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Original Article
Silodosin inhibits the growth of bladder cancer cells and enhances the cytotoxic activity of cisplatin via ELK1 inactivation

Takashi Kawahara1,2, Hiroki Ide1, Eiji Kashiwagi1, John D Patterson1, Satoshi Inoue1, Hasanain Khaleel Shawreef1,3, Ali Kadhim Aljarah1,4, Yichun Zheng1, Alexander S Baras1, Hiroshi Miyamoto1

1Departments of Pathology and Urology, Johns Hopkins University School of Medicine, Baltimore, MD, USA; 2Department of Urology, Yokohama City University Graduate School of Medicine, Yokohama, Japan; 3Department of Biology, University of Babylon College of Science for Women, Babylon, Iraq; 4Department of Biology, University of Baghdad College of Science, Baghdad, Iraq

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Abstract: Silodosin, a selective α1A-adrenergic blocker prescribed for the symptomatic treatment of benign prostatic hyperplasia, was previously shown to decrease the expression of ELK1, a c-fos proto-oncogene regulator and a well-described downstream target of the PKC/Raf-1/ERK pathway, in human prostate smooth muscle cells. PKC/Raf-1/ERK activation has also been implicated in drug resistance. In the current study, we assessed the effects of silodosin on ELK1 expression/activity in bladder cancer cells as well as on their proliferation in the presence or absence of chemotherapeutic drugs, including cisplatin and gemcitabine. In bladder cancer cell lines, silodosin reduced the expression of ELK1 (mRNA/protein) and its downstream target, c-fos gene, as well as the transcriptional activity of ELK1. While silodosin alone (up to 10 µM) insignificantly affected the growth of bladder cancer cells cultured in androgen depleted conditions or those expressing ELK1-short hairpin RNA, it considerably inhibited the viability of androgen receptor (AR)-positive/ELK1-positive cells in the presence of androgens. Silodosin also inhibited the migration of ELK1-positive cells with or without a functional AR, but not that of ELK1 knockdown cells. Interestingly, silodosin treatment or ELK1 silencing resulted in increases in drug sensitivity to cisplatin, but not to gemcitabine, even in AR-negative cells or AR-positive cells cultured in an androgen-depleted condition. In addition, silodosin decreased the expression of NF-κB, a key regulator of chemoresistance, and its transcriptional activity. Moreover, immunohistochemistry in bladder cancer specimens from patients who received neoadjuvant chemotherapy revealed that phospho-ELK1 positivity strongly correlated with chemoresistance. Silodosin was thus found to not only inhibit cell viability and migration but also enhance the cytotoxic activity of cisplatin in bladder cancer lines via inactivating ELK1. Our results suggest that combined treatment with silodosin is useful for overcoming chemoresistance in patients with ELK1-positive urothelial carcinoma receiving cisplatin.

Keywords: α1A-adrenergic blocker, bladder cancer, cisplatin, drug sensitivity, ELK1, silodosin

Introduction

Cisplatin (CDDP)-based systemic chemotherapy [e.g., “GC” = gemcitabine (GEM) + CDDP] constitutes the major therapeutic option for muscle-invasive bladder cancer in both neoadjuvant and adjuvant settings as well as for metastatic disease [1, 2]. Indeed, it often leads to an initial therapeutic success. Nonetheless, intrinsic or acquired resistance against CDDP that multiple molecular mechanisms are likely to underlie remains a major clinical problem. It is therefore of great clinical interest to develop strategies for chemosensitization.

As a member of the ETS-domain family of transcription factors, ELK1 has been known to involve cell proliferation, cell cycle control, and apoptosis via regulating the expression of a variety of genes, including c-fos proto-oncogene [3-5]. In bladder cancer cell lines, the expression of ELK1 [6] and its phosphorylated form (p-ELK1) [7] has been detected. We have recently demonstrated that p-ELK1 expression is elevated in bladder tumors, compared with non-neoplastic urothelial tissues, and that patients with p-ELK1-positive bladder cancer have a significantly higher risk of tumor progression after radical cystectomy [8]. We have
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also found that ELK1 signals promote bladder cancer cell proliferation and migration/invasion only for the former of which an activated androgen receptor (AR) is required [8]. Of note is that ELK1 is a well-described downstream target of the PKC/Raf-1/ERK pathway whose activation has been implicated in drug resistance, especially to chemotherapeutic agents [9, 10].

