</sup> MED for 48 h, and EJ-1 cells were treated with 64.6 μmol L<sup>-1</sup> MED for 48 h. Cells were collected after 48 h of treatment and left overnight in 500 μL of ice-cold 70 % ethanol. Cells underwent three times washing by PBS, then resuspended by 500 μL PBS. To the cell suspension, 50 μg mL<sup>-1</sup> RNase A and 25 μL PI (1 mg mL<sup>-1</sup>) (Beyotime Biotechnology Co., Ltd.) was added. Finally, the cell cycle distribution of each well was subsequently detected using Calibur flow cytometry (BD Biosciences, USA) at 561 nm excitation wavelength. FlowJo\_V10 software was used to calculate the percentage of cells in different cell cycle phases.

### *Cell apoptosis assay*

T24 and EJ-1 cells were seeded in 6-well culture plates at a seeding density of  $2 \times 10^5$  cells/well, as mentioned in a previous study (26). After the adherence to the well, T24 cells were treated with 65.9 μmol L<sup>-1</sup> MED for 48 h, and EJ-1 cells were treated with 64.6 μmol L<sup>-1</sup> MED for 48 h. Cells were collected after 48 h of treatment for staining using 5 μL Annexin V-FITC and 10 μL PI solution (Beyotime Biotechnology Co., Ltd.), incubated in the dark at room temperature for 30 min. After the staining was completed, the cells were

centrifuged and the supernatant was removed, the cells were resuspended with 200  $\mu\text{L}$  PBS, and detected with a Calibur flow cytometer (BD Biosciences) at 488 nm and 535 nm. FlowJo\_V10 software was used to calculate the percentage of cells in different apoptotic phases.

### Cell migration assay

Accordingly (23), T24 and EJ-1 cells were seeded in 24 well culture plates at a seeding density of  $4 \times 10^4$  cells/well. After the adherence to the well, cells were lightly scratched in the middle of the well using a 200  $\mu\text{L}$  pipette tip, T24 cells were treated with  $65.9 \mu\text{mol L}^{-1}$  MED for 48 h, and EJ-1 cells were treated with  $64.6 \mu\text{mol L}^{-1}$  MED for 48 h. Images were acquired at 24 h and 48 h by Leica DM IL LED Inverted Phase Contrast Microscope with a camera (Leica Microsystems, Germany) and photographed at  $10\times$  magnification. The scratch healing area was finally analyzed by using ImageJ version 1.8.0 software and contrasted with the DMSO group.

### Quantitative real-time PCR

T24 and EJ-1 cells were seeded in a T25 culture flask at a seeding density of  $5 \times 10^5$  cells/well, as mentioned in a previous study (28). After adherence to the well, T24 cells were treated with  $65.9 \mu\text{mol L}^{-1}$  MED for 48 h, and EJ-1 cells were treated with  $64.6 \mu\text{mol L}^{-1}$  MED for 48 h. The total RNA of T24 cells and EJ-1 cells was extracted using RNAprep pure Cell/Bacteria Kit (DP430, TIANGEN Biotech Co., Ltd., China), and then reverse transcribed into cDNA with FastKing RT Kit (KR116, TIANGEN Biotech Co., Ltd.). qRT-PCR was performed using the SuperReal PreMix Plus (SYBR Green) kit (FP205, TIANGEN Biotech Co., Ltd.) to quantify the relative expression of genes. Finally, quantitative real-time PCR was performed by QuantStudio1 Real-Time PCR System (A40425, Thermo Fisher Scientific). The relative mRNA levels of each gene of interest were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method. The primers were purchased from Tsingke Biotechnology Co., Ltd. (China), and the primer sequences are shown in Table I.

Specifically, the complete qRT-PCR cycling conditions are shown in Table II.

