Isorhapontigenin (ISO) Inhibits Invasive Bladder Cancer Formation In Vivo and Human Bladder Cancer Invasion In Vitro by Targeting STAT1/FOXO1 Axis

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Abstract

Although our most recent studies have identified Isorhapontigenin (ISO), a novel derivative of stilbene that isolated from a Chinese herb Gnetum cleistostachyum, for its inhibition of human bladder cancer growth, nothing is known whether ISO possesses an inhibitory effect on bladder cancer invasion. Thus, we addressed this important question in current study and discovered that ISO treatment could inhibit mouse-invasive bladder cancer development following bladder carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) exposure in vivo. We also found that ISO suppressed human bladder cancer cell invasion accompanied by upregulation of the forkhead box class O 1 (FOXO1) mRNA transcription in vitro. Accordingly, FOXO1 was profoundly downregulated in human bladder cancer tissues and was negatively correlated with bladder cancer invasion. Forced expression of FOXO1 specifically suppressed high-grade human bladder cancer cell invasion, whereas knockdown of FOXO1 promoted noninvasive bladder cancer cells becoming invasive bladder cancer cells. Moreover, knockout of FOXO1 significantly increased bladder cancer cell invasion and abolished the ISO inhibition of invasion in human bladder cancer cells. Further studies showed that the inhibition of Signal transducer and activator of transcription 1 (STAT1) phosphorylation at Tyr701 was crucial for ISO upregulation of FOXO1 transcription. Furthermore, this study revealed that metalloproteasease-2 (MMP-2) was a FOXO1 downstream effector, which was also supported by data obtained from mouse model of ISO inhibition BBN-induced mouse-invasive bladder cancer formation. These findings not only provide a novel insight into the understanding of mechanism of bladder cancer’s propensity to invasion, but also identify a new role and mechanisms underlying the natural compound ISO that specifically suppresses such bladder cancer invasion through targeting the STAT1–FOXO1–MMP-2 axis.

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Introduction

Bladder cancer is the sixth most common cancer in the United States and the number one cause of death in patients with urinary system malignancies. The incidence of bladder cancer has steadily risen in recent decades. It is estimated that more than 74,690 Americans will be diagnosed with bladder cancer and more than 15,580 will die of this disease in 2014 (1). Approximately 20% to 30% of bladder cancers are muscle-invasive, 50% of these patients die from metastasis within 2 years of diagnosis, and the 5-year survival rate for metastatic bladder cancer is only 6% (2). The remaining 70% to 80% of bladder cancers are diagnosed as non-muscular invasion, in which about 20% progress to muscle-invasive bladder cancer and have a 43% relative lower 5-year survival rate (2). In addition, bladder cancer is one of the most costly cancers as a result of necessary lifetime monitoring and treatment. For these reasons, the identification of new natural compounds that specifically suppress human bladder cancer invasion and defining key molecules that are responsible for mediating human bladder cancer invasion and metastasis are of extremely importance for improving the clinical outcome of patients with this disease. Isorhapontigenin (ISO) is a novel derivative of stilbene and is isolated and purified from a Chinese herb Gnetum cleistostachyum (3–5), which has been used for centuries as a Chinese herbal medicine for the treatment of several cancers including bladder cancers. Recent work by our group has demonstrated that ISO treatment induces cell-cycle arrest at G0–G1 phase and inhibits anchorage-independent cell growth through inhibiting cyclin D1 expression in both RT4 human noninvasive bladder cancer cells and UMUC3 human-invasive bladder cancer cells (4). Our studies have also found that ISO exhibits the anticancer activity...
activity accompanied by downregulating X-linked inhibitor of apoptosis protein (XIAP), thus inducing apoptosis in T24T human-invasive bladder cancer cells in a relative high dose (5). Nevertheless, nothing is known whether ISO is able to inhibit bladder cancer invasion at noncytotoxic doses.

FOXO proteins are reported to upregulate negative regulators of the G1–S transition of the cell cycle, such as p27kip1, p21WAF1, and p130, and repress positive regulators, such as cyclin D1 and D2 (6). Overexpression of FOXO proteins also enhances Gadd45x and cyclin G2 promoter activity, resulting in cell-cycle G1–M arrest (8). Besides, activation of FOXOs triggers apoptosis through binding to the promoters of the proapoptotic gene, FasL and 82A, and inducing their expression (9–11). Moreover, FOXO1 has been found to inhibit prostate cancer cell migration and invasion through binding to Runt-domain containing protein RunX2 and repress its transcriptional activity (12). Expression of a constitutive nuclear active form of FOXO1 significantly inhibited matrix metalloproteinase-9 (MMP-9) activation induced by EGF and prevented cell invasion in glioblastoma cells (13). Although the mRNA and protein expression level of FOXO1 are found to be downregulated in high-grade and invasive bladder cancer, nothing is known about the roles, mechanisms, and the upstream regulator/downstream effectors of FOXO1 in human bladder cancer invasion (14). Therefore, we addressed these questions and explored the potential FOXO1-related inhibitory effects of ISO on human bladder cancer invasion in the present study both in vitro and in vivo.