Silodosin is a selective α1A-adrenergic receptor (α1A-AR) antagonist approved by the United States Food and Drug Administration in 2008 for the symptomatic treatment of benign prostatic hyperplasia. Interestingly, silodosin has been shown to reduce the expression of ELK1 in human prostate smooth muscle cells [11] as well as that of c-fos in the rat spinal cord [12]. In the current study, we determined whether silodosin could inactivate ELK1 in bladder cancer cells and thereby inhibited their growth. We also assessed the effects of silodosin on the cytotoxic activity of chemotherapeutic drugs, including CDDP and GEM, in bladder cancer cells.

Materials and methods

Cell culture and chemicals

Human urothelial carcinoma cell lines (TCCSUP, UMUC3, and 5637) were originally obtained from the American Type Culture Collection. 647V cell line was used in our previous studies [13-15]. All these lines were recently authenticated, using GenePrint 10 System (Promega), by the institutional core facility. In addition, UMUC3 sublines stably expressing a short hairpin RNA (shRNA) plasmid targeting human ELK1 (sc-35290-SH; Santa Cruz Biotechnology) or a non-silencing control shRNA plasmid (sc-108060; Santa Cruz Biotechnology) were established (Figure 1), as described previously [8]. These parental and stable cell lines were maintained in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 5-10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 units/mL) at 37°C in a humidified atmosphere of 5% CO₂. Cells were then cultured in phenol red-free medium supplemented with 5% normal FBS or 5% charcoal-stripped FBS at least 24 hours before experimental treatment. We obtained silodosin from Cayman Chemical; and CDDP and GEM from Sigma.

Reverse transcription (RT) and real-time polymerase chain reaction (PCR)

Total RNA (0.5 μg) isolated from cultured cells, using TRizol (Invitrogen), was reverse transcribed, using 1 μM oligo (dT) primers and 4 units of Omniscript reverse transcriptase (Qiagen), in a total volume of 20 μL. Real-time PCR was then performed, using SYBR GreenER qPCR superMix (Bio-Rad) for iCycler (Invitrogen), as described previously [14, 15]. The following primer pairs were used for PCR: ELK1 (forward, 5’-CAGCCAGAGGTGTCTGTTACC-3’; reverse, 5’-GAGCGCATGTACTCGTTCC-3’), α1A-AR (forward, 5’-CGCTACCCAACCATCGTCAC-3’; reverse, 5’-GAACAGGGGTCCAATGGATATG-3’), c-fos (forward 5’-CGAGATGGAGATCGGTATGGT-3’; reverse, 5’-GGGTCTTCTTACCCGGCTTG-3’), NF-κB (forward, 5’-AACAGAGAGGATTTCGTTTCC-3’; reverse, 5’-TTTGACCTGAGGGTAAGACTTCT-3’).

GAPDH (forward, 5’-TTACACCCCTTCTTTGACGTTG-3’; reverse, 5’-CATACCAGGAATGAGCTTGG-CAA-3’) was used as an internal control.

Western blotting

Protein extraction and western blotting were performed, as described previously [14, 15] with minor modifications. Briefly, equal amounts of protein (50 μg) obtained from cell extracts were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Immun-Blot PVDF Membrane, Bio-Rad) by electroblotting. Specific antibody binding was detected, using an anti-ELK1 antibody (clone I-20; dilution 1:50; Santa Cruz Biotechnology), an anti-NF-κB/p65 antibody (clone F-6; dilution 1:1000; Santa Cruz Biotechnology), or an anti-GAPDH antibody (clone 6C5; dilution 1:5000; Santa Cruz Biotechnology), and a secondary
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Figure 2. Effects of silodosin on ELK1 in bladder cancer cells. Quantitative RT-PCR of ELK1 (A) and c-fos (D). TCCSUP, UMUC3, or 647V cells treated with ethanol (mock) or silodosin (0.1, 0.5, 3.0, or 10 µM) for 24 hours were subjected to RNA extraction and subsequent real-time RT-PCR. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that of mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *P<0.05 (vs. mock treatment). **P<0.01 (vs. mock treatment). (B) Western blotting of ELK1. Total proteins extracted from TCCSUP cells treated with ethanol (mock) or silodosin (0.1, 0.5, 3.0, or 10 µM) for 24 hours were immunoblotted for ELK1 (62 kDa). GAPDH (37 kDa) served as an internal control. (C) Luciferase reporter activity of ELK1. TCCSUP cells were transfected with pELK1-Luc and pRL TK and subsequently cultured with ethanol (mock) or silodosin (1 or 10 µM) for 24 hours. Luciferase activity is presented relative to that with mock treatment in each cell line. Each value represents the mean (+SD) from at least three independent experiments. *P<0.05 (vs. mock treatment). **P<0.01 (vs. mock treatment). ***P<0.001 (vs. mock treatment).

