Cyclin D1 Downregulation Contributes to Anticancer Effect of Isorhapontigenin on Human Bladder Cancer Cells

Yong Fang, Zipeng Cao, Qi Hou, Chen Ma, Chunsuo Yao, Jingxia Li, Xue-Ru Wu, and Chuanshu Huang

Abstract
Isorhapontigenin (ISO) is a new derivative of stilbene compound that was isolated from the Chinese herb Gnetum Cleistostachyum and has been used for treatment of bladder cancers for centuries. In our current studies, we have explored the potential inhibitory effect and molecular mechanisms underlying isorhapontigenin anticancer effects on anchorage-independent growth of human bladder cancer cell lines. We found that isorhapontigenin showed a significant inhibitory effect on human bladder cancer cell growth and was accompanied with related cell cycle G0-G1 arrest as well as downregulation of cyclin D1 expression at the transcriptional level in UMUC3 and RT112 cells. Further studies identified that isorhapontigenin down-regulated cyclin D1 gene transcription via inhibition of specific protein 1 (SP1) transactivation. Moreover, ectopic expression of GFP-cyclin D1 rendered UMUC3 cells resistant to induction of cell-cycle G0-G1 arrest and inhibition of cancer cell anchorage-independent growth by isorhapontigenin treatment. Together, our studies show that isorhapontigenin is an active compound that mediates Gnetum Cleistostachyum’s induction of cell-cycle G0-G1 arrest and inhibition of cancer cell anchorage-independent growth through down-regulating SP1/cyclin D1 axis in bladder cancer cells. Our studies provide a novel insight into understanding the anticancer activity of the Chinese herb Gnetum Cleistostachyum and its isolate isorhapontigenin.

Introduction
Bladder cancer is one of the most common cancers in the Western world and the fifth most common cancer in the United States (1). According to the American Cancer Society, 73,510 new cases of bladder cancer are expected to be diagnosed and 14,880 patients will die from this disease in the United States in 2012. Because high-grade invasive bladder cancers can progress to life threatening metastases and are responsible for almost 100% of death from this disease (2, 3), identifying a natural compound that specifically inhibits bladder cancer invasion and metastasis is of tremendous importance for potentially reducing mortality as a result of this disease. Previous studies have addressed the clinical relevance of cyclin D1 alteration in bladder cancer development (4, 5). A significant proportion of bladder cancer cases showed that overexpression of the cyclin D1 gene and increased cyclin D1 expression were associated with poor prognosis and decreased postoperative patient survival (4, 6). Aberrant cyclin D1 expression has been observed early in carcinogenesis as well (7). Cyclin D1 is a key cell-cycle regulatory protein, playing a critical role in the G1-to-S transition of the cell-cycle progression through binding to cyclin-dependent kinase 4 (CDK4) to phosphorylate (8) and inactivate the retinoblastoma protein (pRb; ref. 9), heterozygous deletion of which occurs in approximately 50% of human muscle-invasive bladder cancer. Thus, identifying a new anticancer drug targeting and downregulating cyclin D1 expression and function is one of the first priorities in the field of anticancer research.

Because the multifaced biologic activities of natural oligostibenes, in the past 2 decades, more and more attention has been focused on the anticancer activities of this kind of compound (10, 11). Isorhapontigenin (ISO) is a new derivative of stilbene compound that was isolated from the Chinese herb Gnetum Cleistostachyum, which has been used for treatment of bladder cancers for centuries (12). To determine the anticancer activity and mechanisms of this Chinese herb, in this study, the potential anticancer activity, inhibition of cyclin D1 expression as well as molecular events implicated in these activities were elucidated in human bladder cancer cells.
Materials and Methods