Table I. primer list of genes used for qRT-PCR

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>BAK1</i>	TCTGGCCCTACACGTCTACC	ACAAACTGGCCCAACAGAAC
<i>BCL2</i>	CTAAAACCCCTGCCACCTCAA	CTCAGTGCTGAGTCCATCCA
<i>BCL2L11</i>	CTGAGTGTGACCGAGAAG	GATTACCTTGTGGCTCTGT
<i>CASP3</i>	AAGCGAATCAATGGACTCT	TGTACCAGACCGAGATGT
<i>CASP7</i>	TTGTACCGTCCCTCTCAGC	GAACGCCCATACCTGTCACT
<i>CASP9</i>	CGAACTAACAGGCAAGCA	AATCCTCCAGAACCAATGTC
<i>CCND1</i>	CAATGACCCCGCACGATTTC	CATGGAGGGCGGATTGGAA
<i>CYCS</i>	GGTGATGTTGAGAAAGGCAA	GTTCTATTGGCGGCTGTGT
<i>GAPDH</i>	AAGGTGAAGGTCGGAGTCAA	GGAAGATGGTGATGGGATT

Table II. qRT-PCR cycling conditions

Steps	Temperature (°C)	Time (s)	Cycles
Hold stage	50	120	1
	95	600	
PCR stage	95	15	40 cycles
	60	60	
	95	15	
Melt curve stage	60	60	1
	95	15	

### Western blot analysis

T24 and EJ-1 cells were seeded in 6-well culture plates at a seeding density of  $2 \times 10^5$  cells/well, as mentioned in a previous study (29). After adherence to the well, T24 cells were treated with  $65.9 \mu\text{mol L}^{-1}$  MED for 48 h, and EJ-1 cells were treated with  $64.6 \mu\text{mol L}^{-1}$  MED for 48 h. Floating and adherent cells were all collected after 48 h of treatment, and proteins were collected by lysing the cells using Radio Immunoprecipitation Assay (RIPA) buffer (high) (R0010, Solarbio Science & Technology Co., Ltd.). Protein concentrations were detected using bicinchoninic acid (BCA) protein concentration assay kit (P0010, Beyotime Biotechnology Co., Ltd., China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and after completion, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (FFP32, Beyotime Biotechnology Co., Ltd.). PVDF membranes were subsequently incubated in 7 % nonfat milk for 1 h at room temperature and then incubated with primary antibodies against corresponding proteins overnight. After washing three times in  $1 \times$  TBST buffer, the secondary antibodies were added and incubated at room temperature for 1 h. The membranes were washed in  $1 \times$  TBST buffer three times for about half an hour each time. Finally, the proteins were detected by the ECL kit (PE0010, Solarbio, China) and pictures were obtained by using the automatic exposure process of the Tanon 5200 series automatic chemiluminescence/fluorescence image analysis system (Tanon, China). The primary antibodies were as follows: anti- $\beta$ -actin (3700T, 1:5000), anti-Bcl2-L-11 (2933S, 1:1000), anti-BAK1 (12105S, 1:1000) and anti-Caspase-3 (9662S, 1:1000), all from Cell Signaling Technology (USA). The secondary antibodies were as follows: goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP) (LK2003, 1:3000) and goat anti-rabbit IgG (H+L)-horseradish peroxidase (HRP) (LK2001, 1:3000), both from Tianjin Sungene Biotech Co., Ltd. (China).

Finally, the pictures of quantification analysis methods were developed using the National Institutes of Health (NIH) ImageJ software.

### Animal experiments

According to the previous study (30), BALB/c nude mice (six weeks old) were purchased from SiPeiFu Life Technology Co., Ltd. (Beijing, China). In order to establish xenograft mouse models, 20 female BALB/c nude mice (six weeks old) were divided into the T24 group and the EJ-1 group. Each group had 10 mice and was injected with  $6 \times 10^6$  T24 or  $6 \times 10^6$  EJ-1 cells subcutaneously into the back of mice. When tumors reached a diameter of 3 to 5 mm, mice were randomly divided into two subgroups ( $n = 5$ ) and administered intraperitoneally  $10 \text{ mg kg}^{-1}$

MED or PBS every two days for 30 days. During the treatment, tumor sizes were measured by a vernier caliper every two days and were calculated by the formula: volume = (length × width<sup>2</sup>)/2. Finally, the xenograft-bearing mice were euthanized when the volume of tumors was 1000 mm<sup>3</sup> and the mass of tumors was detected by scale. All animal experimental protocols were reviewed and approved by the Animal Care Review Committee of the China-Japan Friendship Hospital (2021-42-K26). The mice were maintained under standard conditions according to the institutional guidelines for animal care.

### *Statistical analysis*

Statistical analysis was conducted by using GraphPad Prism version 5.0 software for windows (GraphPad Software, USA) (RRID: SCR\_002798). The differences between the two groups were analyzed by the Student *t*-test. Cell migration and WB pictures of quantification analysis methods were developed using ImageJ software version 1.8.0 software (25) (National Institutes of Health, USA) (RRID: SCR\_003070). *p* < 0.05 was used as the criterion for statistical significance.

