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Inhibitory effects of 2-methoxyestradiol on cell growth and invasion in human bladder cancer T-24 cells

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Received September 19, 2016, accepted October 21, 2016

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Pharmazie 72: 87–90 (2017) *doi: 10.1691/ph.2017.6839*

Recently, 2-methoxyestradiol (2-ME) has been considered to be a potential anticancer agent but has not been investigated in bladder cancer. This study was conducted to clarify the role of 2-ME in bladder cancer cells. The bladder cancer cell line T-24 was treated with 2 μm 2-ME for 2 d. The T-24 cell viability, colony formation, invasion and apoptosis were observed in 2-ME-treated and control cells. The expression of hypoxia-inducible factor 1 alpha (HIF-1α) was detected using reverse transcription-polymerase chain reaction (RT-PCR). Then western blotting assay was applied to assess expressions of HIF-1α and apoptosis factors caspase-3 and Bcl-x proteins. The mRNA and protein expressions of HIF-1α in 2-ME-treated T-24 cells were remarkably lower than that of the control cells ($P < 0.05$). Treatment of 2-ME could significantly inhibit T-24 the cell viability, colony formation, invasion, and promote apoptosis (all $P < 0.05$). In addition, the protein expression of Caspase-3 was higher and that of Bcl-x protein was lower after administration of 2-ME compared to control (both $P < 0.05$). Collectively, we characterized the efficacy of 2-ME on bladder cancer T-24 cells as being mediated by inhibition of cell viability, colony fomation, invasion and promoting cell apoptosis, which may be achieved by suppressing HIF-1α levels. This study suggests 2-ME as a potential drug for bladder cancer therapy.

1. Introduction

Bladder cancer is the second most common malignancy of the genitourinary tract with approximately 330,000 new cases each year (Witjes et al. 2014). Bladder cancer is a devastating disease causing more than 30,000 deaths annually (Chiyomaru et al. 2010). So far, the established therapy for invasive bladder cancer is surgery, whereas conventional systemic chemotherapy and radiotherapy have remained the feasible approaches against tumor metastasis for the past 30 years (Inoue et al. 2000). Chemotherapy regimen options for metastatic carcinoma from the bladder mainly contain gemcitabine plus cisplatin (GC) and methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) (Kim 2012; Lombard and Mudryj 2015). Most patients succumb to bladder cancer on account of the development of drug resistance (Wen et al. 2015). Novel drugs are desired for them since chemotherapy is related to the high local recurrence rate and low living quality.

Recently, 2-methoxyestradiol (2-ME) has been considered to be a potential anticancer agent and is under investigation in clinical trials (Lakhani et al. 2003). Substantial studies have implicated that 2-ME possessed potent anti-proliferative, antiangiogenic and pro-apoptotic effects on breast cancer (BC), hepatocellular carcinoma (HCC) and pancreatic cancer (Cho et al. 2011; El Naga et al. 2009; Ma et al. 2014; Zhou and Du 2012). Accounting for the anticancer features of 2-ME, primary mechanisms lie in hypoxia-inducible factor (HIF)-1 dysregulation and microtubule disruption (Mabjeesh et al. 2003). HIF-1 alpha (HIF-1α), a member of the helix-loop-helix family, has been a major stress factor under hypoxic conditions, which is involved in angiogenesis, tumor growth and metastasis (Batmunkh et al. 2010; Huang et al. 2005). It has been reported that the high expression of HIF-1α were closely related to the promotion of cell proliferation and prognosis in bladder cancer (Ioachim et al. 2006; Sun et al. 2016; Zhang et al. 2012). However, as an acknowledged inhibitor of HIF-1α, effects of 2-ME on bladder cancer have been elusive.

This study was conducted to clarify the role of 2-ME in bladder cancer cells, which is expected to contribute to the therapy of bladder cancer. We detected whether 2-ME played a role in the cell viability, colony formation, invasion and apoptosis of bladder cancer T-24 cells, as well as its underlying mechanism.