Plasmids, antibodies, and reagents

The GFP-tagged cyclin D1 expression construct was described in our previous publication (13). The cyclin D1 promoter-driven luciferase reporter (cyclin D1 Luc) came from Dr. Anil Rustgi (Gastroenterology Division, University of Pennsylvania, PA; ref. 14). Human cyclin D1 −163 and −163 mSP1 (point mutation at −130 of SP1 binding site) promoter-driven luciferase reporter was a gift from Dr. Richard G. Pestell (Kimmel Cancer Center, Thomas Jefferson University, PA; ref. 15). The transcription factor Specific protein 1 (SP1) luciferase reporter, containing 3 consensus SP1 binding sites, was kindly provided by Dr. Farnham Peggy J (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI; ref. 16). The antibodies against p53, P-ATFII, were purchased from Cell Signaling Technology. The antibodies against CDK4, CDK6, FOS (C-FOS), cyclin A, cyclin B1, cyclin D1, cyclin E, p21, and SP1 were obtained from Santa Cruz Biotechnology. The antibodies against c-Jun, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), nuclear factor kappa B (NF-kB) p65, p-c-Jun Ser 63, p-c-Jun Ser 73, and p-NF-kB p65 were obtained from Cell Signaling Technology. The antibody against heat shock factor-1 (HSF-1) was obtained from Stressgen Biotechnologies Inc.. The antibody against p27 was obtained from Abcam Inc.. Isorhapontigenin with purity more than 99% was obtained from Dr. Qi Hou (Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China). Isorhapontigenin was dissolved in dimethyl sulfoxide (DMSO) to make a stock concentration at 10 mmol/L and the same concentration (0.1%, v/v) of DMSO was used as a negative control in all experiments.

Cell culture and transfection

Human bladder cancer cell line RT4, RT112, and UMUC3 were provided by Dr. Xue-Ru Wu (Departments of Urology and Pathology, New York University School of Medicine, New York, NY; ref. 17). Normal mouse epidermal cell line C141 cells were provided by Dr. Zigang Dong (Hormel Institute, University of Minnesota, Austin, MN; ref. 18–20) and was cultured with Eagle’s Minimum Essential Medium with 5% FBS, 2 mmol/L L-glutamine, and 25 μg/mL gentamycin. All cell lines were subjected to DNA tests and was cultured with accessing water ad libitum overnight. Mice were then administered with isorhapontigenin (150 mg/kg) via gastric gavage. Three mice were sacrificed and blood samples were taken at each time points of 0.033, 0.083, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, and 4 hours after isorhapontigenin was given. The serum was collected from each mouse by centrifuging of blood sample at 4,000 rpm for 30 minutes and stored at −20°C for further analyses. To determine pharmacokinetics of isorhapontigenin in serum of mice, a 50 μL aliquot of each serum sample was transferred to 1.5 mL polypropylene tubes, and 300 μL methanol (LC grade) was added to each sample with vortex for 5 minutes. After centrifugation for 10 minutes at 10,000 rpm, the supernatant was filtered through 0.45 μm filter membrane and then applied to the liquid chromatography/tandem mass spectrometry (LC/MS-MS). The LC/MS-MS system that was used consisted of an Applied Biosystems Sciex QTrap 5500 mass spectrometer (Thornhill) coupled to a Shimadzu UPLC system (Shimadzu). Isorhapontigenin and IS naringenin were separated on a Shimpack C18 ODS column (150 mm × 2.3 mm id, 3 μm particle size) with a gradient elution of the mobile phase system consisting of 0.1% acetic acid solution (A) and methanol (B). The elution

DMEM with 2 mmol/L L-glutamine and 25 μg/mL gentamycin and cultured for 24 hours. The cells were then exposed to isorhapontigenin (5 μmol/L) for the indicated time. The isorhapontigenin-treated and control cells were harvested and fixed in 75% ethanol overnight. The cells were then suspended in staining buffer [containing 0.1% Triton X-100, 0.2 mg/mL RNase A, and 50 μg/mL propidium iodide (PI)] at 4°C for 1 hour and then DNA content was determined by flow cytometry using a Epics XL flow cytometer (Beckman Coulter Inc.) and EXPO32 software as previously described in ref. 13.

Anchorage-independent growth assay

The potential isorhapontigenin inhibitory effect of anchorage-independent growth (soft agar assay) on human bladder cancer cells was determined in UMUC3 cell line (21). In brief, 1 × 10⁴ UMUC3 cells were exposed to various concentrations of isorhapontigenin in 10% FBS Basal Medium Eagle (BME) containing 0.33% soft agar and were seeded over bottom layer of 0.5% agar in 10% FBS/BME in each well of 6-well plates. The cultures were maintained at 37°C in 5% CO₂ incubator for 21 days and the cell colonies with more than 32 cells were scored, as described in our previous studies (21, 22). Colonies were observed and counted under microscope. The results were presented as mean ± SD of colony number per 10,000 seeded cells in soft agar from 3 independent experiment wells.