## RESULTS AND DISCUSSION

### *Medicarpin inhibits the proliferation of bladder cancer cells in vitro*

T24 and EJ-1 cells were derived from grade 3 BC tumor and they are representatives of urinary bladder transitional cells. They were selected in this study to evaluate the effect of MED on BC cells. Firstly, T24 and EJ-1 cells were treated with different concentrations of MED and cultured for 24 and 48 h, respectively. The MED concentration gradient ranges from 0 to 200.0 μmol L<sup>-1</sup>. Cell viability was analyzed by cell counting kit-8 (CCK-8). The results demonstrated that there was an inhibitory effect of MED on BC cells in a dose-dependent manner, with cell viability of EJ-1 and T24 cells decreasing with increasing concentrations of MED, and the inhibitory effect of MED treatment on BC cells at 48 h was stronger than that of 24 h. The half maximal inhibitory concentration (*IC*<sub>50</sub>) values were 80.3 and 65.9 μmol L<sup>-1</sup> for T24 cells treated with MED for 24 and 48 h, respectively, while EJ-1 cells treated with MED for 24 and 48 h were 79.1 and 64.6 μmol L<sup>-1</sup>, respectively (Fig. 1a,b). Meanwhile, we checked the cytotoxicity of MED against SV-HUC-1, a normal immortalized human bladder epithelial cell line, and it is obvious from the results, SV-HUC-1 is insensitive to MED (Fig. 1a,b). The results implied that MED not only effectively inhibited the proliferation of BC cells *in vitro*, but also possessed lower side toxicity against normal bladder epithelial cells.

### *Medicarpin induces bladder cancer cells apoptosis and arrested bladder cancer cells at the G1 phase in vitro*

To further understand the specific mechanism underlying the inhibitory effect of MED on BC cell lines, we examined the apoptosis of T24 and EJ-1 cells treated with MED for 48 h, using flow cytometry. It was found that the results of these two cell lines were basically consistent, MED could significantly increase the apoptosis of both cell lines from less than 5 % to around 20 % (Fig. 2a,b). In addition, the cell cycle of T24 and EJ-1 cells after 48 h of MED treatment showed an increase in the G1 phase, a decrease in the S phase, and no change in the G2/M phase (Fig. 1c,d). These results indicated that MED significantly

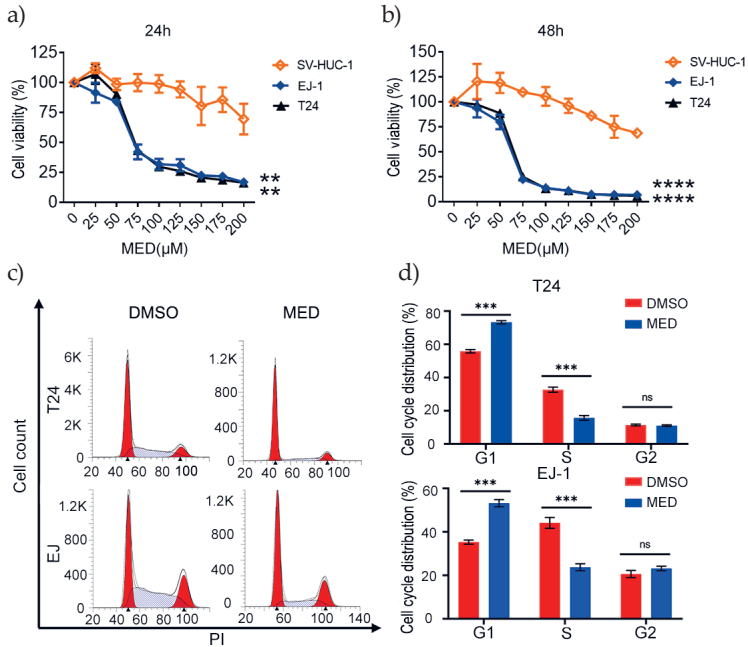


Fig. 1. Medicarpin inhibits proliferation and arrests the cell cycle of bladder cancer cells *in vitro*. a) and b) CCK8 assay was used to detect the chemo-sensitivity of T24, EJ-1 and SV-HUC-1 cells to MED treatment. The survival inhibition effect of MED demonstrated a dose Student *t*-test ( $n = 3$ , \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ ); c) and d) effects of MED on cell cycle of bladder cancer T24 and EJ-1 cell lines by cell cycle test, Student *t*-test. Data is represented as mean  $\pm$  SD of three independent experiments ( $n = 3$ , ns – no significance, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