antibody (mouse IRDye 680LT or rabbit IRDye 800CW, LI-COR), followed by scanning with an infrared imaging system (Odyssey, LI-COR).

**Reporter gene assay**

Cells at a density of 50-70% confluence in 24-well plates were co-transfected with 250 ng of pELK-Luc (LR-2061, Signosis) or pNFκB-Luc (LR-2001, Signosis) reporter plasmid DNA and 2.5 ng of pRL-TK plasmid DNA, using GeneJuice (Novagen). After 18 hours of transfection, the cells were cultured in the presence or absence of silodosin for 24 hours. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit (Promega) and luminometer (FLUOstar Omega, BMG Labtech).

**Cell proliferation**

We used methyl thiazolyl disphenyl tetrazolium bromide (MTT) assay to assess cell viability. Cells (3-5 × 10^3) seeded in 96-well tissue culture plates were incubated for 96 hours, and at the end of the culture 10 µL MTT stock solution (5 mg/mL; Sigma) was added to each well with 100 µL of medium for 4 hours at 37°C. The medium was replaced with 100 µL dimethyl sulfoxide, followed by incubation for 5 minutes at room temperature. The absorbance was then measured at a wavelength of 570 nm with background subtraction at 655 nm.

**Cell migration**

In order to evaluate the ability of cell migration, a scratch wound healing assay was performed.
Cells at a density of 90-100% confluence in 12-well plates were scratched manually with a sterile 200 µL plastic pipette tip, cultured for 24 hours, fixed with methanol, and stained with 0.1% crystal violet. The width of the wound area was quantitated, using ImageJ software (National Institutes of Health).

**Bladder tissue microarray (TMA) and immunohistochemistry**

We retrieved bladder tissue specimens obtained by transurethral resection performed at the Johns Hopkins Hospital. Appropriate approval from the institutional review board was obtained before construction and use of the TMA. These bladder TMAs constructed previously [16] included cases of high-grade muscle-invasive urothelial carcinoma that received GC neoadjuvant chemotherapy prior to radical cystectomy. Patients who received only 3 cycles of GC with dose reduction or ≤2 cycles of GC were excluded from the analysis. Responders (n=16) and non-responders (n=21) to the neoadjuvant therapy were pathologically defined as the absence (≤pT1N0M0) and presence (≥pT2, pN1-3, and/or M1) of muscle-invasive, extravesical, or metastatic disease at the time of cystectomy, respectively [17].

Immunohistochemistry was performed on the sections (5 µm thick) from the bladder TMAs, using a primary antibody to p-ELK1 (clone B-4 phosphorylated at serine 383; dilution 1:30; Santa Cruz Biotechnology) and a broad spectrum secondary antibody (Invitrogen), as described previously [8]. All stains were manually quantified by a single pathologist (H.M.) blinded
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Figure 4. Wound healing assay in UMUC3 (A), 647V (B), and UMUC3-ELK1-shRNA (C). The cells grown to confluence were gently scratched and the wound area was measured after 24-hour culture in the presence of ethanol (mock) or silodosin (0.1, 0.5, 3.0, or 10 µM). The migration determined by the rate of cells filling the wound area is presented relative to that with control in each line. Each value represents the mean (+SD) from at least three independent experiments. **P<0.01 (vs. mock treatment), ***P<0.001 (vs. mock treatment).

to sample identify. As described [8], the expression of p-ELK1 was considered positive when >10% of tumor cells were immunoreactive or at least 1% of tumor cells showed moderate to strong intensity.