Animal experiment and isorhapontigenin pharmacokinetics analysis in vivo

Thirty Wistar male mice, weighing 20 to 25 g, were purchased from Experimental Animal Center of the Chinese Academy of Military Medical Sciences and kept under controlled conditions with a 12-hour light cycle with accessing water ad libitum overnight. Mice were then administered with isorhapontigenin (150 mg/kg) via gastric gavage. Three mice were sacrificed and blood samples were taken at each time points of 0.033, 0.083, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, and 4 hours after isorhapontigenin was given. The serum was collected from each mouse by centrifuging of blood sample at 4,000 rpm for 30 minutes and stored at −20°C for further analyses. To determine pharmacokinetics of isorhapontigenin in serum of mice, a 50 μL aliquot of each serum sample was transferred to 1.5 mL polypropylene tubes, and 300 μL methanol (LC grade) was added to each sample with vortex for 5 minutes. After centrifugation for 10 minutes at 10,000 rpm, the supernatant was filtered through 0.45 μm filter membrane and then applied to the liquid chromatography/tandem mass spectrometry (LC/MS-MS). The LC/MS-MS system that was used consisted of an Applied Biosystems Sciex QTrap 5500 mass spectrometer (Thornhill) coupled to a Shimadzu UPLC system (Shimadzu). Isorhapontigenin and IS naringenin were separated on a Shimpack C18 ODS column (150 mm × 2.3 mm id, 3 μm particle size) with a gradient elution of the mobile phase system consisting of 0.1% acetic acid solution (A) and methanol (B). The elution
program was conducted with flow rate at 0.2 mL/minute under column temperature at 30°C. The mass spectrometer was conducted using electrospray ionization (ESI) with an ionspray voltage of –4,500 V and 550°C. The negative ion multiple-reaction-monitoring (MRM) mode analysis was conducted using nitrogen as the collision gas. Precursor/product ion pairs for isorhapontigenin and naringenin were m/z 257.0/241.1 and m/z 271.1/151.1. Data acquisition and processing were carried out using Sciex Analyst 1.5.1 software package (SCIEX).

Western blotting assay
After the cells were exposed to the indicated concentration of isorhapontigenin or for the indicated time with 5 μmol/L isorhapontigenin, cells were extracted in a cell lysis buffer (10 mmol/L Tris-HCl (pH 7.4), 1% SDS, and 1 mmol/L Na3VO4) and total protein was quantified using a DC protein assay kit (Bio-Rad). The membranes were probed with the indicated primary antibodies and the AP-conjugated second antibody. Signals were detected by the ECF Western blotting system, as previously described (23).

Reverse transcription PCR
Total RNA was extracted with TRIzol reagent (Invitrogen Corp.) after isorhapontigenin treatment and the cDNAs were synthesized with the Thermo-Script RT-PCR system (Invitrogen Corp.). The mRNA amount present in the cells was measured by semiquantitative reverse transcription (RT)-PCR. The primers were 5'- AGAACGGCTGGGCTCATTTG-3' and 5'- AGGGGCCCATTCCA CAGTCTTC -3' for human GAPDH, and 5'- GAGGTCTGGGAGGAACA GAAGTG-3' and 5'- GAGGGGCGGATTGAAATGAACCTC-3' for human cyclin D1. The PCR products were separated on 2% agarose gels and stained with ethidium bromide, and the images were scanned with a UV light.

Biinformatic analysis
Cyclin D1 promoter region was analyzed for potential transcription factor binding sites using TFANSFAC Transcription Factor Binding Sites Software (Version 7.0).

Statistical methods
Student t test was used to determine the significance of differences between different groups. The differences were considered to be significant at P < 0.05.