2. Investigations and results

2.1. Effects of 2-ME on HIF-1α levels in T-24 cells

The past decades have seen 2-ME as an acknowledged inhibitor of HIF-1α. We examined the effects of 2-ME on expression of HIF-1α mRNA and protein in T-24 bladder cancer cells. The RT-PCR data showed that the expression of HIF-1α mRNA in 2-ME-treated T-24 cells was remarkably lower than in control cells ($P < 0.05$, Fig. 1A). Further, the western blotting assay showed that the expression of HIF-1α protein in T-24 cells was significantly reduced by 2-ME ($P < 0.05$, Fig. 1B). Thereby, 2-ME could reduce the expressions of HIF-1α mRNA and protein in T-24 cells.

2.2. Effects of 2-ME on T-24 cell viability, colony formation and invasion

The impact of 2-ME on cell viability was analyzed on days 0, 3 and 7. As shown in Fig. 2A, anti-viability effect of 2-ME on T-24 cells began after being cultured for 3 d but without significance ($P > 0.05$); the inhibitory impact was strikingly intensified on day 7 in a time-dependent manner ($P < 0.05$).

The ability of T-24 cells to form colonies in the presence of 2-ME was observed. Our data claimed that 2-ME effectively inhibited colony formation in T-24 bladder cancer cells compared to the control cells (Fig. 2B), suggesting that 2-ME might suppress T-24 cell reproduction.

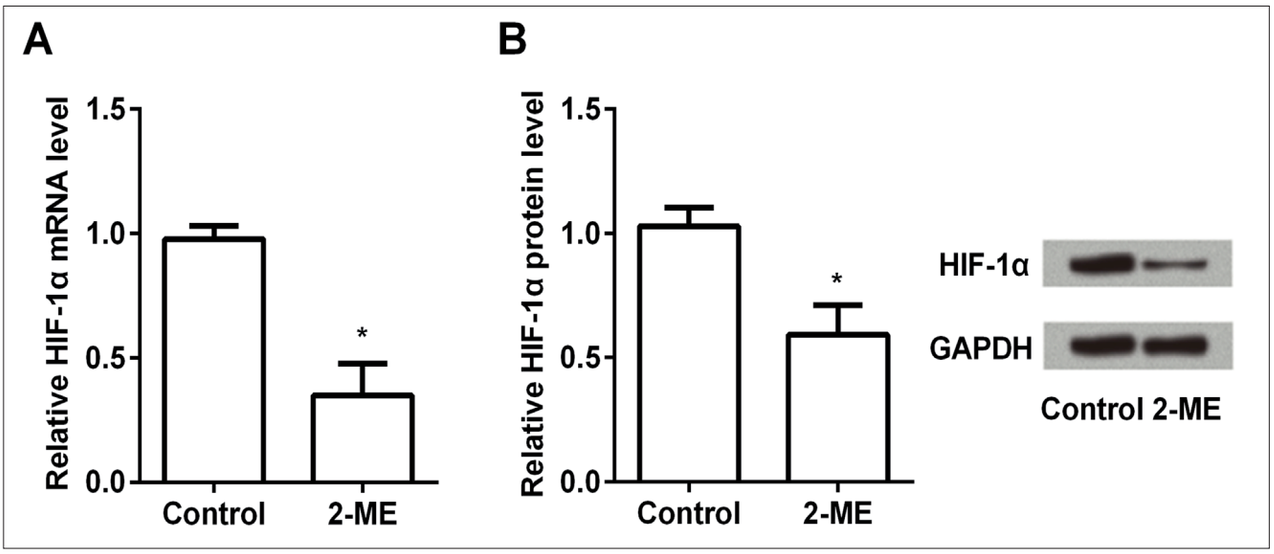

Fig. 1: Effects of 2-ME on HIF-1α mRNA and protein in T-24 cells. A. Expressions of HIF-1α mRNA were reduced by 2-ME treatment in T-24 cells; B. Expressions of HIF-1α protein were reduced by 2-ME treatment in T-24 cells. HIF-1α, hypoxia-inducible factor-1 alpha; 2-ME, 2-methoxyestradiol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.*, $P < 0.05$.