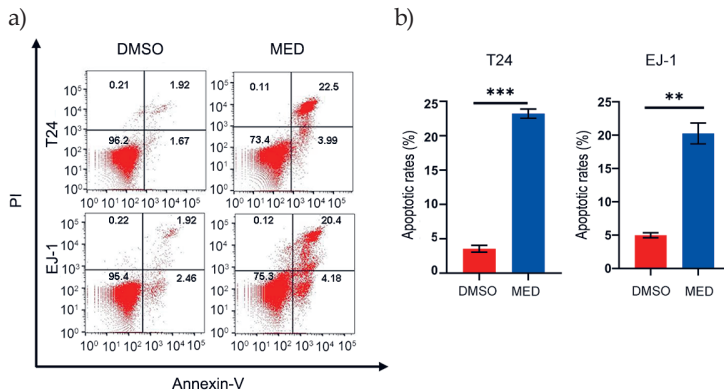


Fig. 2. Medicarpin markedly increases apoptosis in bladder cancer T24 and EJ-1 cells. a) T24 cells were treated with  $65.9 \mu\text{mol L}^{-1}$  MED for 48 h, and EJ-1 cells were treated with  $64.6 \mu\text{mol L}^{-1}$  MED for 48 h. Annexin V-FITC/PI dual staining apoptotic analysis was performed; b) percentage of Annexin V/PI double positive plus Annexin V positive calculated from three independent experiments for two groups ( $n = 3$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

inhibited the proliferation of BC cells by increasing the apoptosis of BC cells and arresting the BC cell cycle in the G1 phase.

### *Medicarpin inhibits the migration of bladder cancer cells in vitro*

We used MED to treat T24 and EJ-1 cells, and the migration rates of BC cells were examined after 24 and 48 h, respectively. The wound area was used to calculate the wound healing rate and finally evaluate the effect of MED on the motility of BC cells. The results showed that the migration of T24 and EJ-1 cells was reduced after treatment with MED for 24 and 48 h (Fig. 3a,b).

### *Medicarpin inhibits bladder cancer cells by upregulating the mitochondrial apoptotic pathway*

To explore the specific mechanism underlying the anti-BC effect of MED, we detected the expression of genes related to cell cycle and apoptosis by qRT-PCR. Compared with the DMSO group, the expression of the intrinsic apoptotic pathway-related genes, such as

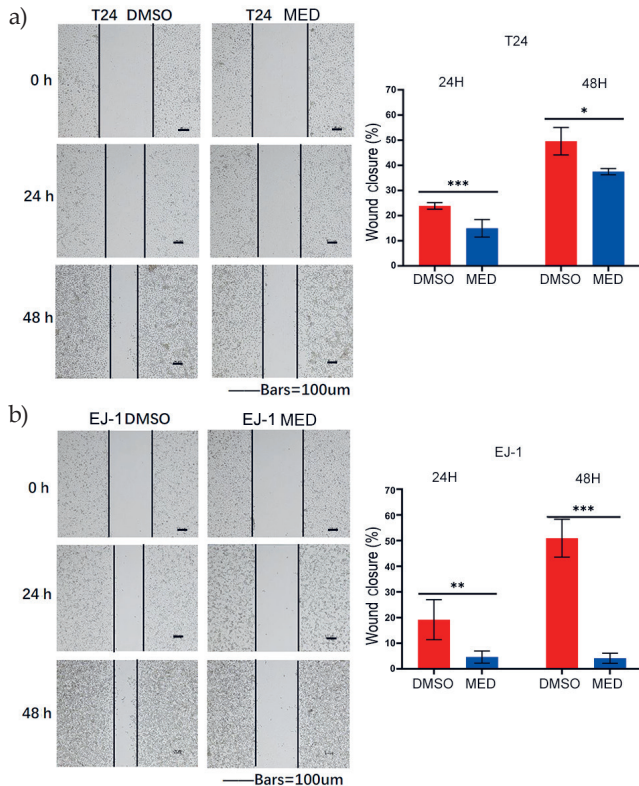


Fig. 3. Cell wound healing assay was performed to measure the migration ability of T24 and EJ-1 cells after medicarpin treatment. a) and b) MED inhibited T24 and EJ-1 cell migration as analyzed by scratch wound assay. The scale bar represents 100  $\mu$ m. Data are represented as mean  $\pm$  SD of three independent experiments ( $n = 5$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).