Materials and Methods

Plasmids, antibodies, and other reagents

The FLAG-tagged human FOXO1 expression construct, Fasl promoter, and IGF/IRS-1 promoter luciferase reporters were constructed and used in previously studies (15). The shRNA sets for human FOXO1 were purchased from Open Biosystems (Thermo Fisher Scientific). The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system–specific targeting FOXO1 was purchased from Applied Biological Materials (ABM) Inc. Human FOXO1 promoter luciferase reporter was cloned into the pGL3 luciferase assay vector and was kindly provided by Dr. Jean-Baptiste Demoulin (De Duve Institute, Catholic University of Louvain, BE-1200 Brussels, Belgium; ref. 16). A mutation of Signal transducer and activator of transcription 1 (STAT1)–binding site in the FOXO1 promoter was created using site-directed mutagenesis by the overlap extension PCR method with mutagenic primers 5′-ACAGAACACCTGCA- GAAGCCGCCATCCAAATATAGAAGCGCCATCCAATAATAGAGATCCAAA-3′ (sense) and 5′-TTTGGATACCTCATTATTGGATGAGCCTCCCTCGGCTGTTCTCCTG-3′ (antisense), and flanking primers 5′-ATTGAGTATGCCTCCGGCCGCCGCCC-3′ (forward) and 5′-TGGACACCTCCTCGGCAGCGTGTCG-3′ (reverse). GFP-STAT1 Y701F was obtained from Adgene. Human MMP-2 promoter luciferase construct was kindly provided by Dr. Jian Cao (Department of Medicine, School of Medicine, State University of New York at Stony Brook, Stony Brook, NY; ref. 17). Human MMP-2 promoter luciferase reporter was a gift from Dr. Ety N. Benveniste (Department of Cell Biology, The University of Alabama at Birmingham, Birmingham, Alabama; ref. 18). The antibodies against FOXO1, p-FOXO1, FOXO4, PARP, STAT1, p-STAT1, STAT3, p-STAT3, and CREB were commercially purchased from Cell Signaling Technology. The antibodies against MMP-2, SOCS1, and GAPDH were bought from Santa Cruz Biotechnology. ISO with purity greater than 99.9% was purchased from Higher Biotech. ISO was dissolved in dimethyl sulfoxide (DMSO; Sigma) to make a 20 mmol/L stock solution, and the same concentration of DMSO was also made and used as a vehicle control in all experiments.

Animal experiments

The C57BL/6j male mice at age of 5 to 6 weeks were randomly divided into three groups, 12 mice in each group, including vehicle-treated control group, N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-treated group, and BBN combined with ISO-treated group. Mice in BBN-treated group received BBN (0.05%) in drinking water for 20 weeks, whereas mice in BBN combined with ISO-treated group received BBN and ISO (150 mg/kg/day) in drinking water for 20 weeks. ISO was given to the mice in drinking water on the day of initial exposure to BBN and continued throughout the tumor induction period. Mice bladder tissues were excised and fixed overnight in 4% paraformaldehyde at 4°C. Fixed tissues were processed for paraffin embedding, and the serial 5-μm-thick sections were then stained by hematoxylin and eosin (H&E) staining.

Cell culture and transfection

Human-invasive bladder cancer cell line UMUC3 and noninvasive bladder cancer cell line RT4 were provided by Dr. Xue-Ru Wu (Departments of Urology and Pathology, New York University School of Medicine, New York, NY) in 2010, and were described and used in our previous studies (4, 19). The human metastatic bladder cancer cell line T24T, which was a lineage-related lung metastatic variant of invasive bladder cancer cell line T24 (20–22), was kindly provided by Dr. Dan Theodorescu (Departments of Urology, University of Virginia, Charlottesville, VA; ref. 20) in 2010. All the cell lines were subjected to DNA tests and authenticated in our previous studies (4). The cell lines were regularly authenticated on the basis of viability, recovery, growth, morphology, and chemical response as well and were most recently confirmed 4 to 6 months before use by using a short tandem repeat method. UMUC3 cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mmol/L l-glutamine (Life Technologies). T24T cells were cultured in DMEM/Ham’s F-12 (1:1 volume) mixed medium supplemented with 5% FBS, 1% penicillin/streptomycin, and 2 mmol/L l-glutamine. RT4 cells were maintained in McCoy’s 5A supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mmol/L l-glutamine. Transfections were carried out using PolyJet DNA In Vitro Transfection Reagent (SignaGen Laboratories) according to the manufacturer’s instructions. The transfected cells were then respectively selected with G418, hygromycin, or puromycin (Life Technologies) for 4 to 6 weeks. Surviving cells were pooled as stable mass transfectants as described in our previous studies (23, 24).

Human tissue specimens

Ninety-eight pairs of primary bladder cancer samples and their paired adjacent normal bladder tissues were obtained from patients who underwent radical cystectomy at Department of Urology of the Union Hospital of Tongji Medical College (Wuhan, China) between 2012 and 2015. All specimens were...
immediately snap-frozen in liquid nitrogen after surgical removal. Histologic and pathologic diagnoses were confirmed, and the specimens were classified by a certified clinical pathologist according to the 2004 World Health Organization Consensus Classification and Staging System for bladder neoplasms. All specimens were obtained with appropriate informed consent from the patients and a supportive grant obtained from the Medical Ethics Committee of China.

**ATP cell viability assay**

Cell viability was measured utilizing the CellTiter-Glo Luminiscent Cell Viability Assay Kit (Promega Corp.) according to the manufacturer’s instructions as described in our previous studies (25). Briefly, cells were plated in 96-well plates at a density of 10,000 cells per well and allowed to adhere overnight. The cell culture medium was then replaced with 0.1% FBS DMEM and cultured for 12 hours. After ISO treatment for the indicated time and doses, 50 μL of CellTiter-Glo assay reagent was added to each well. The contents were mixed on an orbital shaker for 2 minutes to induce cell lysis and then incubated at room temperature for 10 minutes to stabilize the luminescent signal. Results were read on a microplate luminometer LB 96V (Berthold GmbH & Co. KG). Cell viability (%) was defined as the relative absorbance of treated samples versus that of the untreated control. All experiments were performed with six wells for each experiment and repeated at least three times.

**In vitro cellular migration and invasion assays**

*In vitro* migration and invasion assays were conducted by using Transwell chamber (for migration assay) or Transwell precoated Matrigel chamber (for invasion assay) according to the manufacturer’s protocol (BD Biosciences) as described previously (26, 27). Briefly, 700 μL of medium containing 10% FBS (for UMUC3 and T24T cells) or 40% FBS (for RT4 cells) was added to the lower chambers, whereas homogenous single-cell suspensions (5 × 10⁴ cells/well) in 0.1% FBS medium with or without ISO as indicated were added to the upper chambers. The Transwell plates were incubated in 5% CO₂ incubator at 37°C for 24 hours, and thereafter were washed by PBS, fixed with 4% formaldehyde, and stained with Giemsa stain. The nonmigration or noninvading cells were scrapped off on the top of chamber. The migration and invasion rates were quantified by counting the migration and invaded cells at least three random fields under a light microscope (Olympus).