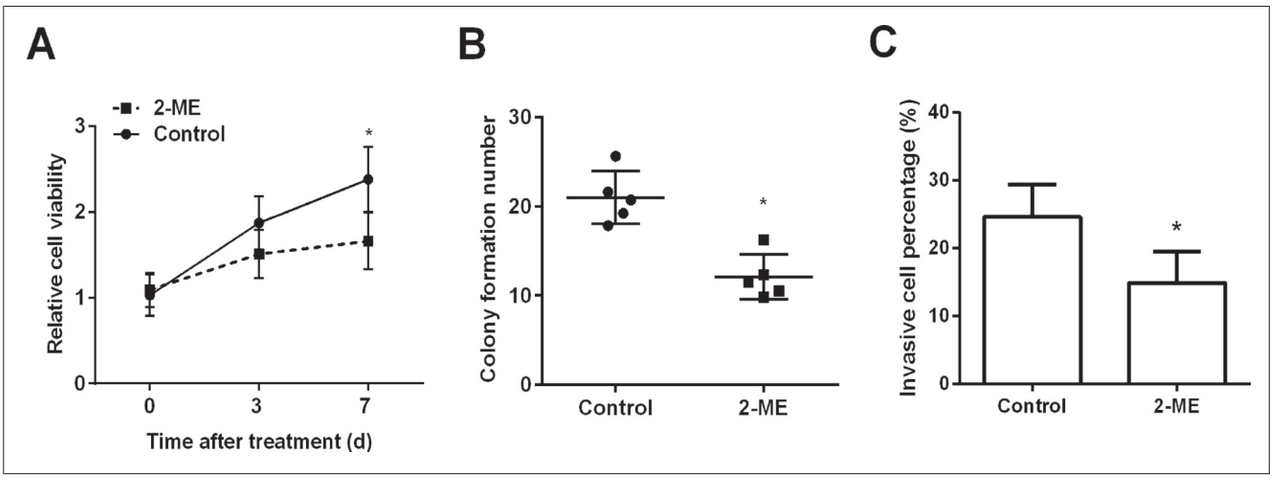

Fig. 2: Effects of 2-ME on T-24 cell viability, colony formation and invasion. The 2-ME suppressed T-24 cell viability; The 2-ME inhibited T-24 cell colony formation; The 2-ME suppressed T-24 cell invasion. 2-ME, 2-methoxyestradiol.*, $P < 0.05$.

We employed a transwell assay to evaluate cell invasion of bladder carcinoma cells. T-24 cells in control group passed better across the membrane and had more invasive capability *in vitro* ($P < 0.05$, Fig. 2C), indicating that 2-ME inhibited T-24 cell invasion.

2.3. Effects of 2-ME on T-24 cell apoptosis

Our data revealed that 2-ME was associated with cell viability of bladder carcinoma cells. We next investigated whether 2-ME treatment could also promote T-24 cell apoptosis. Flow cytometry was used to quantify the effects of 2-ME on cell apoptosis and the expressions of apoptosis key factors caspase-3 and Bcl-x proteins were determined by western blot. As shown in Fig. 3A, compared with the control, 2-ME treatment caused significant promotion of the percentage of T-24 cell apoptosis ($P < 0.05$). The expression of caspase-3 protein was higher and that of Bcl-x protein was lower by 2-ME compared to control (both $P < 0.05$, Fig. 3B and C). We therefore concluded that 2-ME potently promoted apoptosis in T-24 carcinoma cells.

3. Discussion

To our knowledge, this study provides the first insight into the efficacy of 2-ME in bladder cancer T-24 cells. With 2-ME treatment, expressions of HIF-1α mRNA and protein were reduced in T-24 cells, which indicates a protective mechanism for bladder cancer of 2-ME. T-24 cell viability, colony formation and invasion were statistically suppressed and cell apoptosis was promoted by administration of 2-ME. Taken together, our results indicate that 2-ME might possess a protective effect on bladder cancer by indicate that inhibing cell growth and invasion and promoting apoptosis.