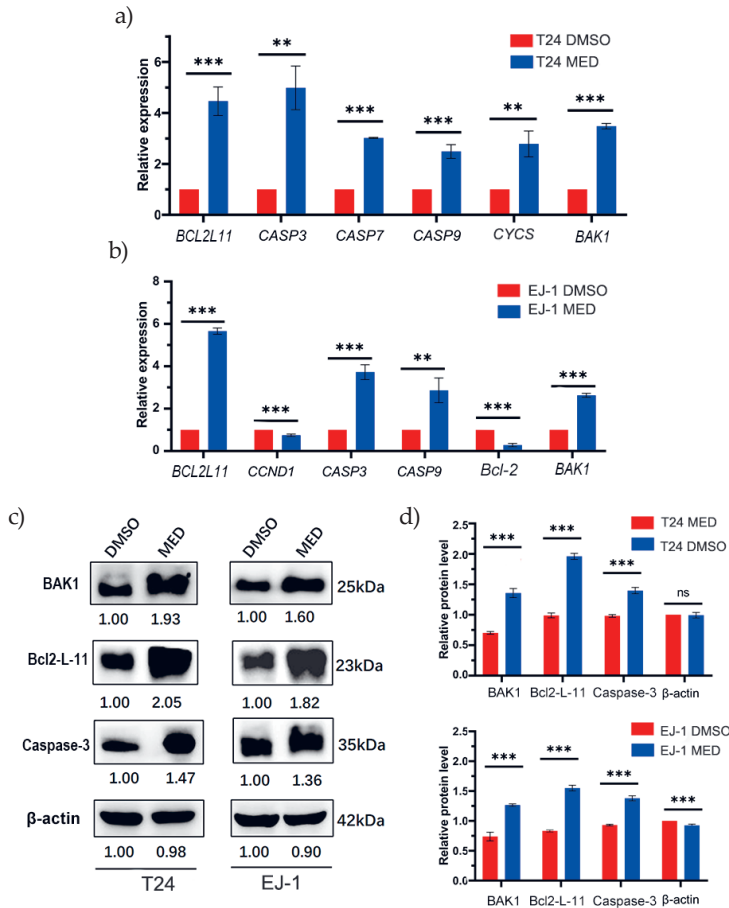


Fig. 4. Effect of medicarpin on apoptosis and cell cycle-related genes in bladder cancer T24 and EJ-1 cell lines. a) and b) Analysis of mRNA levels of apoptosis and cell cycle-related genes using qRT-PCR. GAPDH was elected as an internal control; c) and d) Western blot analysis of apoptosis-related proteins.  $\beta$ -actin was chosen as the loading control. Data are represented as mean  $\pm$  SD of three independent experiments ( $n = 3$ , ns – no significance, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

*BCL2L11*, *BAK1*, *CASP3*, *CASP7*, and *CASP9*, were consistently upregulated in both T24 and EJ-1 cells (Fig. 4a,b). Therefore, MED induces apoptosis of BC cells by upregulating the intrinsic apoptotic pathway. Thereafter, we treated T24 and EJ-1 cells with MED for 48 h and measured the expression levels of *BAK1*, *Bcl2-L-11* and caspase-3 proteins. The expression of *BAK1*, *Bcl2-L-11* and caspase-3 were significantly upregulated after MED treatment, which is consistent with the results of qRT-PCR (Fig. 4c,d).