**Western blotting**

Western blot assay was assessed as previously described (28). Briefly, cells were plated in 6-well plates and cultured in normal FBS medium until 70% to 80% confluence. The cells were then cultured in 0.1% FBS medium for 12 hours, followed by treatment with different doses of ISO for the indicated time. The cells were washed once with ice-cold PBS, and cell lysates were prepared with a lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 1% SDS, and 1 mmol/L Na₂VO₃]. An equal amount (80 μg) of total protein from each cell lysate was subjected to Western blot with the indicated antibody as described in previous studies (25, 26). Immunoreactive bands were detected by using the alkaline phosphatase-linked secondary antibody and ECF Western blotting system (Amersham Biosciences). The images were acquired by using Typhoon FLA 7000 imager (GE Healthcare).

**Nuclear extract preparation**

Nuclear extracts were prepared as previously described (23). UMUC3 cells were seeded into 10-cm culture dishes at 70% to 80% confluence, cultured in 0.1% FBS medium for 12 hours, and then treated either with vehicle (0.1% DMSO) or with 10 μmol/L of ISO for 12 hours. The nuclear proteins were extracted by the Nuclear/Cytosol Fractionation Kit (BioVison Technologies) following the manufacturer’s protocols. Equal Protein concentrations were measured by a protein quantification assay kit (Bio-Rad). Nuclear extracts were stored at −80°C before they were used.

**Reverse transcription PCR and qRT-PCR**

Total RNA was extracted with TRizol reagent (Invitrogen Corp.), and cDNAs were synthesized with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corp.). A pair of oligonucleotides (Forward: 5′-GATG ATCTTGAGGC- GTGTGTC-3′ and Reverse: 5′-CAGGGCTCTTTTAAACTCTG-3′) were used to amplify human gapdh cDNA as loading control. The human foxo1 cDNA fragments were amplified by a pair of human foxo1-specific PCR primers (Forward: 5′-AACCT GCCAT- TACAGTTGGC-3′; Reverse: 5′-AAATGACGAGCTGAACATATGC- GT-3′). The flag-foxo1 fragments were amplified by primers 5′- ACAAGGAGGAGGTAGCAAGG-3′ (Forward) and 5′-GCCGAG- AGITGAGACATGGCTAAA-3′ (Reverse). The human mmp-2 cDNA fragments were amplified by 5′-CAATGCAGCAGAAGAAGAAGA-3′ (Forward) and 5′-CCA AAGTITCTTGATAGCAAGTC-3′ (Reverse). The human mmp-9 cDNA fragments were amplified by 5′-GGCAGG- CAGACATCGTCAC-3′ (Forward) and 5′-TGCTCAGCCCTCCGA- TGCC-3′ (Reverse). The PCR products were separated on 2% agarose gels and stained with ethidium bromide. The images were visualized and scanned with UV lights with FluorChem SP imaging system (Alpha Innotech Inc.) as described previously (29). The Quantitative reverse transcription PCR (qRT-PCR) analysis was carried out using the SYBR Green PCR Kit (Qiagen) and the 7900HT Fast Real-time PCR system (Applied Biosystems).

**Luciferase assay**

As described in our previous studies (4, 5), dual-luciferase reporter assay was performed by using the luciferase assay system (Promega Corp.). Briefly, Human Fasl, promoter, IGFβ1 (3×1RS) promoter, FOXO1 promoter, and MMP-2 promoter luciferase reporters were transfected into the indicated human bladder cancer cells, respectively. After ISO treatment, cells were extracted with passive lysis buffer [25 mmol/L Tris-phosphate (pH 7.8), 2 mmol/L EDTA, 1% Triton X-100, and 10% glycerol]. The luciferase activity was measured with a microplate luminometer LB 96V (Berthold GmbH & Co. KG). The Renilla luciferase signal was normalized to the internal firefly luciferase transfection control.

**Immunohistochemistry paraffin of mice bladder tissues**

Mice bladder tissues were immunostained by antibodies specific against FOXO1 (Cell Signaling Technology) and MMP-2 (Santa Cruz Biotechnology), respectively. The resultant immunostaining images were captured using the AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss). Protein expression levels were analyzed by calculating the
integrated optical density per stained area (IOD/area) using Image-Pro Plus version 6.0 (Media Cybernetics). Briefly, the IHC-stained sections were evaluated at 400-fold magnifications, at least 5 representative staining fields of each section were analyzed to calculate the optical density based on typical photographs that had been captured.

Statistical methods

Associations between categorical variables were assessed using the \( \chi^2 \) test. The Student \( t \) test was utilized to compare continuous variables, summarized as mean/SD, between different groups. Paired \( t \) test was performed to compare the difference between paired tissues using real-time PCR analyze. \( P < 0.05 \) was considered statistically.

Results

ISO treatment inhibited BBN-induced mouse-invasive bladder cancer formation \textit{in vivo} and human bladder cancer invasion \textit{in vitro}

ISO has been shown to inhibit growth and induce apoptosis in human bladder cancer cells in our recent studies (4, 5). BBN is a well-characterized bladder carcinogen for its induction of 100% invasive bladder cancer in mouse model (30). To explore whether ISO exhibit bladder cancer invasion, we first employed BBN-induced invasive bladder cancer mouse model and examined the effects of ISO on BBN-induced mouse-invasive bladder cancer formation. As shown in Table 1 and Fig. 1A, none of the vehicle-treated control mice developed bladder cancer, whereas

![Graph](image_url)

**Table 1.** Effect of ISO treatment on BBN-induced mouse-invasive bladder cancer formation

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Carcinogen</th>
<th>Treatment</th>
<th>Number (%) of papillomas</th>
<th>Number (%) of noninvasive bladder cancer</th>
<th>Number (%) of invasive bladder cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>BBN</td>
<td>Vehicle</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>12 (100)*</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>BBN</td>
<td>ISO</td>
<td>7 (58.3)</td>
<td>3 (25)</td>
<td>2 (16.7)*</td>
</tr>
</tbody>
</table>

*Significant difference between vehicle control group and BBN-treated group (\( P < 0.05 \)).