Results
Isorhapontigenin inhibited cell proliferation and anchorage-independent growth, and induced G0-G1 growth arrest in human bladder cancer UMUC3 cell line
The chemical structure of isorhapontigenin is a chemical compound 4-methoxyresveratrol with a molecular weight of 258 as described in our published study (Fig. 1A; ref. 21). To evaluate the potential inhibition of isorhapontigenin in human bladder cancer, we first examined the effects of isorhapontigenin on cell viability in noncancerous C141 cells, noninvasive human bladder tumor cell line RT4, and high invasive human bladder cancer cell line UMUC3. As shown in Fig. 1B, UMUC3 and RT4 cells with isorhapontigenin treatment at concentration of 5 to 60 μmol/L for 48 hours resulted in significant reduction of cell viability in a concentration-dependent manner in ATPase activity assays. The IC50 of the UMUC3 and RT4 cell lines was 22.4 ± 3.3 μmol/L (n = 3) and 38.6 ± 2.9 μmol/L (n = 3) respectively, whereas there was no obvious reduction of cell viability in normal C141 cells. The cell morphology showed that isorhapontigenin at 20 μmol/L induced UMUC3 cells undergoing markedly morphologic changes such as shrinkage, rounding, detachment, and membrane blebbing (Fig. 1C), which is consistent with our most recent findings that isorhapontigenin induced apoptosis in UMUC3 and other invasive
bladder cancer cells at 20 μmol/L (21). More importantly, isorhapontigenin at concentration of 5 μmol/L did show inhibition of cell proliferation (Fig. 1B and C) without induction of observable apoptosis in UMUC3 cells (Fig. 1D). This notion was further verified with the results obtained from cell-cycle and apoptotic analyses by flow cytometry. Exposure of subconfluent UMUC3 cells to 5 μmol/L isorhapontigenin led to significant induction of G₀–G₁ growth arrest at both 12 (47.58% vs. 57.98%) and 24 hours (47.58% vs. 62.62%; Fig. 1D and E) respectively, whereas it did not induce any increases of apoptotic cells (Fig. 1D). These results suggested that the inhibition of high invasive bladder cancer MUMC3 cell proliferation by low concentration (5 μmol/L) of isorhapontigenin was associated with its induction of cell G₀–G₁ growth arrest. To determine whether a low concentration of isorhapontigenin was able to inhibit anchorage-independent growth of bladder cancer cells, UMUC3 was exposed to isorhapontigenin in soft agar. As shown in Fig. 2A and B, isorhapontigenin also markedly inhibited anchorage-independent growth in a concentration-dependent manner at concentration as low as 5 μmol/L (P < 0.01), indicating that isorhapontigenin induction of cell G₀–G₁ growth arrest might be associated with its anticancer activity in high invasive human bladder cancers.

To determine whether isorhapontigenin concentrations (5–20 μmol/L) used in current in vitro studies are reachable animal models in vivo, 30 Wistar male mice were administered via gastric gavage with isorhapontigenin (150 mg/kg). Blood samples from each group (n = 3) were taken at each time points of 0.033, 0.083, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, and 4 hours after isorhapontigenin was given. The serum was collected for determination of isorhapontigenin concentration in serum of mice using LC/MS-MS system. The mean of isorhapontigenin concentration versus time profiles was shown in Table 1 and the corresponding curve is shown in Fig. 2C following oral administration of 150 mg/kg of isorhapontigenin. The pharmacokinetic parameters of isorhapontigenin were obtained by DAS 3.0 computer software analysis using noncompartmental model and summarized in Table 2. Maximum observed concentration (C_max) at 12.35 μg/mL (47.9 μmol/L) in mouse serum rapidly reached at 0.17 hours (10 minutes). The elimination half-life of isorhapontigenin was 1.7 hours and the MRT was 0.7 hours in vivo. The results showed that isorhapontigenin oral administration could result in a rapid absorption in mice, and 5 to 20 μmol/L of isorhapontigenin concentrations applied in current in vitro studies are reachable in vivo mice.
Isorhapontigenin treatment downregulated cyclin D1 protein expression in human bladder cancer cells

The results above showed that isorhapontigenin pre-treatment led to a G0–G1 phase growth arrest. To elucidate the molecular mechanisms underlying this biological effect of isorhapontigenin, we determined the alteration in cyclin D1 expression upon isorhapontigenin treatment. Treatment of UMUC3 with different concentrations of isorhapontigenin for 24 hours resulted in a concentration-dependent reduction of cyclin D1 protein expression compared with the DMSO-treated cells (Fig. 3A and E), whereas it did not show observable inhibition of other cycle regulators, including cyclin A, cyclin E, cyclin B1, CDK4, CDK6, p53, p27, and p21 (Fig. 3A). As isorhapontigenin at 5 μmol/L showed the induction of cell-cycle arrest without any apoptotic effect, it was used for the time course investigation and in the following experiment. Similarly, the isorhapontigenin showed a markedly inhibition of cyclin D1 expression in a high-grade RT112 cell line (Fig. 3B), a slight inhibition in a low-grade human RT4 cell line (Fig. 3C), and marginal induction of cyclin D1 in a normal Cl41 cell line (Fig. 3D). The significant reduction in cell proliferation was also observed in soft agar assay (Fig. 2A–C). As expected, isorhapontigenin induced a dose-dependent growth arrest in human bladder cancer cells.