Statistical analysis

Student’s t-test or Mann-Whitney U test was used to assess differences in variables with a continuous distribution across dichotomous categories. Chi-square test was used to evaluate the associations between categorized variables. P values less than 0.05 were considered to be statistically significant.

Results

Expression of α1A-AR in human bladder cancer cells

We first investigated the expression of α1A-AR in human urothelial carcinoma cell lines by real-time RT-PCR and found that TCCSUP, UMUC3, 647V, and 5637 expressed its mRNA (data not shown). Among the four cell lines, α1A-AR expression was the strongest in TCCSUP (approximately 13-fold higher than that in UMUC3), while its levels were similar between UMUC3 and 647V. The level of α1A-AR expression in 5637 was approximately 7% of that in UMUC3. We then found that silodosin, as an α1A-AR antagonist, only marginally reduced the levels of α1A-AR expression by up to 21% at 10 µM in TCCSUP cells.

Silodosin inactivates ELK1 in bladder cancer cells

We assessed the effects of silodosin on ELK1 expression by RT-PCR and western blotting in bladder cancer cells. Silodosin at 0.5-10 µM significantly decreased ELK1 gene expression in all the bladder cancer lines examined (Figure 2A). Similarly, the levels of ELK1 protein expression were diminished by silodosin in TCCSUP cells (Figure 2B). ELK1-mediated transcriptional activity was also determined in the cell extracts with transfection of an ELK1 luciferase reporter plasmid and subsequent treatment with silodosin. Silodosin was found to considerably reduce ELK1 luciferase activity, compared with mock treatment (Figure 2C). To confirm the inhibition of ELK1 activity by silodosin, we measured the expression levels of c-fos, a downstream target of ELK1 signals [3]. Significant decreases in c-fos gene expression by silodosin were then seen in TCCSUP cells (Figure 2D). These results indicate that silodosin down-regulates the expression and activity of ELK1 in bladder cancer cells.

Silodosin affects bladder cancer cell proliferation and migration

We recently showed that ELK1 silencing resulted in inhibition of bladder cancer cell proliferation in an androgen/AR-dependent manner but cell migration even in the absence of activated AR [8]. Consistent with these findings, silodosin that could down-regulate ELK1 as shown above, insignificantly (up to 5% increase or 8% decrease at 0.1-10 µM) changed cell viability of AR-positive UMUC3 (Figure 3A) or TCCSUP (Figure 3B) cultured in an androgen-depleted condition or that of AR-negative 647V (Figure 3C). In contrast, silodosin reduced the growth of UMUC3 cells cultured with normal FBS containing androgens (58% decrease at 10 µM; Figure 3D), but not that of UMUC3-ELK1-shRNA cultured similarly (Figure 3E).
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We also performed a scratch wound healing assay to assess the effects of silodosin on cell migration. Silodosin (at 0.5-10 µM) significantly inhibited wound closure of UMUC3 (Figure 4A).

Figure 5. Effects of silodosin on the cytotoxicity of CDDP and GEM in bladder cancer cells. MTT assay was performed in UMUC3 (A, D), 647V (B, E), and UMUC3-ELK1-shRNA (C) cells cultured with ethanol (mock)/3 µM silodosin and different concentrations (25 nM-25 µM) of CDDP (A-C) or GEM (D, E) for 96 hours. Cell viability is presented relative to that of each line with mock/silodosin treatment only (without CDDP or GEM). Each value represents the mean from three independent experiments. *P<0.05 (mock vs. silodosin). **P<0.01 (mock vs. silodosin). ***P<0.001 (mock vs. silodosin).

Figure 6. Effects of ELK1 silencing on the cytotoxicity of CDDP in bladder cancer cells. MTT assay was performed in UMUC3-control-shRNA and UMUC3-ELK1-shRNA cells cultured in medium containing 5% normal FBS (A) or 5% charcoal-stripped FBS (B) as well as different concentrations (25 nM-25 µM) of CDDP for 96 hours. Cell viability is presented relative to that of each line without CDDP treatment. Each value represents the mean from three independent experiments. **P<0.01 (control-shRNA vs. ELK1-shRNA). ***P<0.001 (control-shRNA vs. ELK1-shRNA).
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and 647V (Figure 4B) 24 hours after wound generation. In contrast, only insignificant inhibition (4% decrease at 10 µM) in UMUC3-ELK1-shRNA cell migration by silodosin was seen (Figure 4C).