*Significant difference between BBN-treated group and BBN combined with ISO-treated group (\( P < 0.05 \)).
BBN induced 100% (12/12) high-grade muscle-invasive bladder cancers formation. Interestingly, only 16.7% (2/12) of the BBN-treated mice developed high-grade muscle-invasive bladder cancer while ISO was administrated, with 7 cases of papillomas and 3 cases of low-grade non-muscle-invasive bladder cancers, demonstrating a novel biologic activity of ISO as an efficient drug that targets at stage of invasive bladder cancer development in vivo (P < 0.05). Subsequently, two human-invasive bladder cancer cell lines UMUC3 and T24T were employed for Transwell cancer cell invasion assay in the presence of relative low dosages, 10 µmol/L and 20 µmol/L, of ISO treatment, according to the dosages applied in cell proliferation analyses described previously (4, 5). As shown in Fig. 1B and C, the relative invasion rates of UMUC3 and T24T cells were reduced by 63.1% and 61.2% after 10 µmol/L and 20 µmol/L ISO treatment, respectively, in comparison with the vehicle control, whereas ISO did not show observed effects on the bladder cancer cell migration under same experimental conditions. To further exclude any possible involvement of cellular toxicity, the cells were treated with the same dosages of ISO as shown in Fig. 1D and E for different time points from 6 hours to 36 hours, and cell survival rates were analyzed by ATPase assay. As expected, the selected dosages of ISO did not show any observable cellular toxicity to either UMUC3 (Fig. 1D) or T24T (Fig. 1E) cells at the time point (24 hours) when cell migration and invasion were measured. Taken together, these data, for the first time, demonstrate that ISO is a new natural compound that can specifically inhibit mouse-invasive bladder cancer development in vivo following BBN exposure and suppress bladder cancer cell invasion in vitro.

ISO induced FOXO1 protein expression and FOXO1-dependent transcriptional activity in human bladder cancer cells

FOXO1 has been proposed as a potential prognostic marker for bladder cancer since an increased foxo1 mRNA expression is associated with the reduced bladder cancer disease progression and a significantly prolonged survival of the patients (14). To elucidate the mechanisms underlying ISO inhibitory effect on human bladder cancer, we investigated whether ISO could regulate the expression of FOXO1 in human-invasive bladder cancer cell lines, UMUC3, and T24T. As shown in Fig. 2A, ISO treatment induced FOXO1 protein expression in a dose-dependent manner in UMUC3 cells. Treatment of UMUC3 cells with 10 µmol/L ISO also resulted in a gradual induction in FOXO1 protein level over various time points (Fig. 2B). Consistently, similar FOXO1 protein expression profile was also observed in the metastatic human bladder cancer T24T cells upon ISO treatment (Fig. 2C). It was noted that ISO treatments at dose of 10 µmol/L for UMUC3 cells and 20 µmol/L for T24T cells could markedly induce FOXO1 protein expression (Fig. 2A–C) with a profoundly specific inhibition of cancer cell invasion without affecting their migration (Fig. 1C and D). To unravel the function of ISO-induced FOXO1 protein, we determine FOXO1 protein location and its dependent transcriptional activation in ISO-treated cells. As shown in Fig. 2D, FOXO1 protein was almost all located in nuclear fraction, while it was not observable in cytoplasmatic fraction. Consistent with its location, ISO treatment remarkably enhanced FOXO1-dependent IGFBG-1 promoter and Fasl promoter transcription activities (Fig. 2E and F), strongly demonstrating that ISO-induced FOXO1 proteins are not only located in nuclear, but also functional in human bladder cancer cells.

FOXO1 was downregulated in human bladder cancer tissues and acted as an effective suppressor of invasion of human bladder cancer cells

To find out the clinical relevance of therapeutic effect of ISO in human bladder cancer, we evaluated FOXO1 expression in 98 pairs of bladder cancer tissues and their adjacent normal bladder tissues surgically removed from patients who were diagnosed with bladder cancers. As shown in Fig. 3A, a profound reduction of foxo1 mRNA expression was observed in human bladder cancer tissues with an overall average 2.4-fold lower relative foxo1 mRNA level in comparison with their adjacent normal bladder tissues (P < 0.001). Moreover, a significant negative relationship was observed between foxo1 expression and bladder cancer grade, invasion, as well as the
FOXO1 was downregulated in human bladder cancer tissues and acted as a regulator of human bladder cancer cell invasion. Total RNA and protein lysates were prepared from human normal (N) and paired cancerous (T) tissues among 98 patients diagnosed with bladder cancers, and subjected to qRT-PCR and Western blotting analyses for determining FOXO1 mRNA (A) and protein (B) expression profiles, respectively. Flag-FOXO1 expression construct was used to stably transfect into UMUC3 and T24T cells, respectively. The stable transfectants, UMUC3(Flag-FOXO1) and T24T(Flag-FOXO1), were identified by Western blotting (C) and then used for determination of their abilities of cell invasion and migration as compared with their vector control transfectants (D & E) as described in the “Materials and Methods” section. F, RT4 cells were stably transfected with nonsense shRNA or two FOXO1 shRNA constructs (shFOXO1-4 and shFOXO1-6), respectively, and the knockdown efficiency of FOXO1 protein was evaluated by Western blot. G, FOXO1 stably knockdown transfectants, RT4(shFOXO1-4) and RT4(shFOXO1-6), as well as Nonsense transfectant RT4(Nonsense) were then used for determination of their migration and invasion abilities. Results are the mean ± SD of triplicates. Symbol "*" indicates a significant difference between vector control and FOXO1 overexpression group (P < 0.05). Symbol "**" indicates a significant difference between NONSENSE transfectant and shRNA transfectants (P < 0.05).