Evidence points to a considerable role for 2-ME in anticancer therapy and its applications have focused on solid tumor cells and animal models. Schumacher et al. (1999) reported that 2-ME possessed antitumor activity in human pancreatic cancer cells by means of inhibiting cell growth and lung colonies *in vivo*. Additionally, 2-ME inflicted cell apoptosis and weakened the vascularization at multi-stages in the angiogenic cascade without clinical signs of toxicity. The effect of 2-ME on the suppression of cancer spread enhanced its therapeutic value for tumor treatment (Pribluda et al. 2000). In clinical trials, administration of 2-ME reduced ostealgia

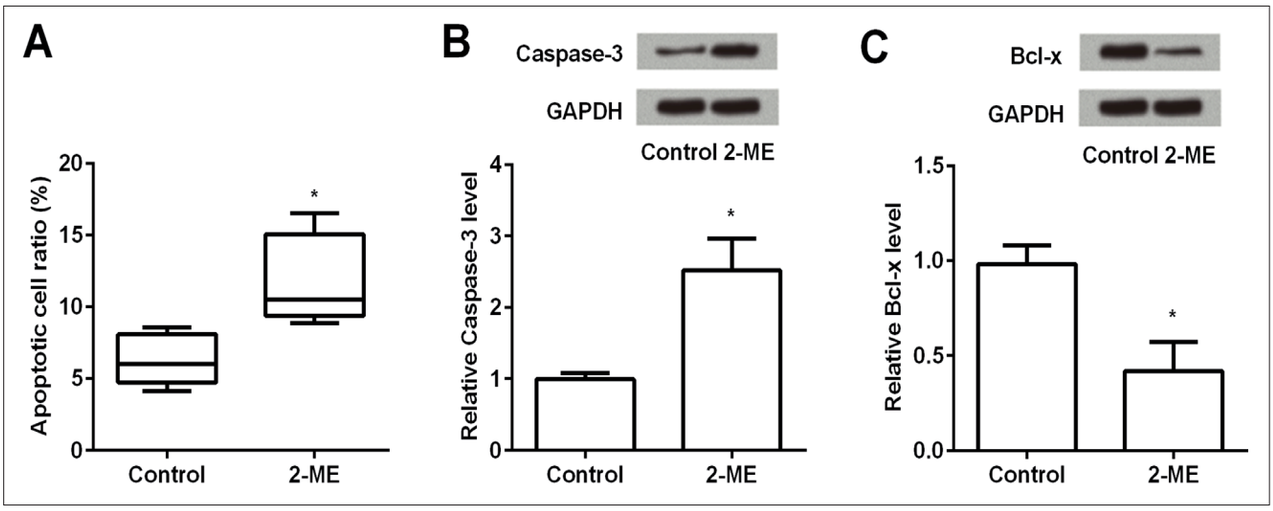

Fig. 3: Effects of 2-ME on T-24 cell apoptosis. A. The flow cytometry data revealed that 2-ME promoted T-24 cell apoptosis; B. The expressions of apoptosis key factors Caspase-3 protein were increased by 2-ME treatment; C. The expressions of anti-apoptotic Bcl-x protein were decreased by 2-ME treatment. 2-ME, 2-methoxyestradiol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *, $P < 0.05$.

and analgesic intake in breast cancer patients, and dropped doses of specific antigens in prostate cancer patients (Lakhani et al. 2003). Nevertheless, to date 2-ME, has not been investigated as a treatment agent for bladder cancer. In the current study, we confirmed the role of 2-ME in bladder cancer T-24 cell growth, invasion and apoptosis, predicting the potential anticancer role in bladder cancer.