Table 1.

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<tr>
<th>Time (hour)</th>
<th>C_{average} \pm SD (μg/mL)</th>
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<tr>
<td>0.033</td>
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<td>0.083</td>
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<td>0.750</td>
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<td>1.000</td>
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<td>1.500</td>
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<td>2.000</td>
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<td>4.000</td>
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*Results represent the mean ± SD of three independent experiments.*

AUC(0–t), area under the curve from the time of dosing to the last measurable concentration or the time of the last observation.
MRT(0–t), mean residence time.
t1/2z, terminal half-life.
T_{max}, time of maximum observed concentration.
CLz/F, apparent clearance.
C_{max}, maximum observed concentration.
of cyclin D1 expression by isorhapontigenin could be observed as early as 6 hours upon isorhapontigenin treatment in both UMUC3 cells (Fig. 4A and B) and RT112 cells (Fig. 4C). Consistently, expression of cyclin A, cyclin E, CDK4, CDK6, p53, p27, and p21 were not affected under the same experimental conditions and cyclin B1 expression was slightly reduced at 24 hours of treatment by isorhapontigenin in UMUC3 cells (Fig. 4A). These results suggest that isorhapontigenin downregulates cyclin D1 expression, and that might be associated with its induction of G0–G1 growth arrest in human bladder cancer cells.

**Ectopic expression of GFP-cyclin D1 in UMUC3 cells rendered the transfectant resistant to G0–G1 growth arrest induction and anchorage-independent growth inhibition by isorhapontigenin**

To evaluate the contribution of cyclin D1 downregulation by isorhapontigenin to cell-cycle and anchorage-independent growth inhibition, UMUC3 cells were transfected with a plasmid encoding a GFP-cyclin D1 fusion protein. Ectopic expression of GFP-cyclin D1 in UMUC3 cells rendered the transfectant resistant to isorhapontigenin-induced G0–G1 growth arrest and anchorage-independent growth inhibition.

**Figure 3.** Isorhapontigenin downregulated cyclin D1 protein expression. A–D, protein expressions, as indicated in UMUC3, RT112, RT4, and CI41 cells, were determined with Western blotting after cells were treated with indicated concentrations of isorhapontigenin for 24 hours. E, quantitative analysis of cyclin D1 expression relative to GAPDH (ratio of cyclin D1/GAPDH) in isorhapontigenin-treated UMUC3 cells using the scanning software. *, indicates a significant difference from medium control (P < 0.01, n = 3).

**Figure 4.** Isorhapontigenin downregulated cyclin D1 protein expression in UMUC3 and RT112 cells. Protein expression as indicated in UMUC3 (A) and RT112 (C) cells were determined with Western blotting after cells were treated with 5 μmol/L of isorhapontigenin for the indicated time periods. GAPDH was used as the protein loading control. B, quantitative analysis of cyclin D1 expression relative to GAPDH (ratio of cyclin D1/GAPDH) in 5 μmol/L isorhapontigenin-treated UMUC3 cells using the scanning software. *, **, indicates a significant difference from medium control (n = 3).
independent growth regulation, we stably transfected GFP-cyclin D1 expression construct into UMUC3 cells and the stable transfectant UMUC3 (GFP-cyclin D1) was established, as indicated in Fig. 5A. UMUC3 (GFP-cyclin D1) and its vector control transfectant UMUC3 (GFP) were exposed to isorhapontigenin for determination of ectopic expression of GFP-cyclin D1 on regulation of cell-cycle and anchorage-independent growth. As shown in Fig. 5A, isorhapontigenin treatment only downregulated endogenous cyclin D1 protein expression, and not exogenous GFP-cyclin D1 expression. Consistent with isorhapontigenin effects on endogenous cyclin D1 and exogenous GFP-cyclin D1 protein expression, isorhapontigenin-induced a G0–G1 growth arrest in UMUC3(GFP) cells (62.74% vs. 74.60%) was impaired by ectopic expression of GFP-cyclin D1 in UMUC3 cells (Fig. 5B). More importantly, isorhapontigenin inhibition of anchorage-independent growth in UMUC3 (GFP) cells was reversed by ectopic expression of GFP-cyclin D1 in UMUC3 cells (Fig. 5C). These results show that downregulating of cyclin D1 expression mediates isorhapontigenin induction of G0-G1 growth arrest and inhibition of anchorage-independent growth of UMUC3 cells.