#### Medicarpin inhibits tumor growth in vivo

Finally, we investigated whether MED could inhibit BC cell growth *in vivo* to achieve antitumor effect. Xenograft cancer models in female mice were employed. Compared with

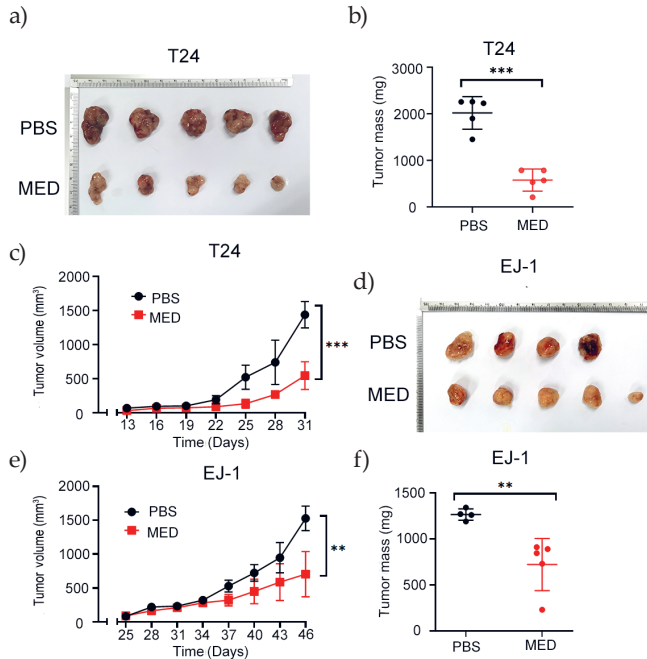


Fig. 5. The effect of Medicarpin on T24 and EJ-1 cells growth and tumorigenicity *in vivo*. a)–c) In T24 cells, tumors collected from mice are shown. Tumor mass of the PBS and MED treatment groups was measured and analyzed, and the average tumor mass in each group was counted at the end of the Experiment Tumor mass. Tumor volume curves were measured e. (d–f) As mentioned above, the tumors of EJ-1 cells that were collected from mice are treated similarly. Data are represented as the mean of 5 determinations per condition repeated 5 times. Results are shown as mean  $\pm$  SD ( $n = 5$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared with the PBS group).

the PBS group, MED significantly inhibited the growth rate of tumor and reduced the tumor mass of T24 cells *in vivo* (Fig. 5a,b,c). Similarly, MED also exhibited the same anti-BC effect for EJ-1 cells *in vivo* (Fig. 5d,e,f). Taken together, MED could significantly inhibit the tumor growth rate and reduce tumor mass of BC cells *in vivo*.

BC is one of the most common tumors in the urinary system, and the leading risk factor of BC is tobacco smoking (26). Although the pathological type of 75 % of patients is NMIBC that belongs to lower malignancy, and the 5-year survival rate after surgery is high, BC easily recurrence after surgery. Most of the recurrence cases come with a deepening of malignancy, and drug resistance, which are the main causes of metastasis and death in BC patients (27).

TCM offers many advantages such as convenience, cheapness, and low toxicity, while natural compounds extracted from TCMs, integrated with advanced modern pharmaceutical technology, have made progress in the treatment of hypertension, diabetes, hyperlipidemia as well as tumors (28–31). MED, an active substance, extracted from *Medicago sativa*, has been shown to be effective against bacteria and fungi as well as against tumors, particularly hematological tumors, where it can induce tumor cell apoptosis as well as reverse

chemoresistance (18, 21, 22). Nonetheless, whether it has an inhibitory effect on BC remains to explore, and the molecular mechanisms involved are still largely unknown.

In cancer cells, genes controlling cell division are altered, resulting in an unlimited proliferation of tumor cells. T24 and EJ-1 cells were derived from grade 3 BC tumor and they are representatives of urinary bladder transitional cells. These cell lines were selected in this study to evaluate the effect of MED on BC cells. Our results showed that MED significantly inhibited BC cell lines T24 and EJ-1. Specifically, MED could inhibit the proliferation of BC cells in a time and dose-dependent manner. The  $IC_{50}$  values for T24 and EJ-1 cells were 65.9 and 64.6  $\mu\text{mol L}^{-1}$ . However, the  $IC_{50}$  values of puerarin and chloroquine for BC cells were 100.0 and 83.2  $\mu\text{mol L}^{-1}$  (32, 33), respectively. These results indicated that BC cells were more sensitive to MED.

Dysregulation of cell cycle progression is also considered a common feature of cancer (34). In this study, MED remarkably induced cell cycle arrest in the G1 phase and the decrease of the S phase in T24 and EJ-1 cells. Similarly, artesunate, a derivative of artemisinin, could increase the proportion of the G1 phase and decrease the proportion of the S phase in BC cells (35). Moreover, MED significantly inhibited the migration of BC cells in a time-dependent manner.