Table 2. Correlation between foxo1 mRNA level and clinicopathologic factors

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group</th>
<th>Total</th>
<th>Low</th>
<th>High</th>
<th>P value</th>
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<tbody>
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<td>79</td>
<td>60</td>
<td>19</td>
<td>0.561</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>19</td>
<td>13</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
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<td>17</td>
<td>14</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥55</td>
<td>81</td>
<td>59</td>
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<tr>
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<td>Low</td>
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<td>13</td>
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<tr>
<td></td>
<td>High</td>
<td>66</td>
<td>54</td>
<td>12</td>
<td></td>
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<tr>
<td>Tumor stage</td>
<td>T1, T2, T3</td>
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<td>22</td>
<td>17</td>
<td>0.017*</td>
</tr>
<tr>
<td></td>
<td>T4, T1</td>
<td>39</td>
<td>22</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>&lt;3.0 cm</td>
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<td>42</td>
<td>16</td>
<td>0.570</td>
</tr>
<tr>
<td></td>
<td>≥3.0 cm</td>
<td>57</td>
<td>42</td>
<td>16</td>
<td></td>
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<tr>
<td>Tumor multiplicity</td>
<td>Unifocal</td>
<td>25</td>
<td>19</td>
<td>6</td>
<td>0.841</td>
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<tr>
<td></td>
<td>Multifocal</td>
<td>73</td>
<td>54</td>
<td>19</td>
<td></td>
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<tr>
<td>Lymph nodes metastasis</td>
<td>Absent</td>
<td>65</td>
<td>45</td>
<td>20</td>
<td>0.008*</td>
</tr>
<tr>
<td></td>
<td>Present</td>
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<td>30</td>
<td>3</td>
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</table>

*P < 0.05.
Flag-FOXO1 expression, excluding the possibility that FOXO1 protein induction by ISO is at protein degradation level. We, therefore, next evaluated the mRNA levels of foxo1 in the same bladder cancer cells. Consistent with the results obtained from protein levels, endogenous foxo1 mRNA expression was markedly upregulated upon ISO treatment in both UMUC3 and T24T cells (Fig. 4C–E), whereas the flag-tagged exogenous flag-foxo1 mRNA was not affected by ISO treatment (Fig. 4F and G). These results suggest that ISO-induced FOXO1 expression may be regulated at mRNA transcription level. To test this notion, the foxo1 promoter-driven luciferase reporter was transfected into UMUC3 cells, and foxo1 promoter activity was significantly upregulated in a time-dependent manner upon ISO treatment (Fig. 4H). Taken together, these data strongly reveal that ISO-induced FOXO1 protein expression is regulated at mRNA transcriptional level in human bladder cancer cells.

FOXO1 expression was required for ISO inhibition of bladder cancer cell invasion

To address the contribution of FOXO1 induction by ISO to its inhibition of bladder cancer invasion, we transfected various FOXO1 shRNAs into UMUC3 and T24T, respectively, and the stable FOXO1 knockdown transfectants were identified by Western blotting as shown in Fig. 5A and B. The stable transfectants, UMUC3(shFOXO1-4), UMUC3(shFOXO1-6), T24T (shFOXO1-4), and T24T(shFOXO1-6), were used to determine invasion and migration abilities as compared with their Nonsense control transfectants, UMUC3(Nonsense), and T24T (Nonsense), respectively. As shown in Fig. 5C and Supplementary Fig. S1A and S1B, knockdown of FOXO1 by its shRNAs (shFOXO1-4 and shFOXO1-6) showed no effects on cell migration, but significantly promoted invasion in both UMUC3 and T24T cells (Fig. 5D; Supplementary Fig. S1A and S1B), which consistently support that FOXO1 protein exhibits specific inhibition of cancer invasion. Importantly, suppression of FOXO1 protein expression markedly reduced ISO inhibition of cell invasion in both UMUC3 and T24T cells (Fig. 5E; Supplementary Fig. S1A and S1B). To further verify the role of FOXO1 in ISO suppression of bladder cancer invasion, CRISPR/Cas9 systems were used to knockout FOXO1 gene in both UMUC3 and T24T cells. The single-clone stable FOXO1 knockout transfectants, UMUC3(KO FOXO1, clone 2) and T24T(KO FOXO1, clone 5), were selected for our studies (Fig. 5F and G). As shown in Fig. 5H and Supplementary Fig. S2A and S2B, bladder cancer migration was not affected upon FOXO1 knockout, whereas the invasion of UMUC3 and T24T cells was further increased (Fig. 5I and Supplementary Fig. S2A and S2B). Moreover, knockout of FOXO1 successfully diminished the effects of ISO on bladder cancer invasion (Fig. 5J; Supplementary Fig. S2A and S2B). These results greatly demonstrate that FOXO1 protein induction by ISO is crucial for its inhibition of bladder cancer invasion.

ISO treatment enhanced FOXO1 transcription through inhibition of STAT1 phosphorylation at Tyr701

