The Chinese Herb Isolate Isorhapontigenin Induces Apoptosis in Human Cancer Cells by Down-regulating Overexpression of Antiapoptotic Protein XIAP*

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Background: The anti-cancer activity and mechanisms of isorhapontigenin (ISO) have never been explored.

Results: ISO exhibited an anti-cancer activity accompanied by apoptotic induction and XIAP down-regulation through attenuation of SP1 expression.

Conclusion: ISO is an active anti-cancer compound by inducing apoptosis via down-regulation of SP1/XIAP pathway.

Significance: Current studies identify a new promising active compound for therapy of human cancers with XIAP overexpression.

Although the Chinese herb Gnetum cleistostachyum has been used as a remedy for cancers for hundreds of years, the active compounds and molecular mechanisms underlying its anti-cancer activity have not been explored. Recently a new derivative of stilbene compound, isorhapontigenin (ISO), was isolated from this Chinese herb. In the present study, we examined the potential of ISO in anti-cancer activity and the mechanisms involved in human cancer cell lines. We found that ISO exhibited significant inhibitory effects on human bladder cancer cell growth that was accompanied by marked apoptotic induction as well as down-regulation of the X-linked inhibitor of apoptosis protein (XIAP). Further studies have shown that ISO down-regulation of XIAP protein expression was only observed in endogenous XIAP, but not in constitutionally exogenously expressed XIAP in the same cells, excluding the possibility of ISO regulating XIAP expression at the level of protein degradation. We also identified that ISO down-regulated XIAP gene transcription via inhibition of Sp1 transactivation. There was no significant effect of ISO on apoptosis and colony formation of cells transfected with exogenous HA-tagged XIAP. Collectively, current studies, for the first time to the best of our knowledge, identify ISO as a major active compound for the anti-cancer activity of G. cleistostachyum by down-regulation of XIAP expression and induction of apoptosis through specific targeting of a SP1 pathway, and cast new light on the treatment of the cancer patients with XIAP overexpression.

Gnetum cleistostachyum, a Chinese herb that grows in the YunNan province of Southwestern of China, has been used for treatment of arthritis, bronchitis, cardiovascular system disease, and several cancers including bladder cancer for hundreds of years (1). ISO is a new derivative of stilbene, recently isolated from the Gnetum cleistostachyum (2). Increasing attention has been given to elucidating anti-cancer activity of natural oligostilbenes in the last 20 years because more and more of their multifaceted biological properties are being identified. For example, through attenuating the generation of reactive oxygen species and activation of the extracellular signal-regulated kinases (ERKs) pathway, ISO exhibits the inhibitory effect on oxidized low-density lipoprotein-induced proliferation and mitogenesis of bovine aortic smooth muscle cells (3). ISO also inhibits cardiac hypertrophy by antioxidative activity and attenuates oxidative stress-mediated signaling pathways, such as protein kinase C (PKC)-dependent phosphatidylinositol 3-kinases (PI3K)-AKT-GSK3/p70S6K pathway (4). However, the potential anti-cancer activity of ISO has never been explored.

As a potent and ubiquitous caspase inhibitor (5), X-linked inhibitor of apoptosis protein (XIAP)2 has garnered the most attention as a promising therapeutic target for overcoming drug resistance (6). Our most recent studies also demonstrate that there is a novel XIAP function that acts as a crucial regulator for controlling cancer cell motility and invasion via its RING domain interaction with the RhodGDI dissociation inhibitor (RhodGI), and subsequent negative modulation of RhodGI SUMOylation at Lys-138 (7). It was accepted that

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‡ The abbreviations used are: XIAP, X-linked inhibitor of apoptosis protein; ISO, isorhapontigenin; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinases; FRA-1, Fos-related antigen-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSF-1, heat shock factor-1; IAP, inhibitor of apoptosis protein; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PARP, poly(ADP-ribose) polymerase; RhodGI, Rhod GDP dissociation inhibitor; SP1, specific protein 1.
XIAP overexpression in cancer tissues is associated with cancer progression, metastasis, and resistance to cancer therapy such as immunotherapy, chemotherapy, and radiotherapy (8). Thus, identifying a new anti-cancer drug targeting XIAP expression and function is one of the important priorities in the field of anti-cancer research. In the current study, the anti-cancer activity of ISO and the potential molecular mechanisms implicated in its anti-cancer activities were investigated in human cancer cells.