Isorhapontigenin downregulated cyclin D1 expression at transcriptional level

Our above results that isorhapontigenin treatment only downregulated endogenous cyclin D1 protein expression but not exogenous GFP-cyclin D1 expression, excluded the possibility of isorhapontigenin inhibiting cyclin D1 expression at regulation of protein stability. To further elucidate the underlying mechanisms of isorhapontigenin-induced downregulation of cyclin D1 protein expression, we examined the effect of isorhapontigenin on cyclin D1 mRNA expression. As shown in Fig. 6A and B, UMUC3 cells treatment with isorhapontigenin resulted in a marked reduction of cyclin D1 mRNA in concentration- and time-dependent manners, which was consistent with the results obtained at protein levels. These results indicate that isorhapontigenin treatment attenuates cyclin D1 expression at either the transcription level or mRNA stability level. To test whether transcription was involved in cyclin D1 downregulation by isorhapontigenin, the cyclin D1 promoter-driven luciferase reporter was stably transfected into UMUC3 cells. The results showed that treatment of UMUC3 cells with isorhapontigenin led to a marked inhibition of cyclin D1 promoter transcriptional activity in a time-dependent manner (Fig. 6C). These results indicated that isorhapontigenin mainly regulated...
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Isorhapontigenin downregulated transcription factor SP1 expression

To identify the related nuclear transcription factors responsible for the downregulation of cyclin D1 by isorhapontigenin, we used the TFANsFAC Transcription Factor Binding Sites Software (Version 7.0) to bioinformatic analysis of the cyclin D1 promoter region. The results revealed that promoter region of the human cyclin D1 gene contained multiple putative DNA-binding sites of transcription factors, including Activator protein 1 (AP-1), HSF-1, activating transcription factor 2 (ATFII), NF-κB, and SP1. We further determined protein expression and nuclear translocation of those transcription factor components upon isorhapontigenin treatment. The results showed that isorhapontigenin (5 μmol/L) treatment only downregulated SP1 protein expression (Fig. 6D and E), whereas it did not show any observable inhibition of other transcription factor expression, activation, or nuclear translocation, including c-FOS, p-c-JUN(ser 73), p-c-JUN(ser63), c-JUN, HSF-1, p-ATFII, p-NF-xB p65, or NF-xB p65 (Fig. 6D), thus suggesting that SP1 was a major transcription factor that might be targeted by isorhapontigenin for downregulation of cyclin D1 transcription. To determine the effect of isorhapontigenin on SP1-depedent transcriptional activity, SP1-luciferase reporter was transfected into UMUC3 cells to establish the stable transfectant. Isorhapontigenin treatment led to a dramatically inhibition of SP1-dependent transcriptional activity in a time-dependent manner (Fig. 7B). These results indicated that isorhapontigenin not only inhibited SP1 protein expression and its nuclear translocation, it also inhibited its dependent transcriptional activity.

The transcription factor SP1 binding sites in cyclin D1 promoter region was represented in schematic diagram in Fig. 7A. Previous studies reported that deletion of the promoter sequentially from −163 to −22 dramatically reduced cyclin D1 promoter activity (15). To identify the promoter regions that were necessary for isorhapontigenin downregulating cyclin D1 expression, and to understand the mechanisms that regulate this expression, the wild-type −163 cyclin D1(WT- Cyclin D1-Luc) and mutated −163SP1 cyclin D1 (SP1mut-Cyclin D1-Luc) luciferase reporters were cotransfected with pSuper plasmid into UMUC3 cells, respectively, and the stable transfectants UMUC3/WT-Cyclin D1-Luc and UMUC3/SP1mut-Cyclin D1-Luc, were established. As shown in Fig. 7C, isorhapontigenin treatment inhibited cyclin D1 transcription in UMUC3/WT cyclin D1-Luc transfectant, whereas this treatment did not show a significant inhibition of cyclin D1 transcription in UMUC3/SP1mut-cyclin D1-Luc transfectant, suggesting...
that isorhapontigenin’s inhibition of cyclin D1 transcription was specifically targeting SP1.