The above results demonstrate that ISO inhibition of human bladder cancer invasion was dependent on FOXO1 induction via upregulating FOXO1 gene promoter transcription activity. To identify the transcription factor that is responsible for ISO upregulation of FOXO1 transcription, TFTRANSFAC Transcription Factor Binding Sites Software (Biological Database) was used for bioinformatics analysis of the FOXO1 promoter region. The results revealed that an approximate 800-bp promoter region of the human FOXO1 gene contained the putative DNA-binding sites of various transcription factors, including CREB, FOXO4, STAT1, and STAT3 (Fig. 6A). We next examined the changes in both expression and the nuclear translocation of these factors following ISO treatment for 12 hours. As shown in Fig. 6B, treatment of UMUC3 cells with ISO resulted in a dramatic inhibition of STAT1 phosphorylation at Tyr701 in a dose-dependent manner without affecting total protein expression, whereas there was only a slightly effect on phosphorylation of STAT3 and expression of FOXO4 and CREB. The results obtained from distributions of the transcription factors between nuclear and cytoplasm fractions consistently indicated that ISO treatment mainly targeted on STAT1 phosphorylation at Tyr701 (Fig. 6C). To investigate the role of STAT1 phosphorylation in upregulation of FOXO1 by ISO, a dominant-negative mutant form of STAT1(GFP-STAT1 Y701F) was ectopically expressed in UMUC3 cells, and the dominant-negative effect was confirmed by its inhibition of a STAT1-regulated SOCS1 expression (ref. 31; Fig. 6D). The results showed that transcription of
essential MMP member, such as MMP-9 expression, is not correlated with tumor grade, stage, or overall survival of the patients with bladder cancer (33). Our previous study has also confirmed that upregulation of MMP-2, but not MMP-9, participated in mediation of bladder cancer invasion (22). To determine whether MMP-2 is associated with ISO inhibition of human bladder cancer invasion, we evaluated the effect of ISO on MMP-2 protein expression. The results showed that ISO treatment profoundly inhibited MMP-2 protein expression in both UMUC3 and T24T cells (Fig. 7A and B). Moreover, MMP-2 mRNA level and its promoter-driven luciferase reporter activity were also attenuated by ISO treatment (Fig. 7C and D), suggesting that the downregulation of MMP-2 expression by ISO occurred at transcriptional level. Meanwhile, the mRNA level of MMP-9 was not affected upon ISO treatment (Fig. 7C). To test the potential relation of FOXO1 induction to MMP-2 inhibition following ISO treatment in bladder cancer cells, we next evaluated the effects of FOXO1 overexpression and knockdown on MMP-2 expression in T24T cells. As shown in Fig. 7E, enforced expression of FOXO1 impaired MMP-2 expression,
whereas knockdown of FOXO1 upregulated the basal levels of MMP-2 protein and mRNA and effectively reversed ISO downregulation of MMP-2 expression (Fig. 7F). Moreover, the inhibition of STAT1 phosphorylation at Tyr701 by ISO was comparable between T24T(Nonsense) and T24T(shFOXO1) transfectants. Accordingly, the basal level of MMP-2 promoter activity in T24T(shFOXO1) transfectant was also significantly increased in comparison with T24T(Nonsense) transfectant, and ISO inhibition of MMP-2 promoter activity was impaired in T24T(shFOXO1) transfectant (Fig. 7G). It was important to note that ectopic expression of MMP-2 (Fig. 7H) in T24T cells did not affect cell migration (Fig. 7I; Supplementary Fig. S3), but specifically enhanced the basal level of cell invasion (Fig. 7J; Supplementary Fig. S3), while it effectively attenuated ISO inhibition of T24T cell invasion (Fig. 7K; Supplementary Fig. S3). These results revealed that MMP-2 acted as FOXO1 downstream being responsible for ISO inhibition of bladder cancer invasion, and this novel finding was greatly supported by our results obtained from BBN-induced mouse-invasive bladder cancers in vivo showing that ISO treatment downregulated FOXO1 and upregulated MMP-2 expression in mouse bladder tissues (Fig. 8A–C). Taken together, our results demonstrate that FOXO1 mediates ISO inhibition of MMP-2 transcription and protein expression, by which it inhibits bladder cancer invasion in vivo and invasive bladder cancer development in vivo as diagramed in Fig. 7L.

**Discussion**

Muscular invasion of bladder cancer causes 100% death and represents a major therapeutic challenge of this disease. Therefore, an endeavor to identify new anticancer compounds with strong inhibition of cancer invasion and understand the mechanisms underlying their inhibitory effect on bladder cancer invasion is the key step to discover the unmet future medicines against the invasive malignant disease. ISO is a new derivative isolated from a Chinese herb *Gnetum Cleistostachyum* that has been used to treat bladder cancer for many years (3). Our recent in vivo pharmacokinetic study shows that the concentration of ISO in serum can reach to 47.9 µmol/L without any observed toxicity to experimental mice (4). Here, our results obtained from in vivo animal studies show that ISO treatment inhibits BBN-induced mouse-invasive bladder cancer formation, for first time demonstrating the chemopreventive effects of ISO to the best of our knowledge. We also find that ISO at in vivo–relevant concentrations of 10 to 20 µmol/L specifically represses bladder cancer invasion by promoting FOXO1 transcription through inhibition of STAT1 phosphorylation at Tyr701. Our study also identify that MMP-2 is a downstream target of FOXO1 for its mediation of ISO inhibition of bladder cancer invasion. Our results clearly demonstrate that the natural compound ISO specifically inhibits human bladder cancer invasion through targeting STAT1–FOXO1–MMP-2 axis, which is completely distinct from its induction of cancer cell

**Figure 6.**

ISO promotes FOXO1 transcription by inhibition of STAT1 phosphorylation at Tyr701. A, schematic representation of transcription factor binding sites of human FOXO1 gene promoter. B, UMUC3 cells were treated with either vehicle or ISO as the indicated concentrations for 12 hours. Expression of the related transcription factors in the whole-cell lysates was determined by Western blotting, and GAPDH was used as protein loading control. C, the UMUC3 cells were treated with either vehicle or 10 µmol/L of ISO for 12 hours. The cell extracts were used to isolate nuclear and cytoplasm fractions and then subjected to Western blotting with the specific antibodies as indicated. PARP and GAPDH were used as markers for nuclear and cytoplasm fractions, respectively. D, UMUC3 cells were stably transfected with dominant-negative STAT1, GFP-STAT1 Y701F, and the stable transfectants were treated with either vehicle or 10 µmol/L ISO for 12 hours. The expression of FOXO1 and SOCS1 was determined by Western blotting. GAPDH was used as protein loading control. E, FOXO1 promoter-driven luciferase reporter was transfected into UMUC3(EGFP) and UMUC3(STAT1 Y701F) cells, and the stable transfectants were then treated with 10 µmol/L of ISO for the indicated times. Luciferase activity was evaluated by the Dual-Luciferase Reporter Assay System. F, UMUC3 cells were stably transfected with FOXO1 promoter-driven luciferase or the STAT1-binding site mutant, and the stable transfectants were treated with 10 µmol/L of ISO for 18 hours. Dual-Luciferase Reporter Assay System was performed to determine the Luciferase activity. Results are the mean ± SD of triplicates. Symbol ** indicates a significant difference between UMUC3(EGFP-vector) and UMUC3(STAT1 Y701F) in the same time point upon ISO treatment (P < 0.05). Symbol *** indicates a significant difference between vehicle control and ISO-treated group (P < 0.05).
apoptosis and inhibition of cancer cell anchorage-independent growth. Prevention and therapeutic intervention by phytochemicals represents a newer dimension in cancer management. Silymarin, a mixture of flavonoids isolated mainly from milk thistle, has been extensively studied in preclinical models as well as clinically for its efficacy against various cancers including bladder cancer, and it has been shown to effectively inhibit cell proliferation, angiogenesis, epithelial mesenchymal transition, metastasis, as well as promote cell apoptosis (35, 36). Whether ISO exhibits other anticancer effects apart from targeting cell growth, apoptosis, and invasion deserves further investigation.