Silodosin or ELK1 silencing increases sensitivity to CDDP in bladder cancer cells

To determine whether ELK1 down-regulation exerts an influence on the cytotoxic effects of chemotherapeutic agents on the proliferation of bladder cancer cells, we again performed MTT assay that compared the viability of cells with versus without silodosin treatment or ELK1-shRNA expression. Interestingly, silodosin treatment resulted in significant enhancement of CDDP (0.4-12.5 µM) cytotoxicity in UMUC3 (Figure 5A) and 647V (Figure 5B) cells, but not in UMUC3-ELK1-shRNA cells (Figure 5C). However, silodosin did not significantly change GEM cytotoxicity even in these ELK1-positive cells (Figure 5D, 5E). Similarly, UMUC3-ELK1-shRNA cells were significantly more sensitive to CDDP, compared with UMUC-control-shRNA cells cultured in the presence (Figure 6A) or absence (Figure 6B) of androgens. There were no significant differences in the efficacy of GEM between UMUC3-control-shRNA versus UMUC3-ELK1-shRNA (data not shown). In these assays, the effects of silodosin or ELK1 itself, irrespective of CDDP or GEM, on cell growth were excluded by comparing with respective controls without CDDP/GEM treatment.
**Silodosin inhibits NF-κB in bladder cancer cells**

The NF-κB pathway has been known to play a central role in modulating chemosensitivity to CDDP [18, 19]. We therefore assessed the effects of silodosin on the expression of NF-κB and its transcriptional activity in bladder cancer cells. ELK1 silencing in UMUC3 cells resulted in a decrease in NF-κB protein expression (Figure 7A). Silodosin was also found to reduce NF-κB expression at both mRNA (Figure 7B) and protein (Figure 7C) levels in two cell lines. In addition, silodosin inhibited NF-κB luciferase activity, compared with mock treatment, in these cells (Figure 7D).

**p-ELK1 expression correlates with chemoresistance in patients with bladder cancer**

We recently demonstrated the immunohistochemical findings indicating that the expression of p-ELK, but not ELK1, in muscle-invasive bladder cancers precisely predicted disease progression after radical cystectomy [8]. Here, we immunohistochemically stained for p-ELK1 in additional bladder TMAs (Figure 8) consisting of urothelial tumor specimens from patients who subsequently received neoadjuvant GC therapy. We then compared the levels of p-ELK1 expression between responders versus non-responders to chemotherapy. Overall, p-ELK1 was positive in 21 (57%) of 37 cases, including 6 (38%) of 16 responders and 15 (71%) of 21 non-responders. Thus, p-ELK1 positivity was significantly (P=0.039) associated with resistance to chemotherapy.

**Discussion**

We have recently demonstrated in vitro and in vivo data suggesting the involvement of ELK1 in bladder cancer progression via modulating cell proliferation/apoptosis, migration, and invasion [8]. Because previous studies indicated the link between activation of the PKC/Raf-1/ERK pathway, of which ELK1 was known to be a downstream target, and resistance to anticancer therapy [9, 10], we anticipated that ELK1 played a vital role in altering the sensitivity of bladder cancer cells to chemotherapeutic drugs. In the present study, we have investigated whether silodosin inactivates ELK1 in bladder cancer cells, leading to tumor regression as well as chemosensitization.

ELK1 and its downstream target c-fos have been implicated in the growth of smooth muscle cells [20, 21]. On the other hand, because α1A-AR is abundant in smooth muscle cells of the prostate and bladder neck, its blockers are often prescribed to the patients suffering from lower urinary tract symptoms secondary to prostatic enlargement [22]. In studies using human prostate smooth muscle cells [11] and the rat spinal cord [12], reduction of ELK1 and c-fos levels, respectively, by silodosin treatment has been documented. We then showed that silodosin, as an α1A-AR blocker, insidiously down-regulated α1A-AR expression in TCCSUP cells with a relatively high level of α1A-AR, while it could strongly inhibit the expression of ELK1 and c-fos as well as the transcriptional activity of ELK1. Thus, silodosin was found to effectively inactivate ELK1 in bladder cancer cells.