Although 2-ME is a promising agent for cancer therapy, the mechanism of action is still undefined. Mabjeesh et al. (2003) made a strong case for 2-ME acting as an inhibitor of HIF-1α. The preceding report also claimed that 2-ME dropped off the nuclear binding activity of HIF-1α in head and neck squamous cell carcinoma (HNSCC) (Ricker et al. 2004). Moreover, Becker et al. (2008) revealed that 2-ME suppressed the growth of endometriosis lesions in a mouse model via inhibition of HIF-1α-inducible genes vascular endothelial growth factor (VEGF), phosphoglycerate kinase (PGK) and glucose transporter-1 (Glut-1). HIF-1α could serve as an indicator for predicting malignant behavior such as lymph node metastasis and venous invasion for gallbladder cancer (Batmunkh et al. 2010). HIF-1α is conductive to cancer progression by means of various mechanisms, covering improvement of angiogenesis, control of apoptotic cells, and regulation of metabolism by mediating glycolysis flux and oxidative phosphorylation (Hockel and Vaupel 2001; Ryan et al. 2000). Further, elevated expressions of HIF-1α protein are closely related to a poor prognosis in prostate cancer and HNSCC (Beasley et al. 2002; Koukourakis et al. 2002; Zhong et al. 2000). Our study showed reduced levels of HIF-1α in bladder cancer cell line T-24 after treatment of 2-ME. Accordingly, 2-ME-mediated T-24 cell growth and invasion may be associated with the suppression of HIF-1α levels, which is expected to improve prognosis of bladder cancer. However, more work is required to address the mechanism in detail.

In the current study, 2-ME potently inhibited bladder T-24 oncocytes growth by promoting apoptosis and suppressing cell viability. One tentative explanation might be that HIF-1α inhibited c-Myc function, which contributed to both p21-mediated cell cycle arrest and apoptosis in lung and kidney carcinoma (Gordan et al. 2007; Savai et al. 2005). The dysregulated levels of apoptotic factors caspase-3 and Bcl-x protein by 2-ME were observed in this study. The finding appears to be supported by the down-regulation of HIF-1α expressions with malignancy suppressor capability, which could induct the pro-apoptotic genes (Hammond and Giaccia 2006; Rezvani et al. 2011; Sowter et al. 2001). Besides, further detections on vast animal and tumor heterotransplantation models are still needed to determine specific prognosis and 2-ME optimal dose in bladder cancer patients, which will provide the foundation and clues for clinical drug application.

Collectively, we characterized the efficacy of 2-ME on bladder cancer T-24 cells via inhibiting cell viability, colony fomation, invasion and promoting cell apoptosis, which may be achieved by suppressing HIF-1α levels. This study suggests 2-ME as a potential drug for bladder cancer therapy.

4. Experimental

4.1. Cell culture and 2-ME treatment

The human bladder carcinoma cell line T-24 was obtained from the Shanghai Cell Institute Country Cell Bank (Shanghai, China). T-24 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, USA), 100 μg/mL streptomycin, 100 IU/mL penicillin (both from Gibco-Invitrogen Corp., Paisley, UK) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. T-24 cells (2×10^4 cells/well) were seeded in 96-well plates overnight. Then T-24 cells were treated with 2 μm 2-ME (EntreMed Inc., Rockville, USA) or vehicle (0.05% ethanol as control) on the next day for additional 2 d.

4.2. Quantitation of cell viability

The T-24 cell viability was quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) colorimetric assay (Xu et al. 2015). After treatment for 2 d, the control and 2-ME-treated T-24 cells were cultured in DMEM (with 10% FBS) containing 0.5 mg/mL MTT (Sigma, USA). Furthermore, 100 μL dimethylsulfoxide (DMSO; Lonza, USA) was added to dissolve the blue formazan (Sigma, USA) product for 1 h. The percentage of living T-24 cells was determined on 3 and 7 d by absorbance at a wavelength of 490 nm using an automated plate reader (Bio-Tek, Winooski, USA).