Apoptosis refers to spontaneous programmed cell death, and this process involves the sequential activation, expression, regulation, and activation/inhibition of a series of genes within the cell. It is an important link in the normal human body to maintain the stability of the cellular microenvironment. Resistance to the apoptosis mechanism is considered a characteristic of cancer, which may lead to uncontrolled cell proliferation (36). Therefore, inducing apoptosis in cancer cells is an effective way to treat cancer (37, 38). As detected by flow cytometry, the percentage of apoptotic cells increased from 3.6 % to 26.5 % ( $p < 0.001$ ) in T24 cells treated with MED for 48 h, and from 4.4 % to 24.6 % ( $p < 0.01$ ) in EJ-1 cell treated with MED, respectively. Our study highlighted that MED could obviously induce the apoptosis of BC cells.

To further explore the specific mechanism of MED-induced BC cell apoptosis, we detected the changed mRNA expression of apoptosis-related genes in T24 and EJ-1 cells

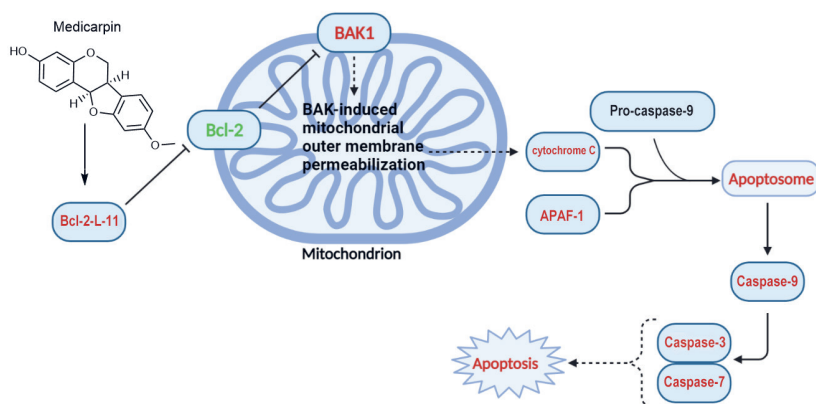


Fig. 6. The anti-bladder cancer mechanism of medicarpin. Red represents upregulation and green represents downregulation.

for 48 h of MED treatment. Our experimental results revealed that genes such as *BCL2L11*, *BAK1*, *CASP3*, *CASP7* and *CASP9* were consistently upregulated in both T24 and EJ-1 cells. Consistent with qRT-PCR, WB analysis indicated that Bcl2-L-11, caspase-3 and BAK1 were upregulated after the treatment of MED. It is well known that in the intrinsic apoptotic pathway, elevated Bcl2-L-11 can inhibit Bcl-2 and then lead to an increased BAK1/Bcl-2 ratio, which relieves the inhibition of BAK1 by Bcl-2. Subsequently, BAK1 leads to loss of mitochondrial membrane potential, decreased mitochondrial permeability and subsequent release of cytochrome C in the cytosol, which induced the formation of apoptotic bodies and activation of caspase-9. Activation of caspase-9 activates caspase-3 through proteolytic cleavage, and caspase-3 can cleave cellular substrates such as RARP and promotes cell apoptosis (Fig. 6). Therefore, MED may induce BC cell apoptosis by activating the intrinsic apoptotic pathway. Our *in vivo* experimental results also showed that compared with the PBS group, the tumor growth rate of mice in the MED-treated group was found significantly decreased, and the final tumor mass was also significantly lower than that in the PBS control group.

In summary, our current study found that MED induces cell apoptosis, inhibits cell proliferation and migration, and attenuates the tumorigenic ability and growth rate of cells *in vivo*. Meanwhile, we also found that MED upregulated the intrinsic apoptotic pathway by upregulating the expression of Bcl2-L-11, BAK1, caspase-3, caspase-9 and so on. Therefore, MED is a promising candidate for BC therapy.

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*Authors' contributions.* – ZY and YC designed the experiments. YC and LY performed the experiments, analyzed the data, and wrote the manuscript. WX, JG, YS, SC, YL, MH and RG prepared the samples and performed the experiments. QW, JZ, JL, RC, QZ and JW revised the manuscript. HW and ZY initiated the study and organized, designed, and revised the manuscript.

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