Our recent studies have revealed that ISO effectively attenuates transcription factor-specific protein 1 (Sp1) expression and transactivation (4, 5), which results in the decreased bindings of Sp1 to promoter region of its regulated genes, cyclin D1 and XIAP, in turn leading to the downregulation of these gene expressions and consequently resulting in suppression of cancer cell anchorage-independent growth and induction of apoptosis in bladder cancer cells (4, 5). Although our group has reported above mechanism underlying anticancer effects of ISO compound, evaluation of whether ISO is a new agent with strong activity for inhibition of cancer invasion and understanding the potential mechanism involved in its inhibition of bladder cancer invasion are of tremendous importance for ISO potentially therapeutic clinical application. In the present study, we demonstrate that ISO at relevant applicable concentrations shows a great inhibition of bladder cancer invasion in vitro and mouse-invasive bladder cancer development in vivo. Accordingly, treatment of ISO significantly promotes FOXO1 expression, whereas suppression of FOXO1 expression completely abolishes ISO inhibition of bladder cancer invasion in both UMLUC3 and T24T cells. Thus, ISO is an effective therapeutic agent for inhibiting bladder cancer invasion, and upregulation of FOXO1 plays a critical role in

Figure 7.
ISO inhibited bladder cancer cell invasion through downregulation of MMP-2 in a FOXO1-dependent manner. UMUC3 (A) and T24T (B) cells were treated with either vehicle or ISO as the indicated concentrations for 18 hours. The protein level of MMP-2 was determined by Western blotting, and GAPDH was used as protein loading control. C, T24T cells were treated with either vehicle or the indicated concentrations of ISO for 18 hours. The MMP-2 and MMP-9 mRNA levels were evaluated by RT-PCR, and gapdh mRNA was used as the internal loading control. D, T24T cells were stably transfected with MMP-2 promoter-driven luciferase reporter, and the stable transfectants were treated with 20 µmol/L of ISO for the indicated times. Dual-Luciferase Reporter Assay System was used to detect the luciferase activity. Results are the mean ± SD of triplicates. Symbol *** indicates a significant difference between vehicle control and ISO-treated groups (P < 0.05). E, the expression of FOXO1 in T24T(Vector) and T24T(Flag-FOXO1) stable transfectants was detected by Western blotting. F, T24T(ShFOXO1-6) and T24T(Nonsense) cells were treated with vehicle or 20 µmol/L of ISO for 18 hours. Cell lysates were subjected to Western blotting with the specific antibodies indicated. G, T24T(ShFOXO1-6) and T24T(Nonsense) cells were transfected with MMP-2 promoter-driven luciferase reporter, and the stable transfectants were then treated with 20 µmol/L of ISO for the indicated times, and cells were then subjected to determine luciferase activity using Dual-Luciferase Reporter Assay. Results are the mean ± SD of triplicates. Symbol *** indicates a significant difference between T24T(ShFOXO1-6) and T24T(Nonsense) cells in the same time point upon ISO treatment (P < 0.05). H, MMP-2 was stably transfected into T24T cells, and the stable transfectant was identified by verification of ectopic expression of MMP-2 protein by Western blotting. The MMP-2 stable transfectant and its vector control transfectant were subjected to cell migration (I and J) and invasion (J and K) assay in the presence of either vehicle or 20 µmol/L of ISO for 24 hours. Results are the mean ± SD of triplicates. Symbol ** indicates a significant difference between Vector transfectant and MMP-2 stable transfectant (P < 0.05). L, the proposed schematic for the cascade underlying ISO inhibition of human bladder cancer cell invasion through downregulation of MMP-2 in FOXO1-dependent manner.
FOXO1 can restore chemosensitivity to anti-EGFR-based therapy may result in improved treatment for muscle-invasive bladder cancers (37, 38). Given that activated EGFR signaling serves as a potential therapeutic target, this biologic effect is highlighted in the previous reports that activated EGFR signaling serves as a potential therapeutic target for muscle-invasive bladder cancers (37, 38). Given that FOXO1 can restore chemosensitivity to anti-EGFR-based therapies both in vitro and in vivo (39), the combination of ISO with anti-EGFR-based therapy may result in improved treatment response through upregulation of FOXO1 expression. FOXO1 has been found to inhibit invasion of other cancers, such as prostate cancer and glioblastoma (12, 13), indicating that treatment of ISO may result in similar therapeutic effects in these cancers. Collectively, our present study provides a valuable information for the design of more effective strategies for utilizing ISO or for the synthesis of other novel conformation-constrained derivatives to treat bladder cancers and other cancers.