4.3. Clonogenicity analysis

T-24 cells with or without 2-ME treatment were liquated using trypsin-Ethylene Diamine Tetraacetic Acid (EDTA; Gibco Lab., Grand Island, New York, USA) solution and then cultured at a density of 500 cells/well without 2-ME-containg medium for 14 d, respectively. Sequentially, T-24 cells were fixed in methanol for 20 min and stained with 0.1% crystal violet (Sigma, USA) for 30 min. The surviving colonies were manually counted under a Leica microscope (DMRBE, Wetzlar, Germany).

4.4. Cell invasion assay

For the effects of 2-ME on T-24 cell invasion ability, transwell assay was performed with 8 μm pore (Costar, Cambridge, USA). Briefly, T-24 control and 2-ME-treated cells were trypsinized (0.25% trypsin; Sigma, USA) and suspended in the culture medium with 10% FBS (1×10^6 cells/mL), respectively. After that, the T-24 cell suspension was transferred to the top surface of the Matrigel invasion chamber with

24-well (0.3 mL/every chamber), while 0.8 mL of DMEM (with 10% FBS) was added to the bottom compartment. Then the T-24 cells were cultured in 5% CO_2 at 37 °C for 1 d. Finally, the number of T-24 cells on the lower layer of the chamber was counted under a light microscope (Pharmacia Biotech, USA).

4.5. Apoptosis assay

Apoptotic T-24 cells were detected by flow cytometry with Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (Biouniquer Technology, Nanjing, China). After incubation for 3 d, 2-ME-treated T-24 and control cells were seeded in 6-well culture plate respectively and washed twice with ice-cold phosphate buffer saline (PBS). In brief, two group cells were co-incubated with serum-free culture medium containing 10 μM dichlorofluorescein diacetate (DCFH-DA; Jiancheng, Nanjing, China) for 15 min at room temperature in the dark. Subsequently, samples were collected by a trypsin digestion approach and centrifuged. Then cell pellets were resuspended in 100 μL annexin-binding buffer and apoptotic T-24 cells measured with flow cytometer according to the manufacturer's protocol.

4.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total HIF-1α RNA of T-24 cells treated with or without 2-ME was isolated respectively using RNA-fast200 Trizol RNA Extract Kit (Fastgene, Shanghai, China). RT-PCR was carried out with the following manufacturer's instructions of NucleoSpin RNA II kit (Macherey Nagel, Dueren, Germany). For the reverse transcription, Multiscribe RT kit (Applied Biosystems, USA) was employed. The HIF-1α primer sequences were forward primer 5'TTCACCTGAGCCTAATAGTCC'3 and reverse primer 5'CAAGTCTAAATCTGTGTCCTG'3 (Qiu et al. 2007). PCR amplification was performed as follows: 25 °C for 10 min, 55 °C for 1 h and 5 min at 94 °C.

4.7. Western blot analysis

The protein used for western blotting was extracted from the control or 2-ME-treated T-24 cells using ice-cold RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitors (Applygen Technologies Inc., Beijing, China). Proteins concentration was quantified by Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Appleton, USA). A Bio-Rad Bis-Tris Gel system was applied to construct the western blot system, in which primary antibodies HIF-1α (ab31358), Caspase-3 (ab2171), Bcl-x (ab98143) and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Abcam (Cambridge, United Kingdom). Each membrane was incubated with the indicated primary antibodies at 4 °C overnight and then secondary antibodies were marked by horseradish peroxidase for 2 h at 37 °C. Further, the polyvinylidenefluoride (PVDF) membrane carrying blots and antibodies was transferred into the Bio-Rad ChemiDoc™ XRS system. The bands were photographed with Gel Doc 2000 (Bio-Rad, Hercules, USA).

4.8. Statistical analysis

All experiments were carried out at least three times, and the results were presented as mean ± standard deviation (SD). Values were performed by one-way analysis of variance (ANOVA) with SPSS 19.0 software (SPSS, Chicago, USA). A statistical significance was defined when $P < 0.05$.

Conflict of interest: The authors report no conflicts of interest.

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