**Isorhapontigenin impaired SP1 binding to its binding site in cyclin D1 promoter**

To test whether downregulation of the SP1 level by isorhapontigenin was associated with its specific binding to cyclin D1 promoter *in vivo*, we conducted ChIP assays followed by PCR with primers, specifically targeting SP1 binding region from −92 to +27 in the human cyclin D1 promoter region in UMUC3 cells (15). As shown in Fig. 7D, SP1 showed its binding to cyclin D1 promoter region between −92 to +27, and this binding was impaired in the cells treated with isorhapontigenin (5 μmol/L). Taken together, the above results showed that isorhapontigenin inhibited cyclin D1 promoter transcription activity in WT cyclin D1 reporter, but not in SP1-mutant reporter (Fig. 7C). We anticipated that downregulation of cyclin D1 transcription induced by isorhapontigenin was mediated by its targeting and inhibiting SP1 expression, transactivation, and specific binding to SP1 binding sites of cyclin D1 promoter region as summarized in Fig. 7E.

**Discussion**

Isorhapontigenin is isolated from the *Gnetum Cleistostachyum*, and belongs to a group of naturally occurring polyhydroxy stilbenes (27). Several studies have indicated that isorhapontigenin exhibits an inhibitory effect on oxidized low-density lipoprotein (oxLDL)-induced proliferation and mitogenesis of bovine aortic smooth muscle cells (28). Isorhapontigenin also inhibits cardiac hypertrophy by anti-oxidative activity and attenuating oxidative stress-mediated signaling pathways (29). Isorhapontigenin has been used for treatment of bladder cancers for centuries. There are reports of side effects from super-high dose (6,000 mg/kg/d) application of the Chinese herb *Gnetum Cleistostachyum* in clinical patients (30), which include dry mouth and dizziness, followed by blurred vision, dry nasopharynx, and stomach pain. Our most recently published results also indicate that isorhapontigenin at concentration over 20 μmol/L show apoptosis in human bladder cancer cells via downregulation of XIAP expression, whereas at concentration at lower than 20 μmol/L, such as the concentration used in the current studies, do not show cytotoxic effect on human bladder...
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cancer cell lines (21). Moreover, the pharmacokinetics of isorhapontigenin in mice indicated that the maximum observed concentration (Cmax) could reach to 47.9 μmol/L in mouse serum, suggesting that 5 to 20 μmol/L of isorhapontigenin concentrations applied in current in vitro studies are relevant to in vivo, and further providing crucial information in future isorhapontigenin application in either animal studies or clinical trials.

We find that isorhapontigenin at concentration within 20 to 60 μmol/L exhibits a significant inhibitory effect on anchorage-independent growth, a marked apoptotic induction, as well as downregulation of X-linked inhibitor of apoptosis protein (XIAP) in human bladder cancer cells, whereas overexpression of exogenous HA-XIAP reverses the apoptotic effects and colony formation inhibition by isorhapontigenin at concentration of 20 to 60 μmol/L (21).

In the current studies, we explored the potential inhibitory effect of isorhapontigenin at nonapoptotic low concentration on anchorage-independent growth, cell-cycle alteration, and the molecular mechanisms underlying these biologic effects in high-grade bladder cancer cell lines, UMUC3 and RT112 cells. We found that isorhapontigenin not only inhibited anchorage-independent cell growth of cancer cell lines, it also induced cell-cycle G0–G1 arrest in a non–cell death concentration of 5 μmol/L in high-grade bladder cancer cell lines, UMUC3 and RT112 cells, whereas it only showed a slight inhibition of cyclin D1 expression in low-grade human bladder tumor RT4 cells. Moreover, we observed that isorhapontigenin had no inhibitory effect on cell proliferation and cyclin D1 expression in noncancerous CH1 cells, suggesting that isorhapontigenin might have a strong inhibitory effect on invasive cancers, rather than low-grade and noncancerous cells. Further studies indicated that the isorhapontigenin anticaner activity was mediated by its downregulation of cyclin D1 expression via direct inhibition of SP1 transactivation and binding activity to cyclin D1 promoter region.