FOXO1, which primarily functions as transcription factor, regulates a large spectrum of cancer-related gene expression and thus plays important biologic roles as tumor suppressor in regulating cell-cycle arrest, apoptosis, DNA damage repair, and/or oxidative stress resistance (6, 7). Nevertheless, the FOXO1 signaling in regulation of cell cancer cell invasion is differential, depending on the cancer types and cellular context. Forced expression of FOXO1 has been reported to suppress Runx2-promoted prostate cancer cell migration and invasion (12). Inhibition of FOXO1 nuclear exclusion prevents the metastatic factor MMP-9 activation and cell invasion induced by EGF in glioblastoma (13). However, FOXO1 has been shown to directly regulate transcription of MMP-1 and is thought to promote breast cancer cell metastasis (40). Moreover, FOXO1 is required for keratinocyte migration and wound healing, which involves upregulation of TGFβ1 and its downstream targets (41). To explore the role of FOXO1 in bladder cancer invasion, the expression of FOXO1 was examined in human bladder cancer tissues as well as the adjacent normal bladder tissues surgically removed from 98 patients diagnosed with bladder cancers. We find that FOXO1 was profoundly downregulated in human bladder cancer tissues, and a significant negative relationship was found between foxo1 mRNA expression and bladder cancer invasion, which is consistently supported by the previous study that FOXO1 expression is reduced in the progressed bladder cancer tissues as compared with nonprogressed patients (14). Moreover, we demonstrate that ectopic expression of FOXO1 suppresses cancer cell invasion in both UMUC3 and T24T human-invasive bladder cancer cells. Furthermore, knockdown of FOXO1 further enhances bladder cancer invasion in the absence of ISO treatment, whereas it impairs ISO inhibition of bladder cancer invasion. Thus, our results demonstrate that downregulation of FOXO1 expression in bladder cancer cells. This notion is greatly supported by the results showing that the exogenous FOXO1 protein is not affected by ISO treatment. Meanwhile, the expressions of endogenous protein and mRNA, as well as promoter activity of FOXO1, are significantly induced upon ISO treatment, demonstrating that the increased nuclear-located FOXO1 was regulated at mRNA transcriptional level.

STAT1 is a cytoplasmic protein that is activated via phosphorylation at Tyr701 by IFNs and other cell signals in lymphocytes (45). Following activation, STAT1 is translocated to bind specific regulatory sequences to activate or repress transcription of its targeted genes (45). As IFN-induced STAT1 signaling is primarily implicated in mediating immune antiviral and/or antipathogen functions, suppression of cell proliferation, and the induction of apoptosis in lymphocytes, which is the concept of STAT1 as a tumor-suppressive function (45, 46). Nevertheless, the tumor suppressor recognition of

Figure 8.
ISO reversed the BBN-induced downregulation of FOXO1 and upregulation of MMP-2 in mice. Mice were divided into vehicle-treated control group (n = 12), BBN-treated group (n = 12), and BBN combined with ISO-treated group (n = 12). A, immunohistochemistry paraffin staining by antibodies specific against FOXO1 and MMP-2 was performed. FOXO1 (B) and MMP-2 (C) protein expression levels were analyzed by calculating the integrated IOD/area using Image-Pro Plus version 6.0. Results are the mean ± SD of 12 mice in each group. Symbol “†” indicates a significant difference between vehicle control group and BBN-treated group (P < 0.05). Symbol “*” indicates a significant difference between BBN-treated group and BBN combined with ISO-treated group (P < 0.05).
STAT1 has been shattered by emerging evidence that constitutively activated STAT1 signaling is involved in the resistance to DNA-damaging therapeutic agents of epithelial cancers (45–47). In line with these observations, muscle-invasive bladder cancer tissues were reported to be characterized by constitutively nuclear expression of phosphorylated STAT1 (48). STAT1 has been thought to exhibit a negative regulatory effect on FOXO1 transcription in pancreatic β cells (49); however, this notion has never been explored in any cancer cells. In addition, nothing is known about the mechanism underlying STAT1 regulation of FOXO1 transcription. In the present study, we demonstrate that ISO treatment increases FOXO1 transcription accompanied with repression of STAT1 phosphorylation at Tyr701 in bladder cancer cells. The inhibition of STAT1 activity by overexpression of dominant-negative STAT1 could mimic the biologic effects of ISO treatment, whereas the mutation of STAT1-binding site in FOXO1 promoter region reversed the increased FOXO1 transcription activity induced by ISO treatment. These results reveal a novel function of phosphorylated STAT1 at Tyr701 for its direct binding to FOXO1 promoter resulting in suppression of FOXO1 transcription, which is also the mechanisms for ISO upregulation of FOXO1 transcription and expression, and in turn inhibiting bladder cancer invasion in vivo and mouse-invasive bladder cancer formation in vitro and providing new information for using ISO as a new agent targeting STAT1 and FOXO1 for the bladder cancer therapy.

FOXO proteins could not only act as transcription factors in the nucleus through sequence-specific interaction with DNA-binding sites, but can also cooperate with or titrate away specific transcription factors or cofactors, and subsequently activate or repress the transcription of the downstream genes that lack consensus binding sequences for FOXO factors (50). FOXO1 has been found to physically interact with transcription factor Runx2 and downregulate the transcription of Runx2-targeted endogenous genes, such as the osteopontin (OP), VEGF, and MMP-13, thereby inhibiting prostate cancer cell migration and invasion (12). The present study shows that transcription of MMP-2, a key player in many cancer cell invasion, is downregulated upon ISO treatment. Moreover, knockdown of FOXO1 upregulates the basal expression level of MMP-2 and effectively reverses ISO inhibition of MMP-2 expression and bladder cancer invasion as well, suggesting that FOXO1 plays a negative role in regulation of MMP-2 transcription. Accordingly, the inhibition of BBN-induced muscle-invasive bladder cancer formation by ISO is accompanied by upregulation of FOXO1 and downregulation of MMP-2 expression. As MMP-2 promoter lacks consensus binding site for FOXO1, we anticipate that FOXO1 probably represses MMP-2 transcription indirectly through interacting with other specific transcription factors. Further elucidating of this hypothesis is currently ongoing in our laboratory.

In conclusion, we demonstrate that FOXO1 acts as a tumor suppressor by inhibiting bladder cancer invasion, and the novel derivative anticancer drug ISO specifically represses

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Isorhapontigenin (ISO) Inhibits Invasive Bladder Cancer Formation In Vivo and Human Bladder Cancer Invasion In Vitro by Targeting STAT1/FOXO1 Axis

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