In bladder [8] and prostate [23] cancers, ELK1 was shown to induce the proliferation of cells only with an activated AR. In addition, we previously demonstrated, using bladder cancer lines, that ELK1 silencing resulted in induction of apoptosis of AR-positive cells as well as that androgens could activate ELK1 via the AR pathway [8]. In contrast, ELK1 promoted cell migration and invasion of bladder [8] and prostate [unpublished data] cancers in androgen/AR-independent manners. Although the precise mechanisms for the difference in the role of ELK1 between cell proliferation and migration/invasion have not been documented, ELK1 appears to require AR as its coactivator [23] for regulating the former whereas it, without AR,
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may modulate the expression of various molecules related to the latter including matrix metalloproteinases [24]. Similar to the findings of ELK1 [8], we demonstrated that silodosin inhibited the proliferation of bladder cancer cells with a functional AR yet the migration of both AR-positive and AR-negative cells. Thus, silodosin was suggested to suppress bladder cancer cell proliferation and migration via inactivating ELK1. This was further supported by the data showing insignificant effects of silodosin on the growth of ELK1 knockdown cells.

Our previous immunohistochemical study in bladder cancer specimens [8] showed up-regulation of ELK1 and p-ELK1 expression in urothelial neoplasms, compared with non-neoplastic urothelial tissues, which was significantly correlated with AR expression. Moreover, patients with p-ELK1-positive tumor had higher risks of tumor recurrence or disease progression. We here demonstrated, using additional bladder TMAs, that p-ELK1 expression correlated with resistance to GC therapy. The current results suggest that ELK1 activity could serve as a predictor of chemosensitivity in patients with bladder cancer. Specifically, those with ELK1-negative tumor are expected to be sensitive to CDDP treatment. In accordance with these findings, in bladder cancer cells both with and without a functional AR, silodosin treatment or ELK1 knockdown via its shRNA resulted in enhancement of the cytotoxic activity of CDDP. However, neither significantly increased the efficacy of GEM in these bladder cancer cells. Because silodosin exhibited only marginal effects on CDDP-treated ELK1-shRNA cells, it enhanced the cytotoxicity of CDDP presumably via down-regulation of ELK1. In addition, silodosin was found to reduce the expression/activity of NF-κB, a key regulator of chemoresistance to CDDP in bladder cancer [18, 19].

α1A-AR antagonists are safe and effective drugs that have been used for the treatment of the signs and symptoms of benign prostatic hyperplasia [20]. In healthy men after receiving oral doses of silodosin (e.g. 8 mg), its plasma levels of approximately 0.1 µM were obtained [25]. In rodents, higher doses of silodosin administration (i.e. up to 125 times of the oral dose in humans corresponding to >10 µM) were not associated with acute toxicity or marked histological changes in the major organs [26]. Therefore, 0.1-10 µM of silodosin we used for our in vitro studies might be tolerable doses in patients.

In conclusion, silodosin was found to inactivate ELK1 in bladder cancer cells and thereby inhibited tumor progression. Silodosin treatment or ELK1 silencing also enhanced the cytotoxic activity of CDDP in bladder cancer cells. Accordingly, ELK1 inactivation by silodosin treatment has the potential of being not only a therapeutic option particularly in patients with ELK1-positive/AR-positive bladder cancer but also a means of chemosensitization in those with ELK1-positive tumor. Further studies are required to determine the therapeutic effects of silodosin, especially in animal models for bladder cancer, and to elucidate the molecular mechanisms responsible for the differences in the effects of ELK1 or silodosin on cell proliferation versus migration/invasion as well as on the cytotoxicities of CDDP versus GEM.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hiroshi Miyamoto, The James Buchanan Brady Urological Institute, The Johns Hopkins Hospital, 600 North Wolfe Street, Marburg 148, Baltimore, MD 21287, USA. Tel: 410-614-1442; E-mail: hmiyamo1@jhmi.edu

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