Growing evidence had indicated that cell-cycle alterations occur in responses of cells to various carcinogens (31, 32). Cyclin D1 is one of the key regulators in the control of cell-cycle progression from G0–G1 to S-phase, and inducible cyclin D1 forms a complex with CDK4/6, which phosphorylates the retinoblastoma tumor suppressor protein (33), sequesters pRb growth inhibitory effects on E2F and enables E2F transcription factors to transcriptional regulate genes required for entry into the DNA synthetic phase (S) of the cell division cycle (34). Cyclin D1 overexpression prevails over that of cyclin D2 and D3 (35), and overexpression of cyclin D1 is one of the cancer features and is responsible for inducing excessive cellular proliferation in many human cancers, including bladder cancer (4), breast (36), cervix (37), colon (38), prostate (39), and skin cancer (40). Thus, cyclin D1 is one of the most frequently altered cell-cycle–regulating protein in cancers and therefore, is a potential therapeutic target (41, 42). For example, Meng and colleagues showed that cyclin D1–associated protein, PACSIN 2, regulates cell spreading and migration, both of which are dependent on cyclin D1 expression (43). Li and colleagues showed cyclin D1-deficient mouse embryo fibroblasts (MEF) exhibited increased adhesion and decreased motility compared with wild-type MEFs (44). Molecular approaches for targeting cyclin D1 expression include cyclin D1α isofrom (full-length cyclin D1) with a small-molecule CDK4/6 inhibitor PD032991 (45), siRNA (42, 46), genomic deletion of cyclin D1 gene (43), and modulation of glycogen synthase kinase 3β (GSK3β) activity (47). However, the major limitations of these genomic therapies are their poor stability, poor membrane permeability, and inadequate stable transfection efficiency (48). While there are no chemical inhibitors targeting cyclin D1 so far, identifying and exploring a natural compound that specifically downregulates cyclin D1 expression is of tremendous importance for cancer therapy and the reduction of mortality as a result of cancers. In current studies, we identified that isorhapontigenin at a concentration as low as 5 μmol/L was able to downregulate cyclin D1 expression at the transcriptional level. At this level, isorhapontigenin exhibited its induction of G0–G1 cell-cycle arrest and inhibition of anchorage-independent growth of human high-grade bladder cancer cells, without affecting cell viability or the other cell-cycle regulators, including cyclin A, cyclin B, cyclin E, CDK4, and CDK6. These findings show isorhapontigenin as a novel mechanism-based cancer therapeutic agent against human bladder cancer, and provide a basis for possible clinical trials exploring the usefulness of isorhapontigenin as a preventive and therapeutic agent against bladder and other cancers with abnormal expression of cyclin D1 in patients.

Cyclin D1 levels could be regulated at transcriptional and posttranscriptional levels (49). The signaling pathways that have been reported to regulate cyclin D1 expression include NF-κB (50), SP1 (15, 51), ras/mitogen-activated protein kinases (MAPK; ref. 52), phosphoinositide 3-kinase (PI3K)/Akt (53, 54), and GSK3β/β-catenin (55). Cyclin D1 promoter was first reported almost 20 years ago (56, 57), and many transcription factors have been identified to directly bind to, or otherwise regulate, the cyclin D1 promoter (36, 58). SP1 was an important transcription factor involved in the regulation of many gene expression and cellular functions, including cyclin D1 (5, 15, 51, 59). Here, we show that the isorhapontigenin-mediated transcriptional downregulation of the cyclin D1 gene was achieved by inhibition of transcription factor SP1. Our results indicate that isorhapontigenin treatment downregulated cyclin D1 expression, accompanied by its inhibition of transcription factor SP1 expression, transcription, and binding activity to the cyclin D1 promoter region. Our studies further showed that putative SP1 binding sites were between −92 and +27 bp with the 5′-untranslated region, which is consistent with the previous finding regarding SP1-mediated regulation of cyclin D1 expression (15). On the basis of our results obtained from ChIP assay, SP1 was found to be a major participant transcription factor binding to the GC-box site of the cyclin
D1 promoter and downregulating cyclin D1 transcription upon isorhapontigenin treatment.

In summary, our studies show that isorhapontigenin is an active compound that is responsible for Gnetum Cleistostachyum inhibition of bladder cancer cell anchorage-independent growth. This anticancer activity of isorhapontigenin is mediated by its downregulation of cyclin D1 expression, and in turn, its induction of cell-cycle G0–G1 arrest via specific targeting of transcription factor SP1 in bladder cancer cells. Our studies provide a novel insight into understanding the anticancer activity of the Chinese herb Gnetum Cleistostachyum isolate, isorhapontigenin, as proposed in Fig. 7E. Although in vivo animal verification and extensive in vitro studies will be required for further translational application of isorhapontigenin in the management of clinical patients, particularly gene models with highly expressed cyclin D1, the understanding of the molecular mechanisms responsible for isorhapontigenin action would provide valuable information for the design of more effective strategies for use of isorhapontigenin in therapy and prevention of high-grade bladder cancers, to substantially impact the field of bladder cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Fang, Q. Hou, X.R. Wu, C. Huang

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