MATERIALS AND METHODS

Plasmids, Antibodies, and Reagents—cDNA constructs expressing HA-tagged XIAP and the pEBB empty vector were gifts from Dr. Colin S. Duckett (University of Michigan) (9). The transcription factor SP1 luciferase reporter, containing three consensus SP1 binding sites, was kindly provided by Dr. Peggy J. Farnham (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison) (10). Human XIAP promoter-driven luciferase reporter was gift from Dr. Taegkyu Kwon (Ajou University School of Medicine, Suwon, South Korea) (11). The antibodies against XIAP were purchased from Cell Signaling Technology (Boston, MA). The antibodies against c-FOS, FRA-1, JUN-D, P85, and SP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against c-FOS, FRA-1, JUN-D, P85, and SP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against AKT, BCL-2, BCL-xl, CASPASE-3, CIAP-1, c-JUN, GAPDH, NF-κB p65, P-AKT 473, P-AKT308, P-JUN (Ser-63), P-c-JUN (Ser-73), P-NF-κB p65, and poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology (Boston, MA). Antibodies against BAX and PKC-α were obtained from Upstate Biotechnology (Lake Placid, NY). Antibodies against cIAP-2 was obtained from R & D Systems Inc. (Minneapolis, MN). The antibody against HA was obtained from Covance Antibody Service Inc. (Princeton, NJ).

ISO with over 99% purity was provided by Dr. Qi Hou, Mater-ria Medica of Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China. The structure of ISO is shown in Fig. 1A. ISO was dissolved in dimethyl sulfoxide (DMSO) to make a stock concentration at 60 mm and further diluted in DMEM with a final DMSO concentration at 0.1% (v/v) for cell culture experiments. The same amount (0.1%, v/v) of DMSO was used as a negative control in all experiments.

Cell Culture and Transfection—Human UMUC3 and RT112 bladder cancer cell lines were obtained from Dr. Xue-Ru Wu (Departments of Urology and Pathology, New York University School of Medicine) (12), and T24 was kindly provided by Dr. Dan Theodorescu (13). These cell lines were maintained at 37 °C in a 5% CO2 incubator in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 medium supplemented with 10% FBS, 2 μM l-glutamine, and 25 μg/ml of gentamycin, respectively. The human colon cancer cell line HCT116 and HCT116 cells were kindly provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins Medical Institutions) (14), and HCT116 XIAP−/− (HA-XIAP) transfection was stably established by transfection with HA-XIAP as described in our previous studies (7). Stable co-transfections were performed with specific cDNA constructs and/or pSUPER vector using PolyJet™ DNA In Vitro Transfection Reagent (SignaGen Laboratories, Gaithersburg, MD) according to the manufacturer’s instructions. For stable transfection selection, cultures were subjected to hygromycin selection for 4–6 weeks, and surviving cells were pooled as stable mass transfectants.

Anchorage-independent Growth Assay—Anchorage-inde-pendent growth (soft agar assay) in soft agar was carried out as described in our previous studies (15). Briefly, 1 × 104 cells mixed with various concentrations of ISO in 10% FBS, β-mer-captoethanol containing 0.33% soft agar, was seeded over the bottom layer of 0.5% agar in 10% FBS, β-mercaptoethanol in each well of 6-well plates. The plates were incubated in 5% CO2 incubator at 37 °C for 3 weeks. Colonies were inspected under a microscope and only colonies with over 32 cells were counted.

 Luciferase Assay—The XIAP promoter-driven luciferase reporter stable transfection or SP1 luciferase reporter stable transfectant cells (1 × 104) were seeded into each well of 96-well plates and cultured in 5% CO2 incubator at 37 °C until 70–80% confluence. The cells were treated with ISO as indicated, then extracted with lysis buffer (25 mmol/liter of Tris phosphate (pH 7.8), 2 mm EDTA, 1% Triton X-100, and 10% glycerol). The luciferase activity was determined by a micro-plate luminometer LB 96V (Berthold GmbH & Co. KG, Bad Wildbad, Germany) using the luciferase assay system (Promega Corp., Madison, WI) as described previously (17).

 Nuclear Extract Preparation—Preparation of nuclear extracts was assessed as previously described in Ref. 18. For cell culture, cancer cells were plated into 10-cm culture dishes at 80% confluence, treated either with DMSO or 60 μM ISO for 12 h. The nuclear proteins were extracted according to the protocol of the Nuclear/Cytosol Fractionation Kit (BioVison Technologies, Mountain View, CA). Equal protein concentrations were determined using a protein quantification assay kit (Bio-Rad). Nuclear extracts were stored at −80 °C until they were used.
Down-regulating XIAP by Isorhapontigenin in Human Cancer Cells

FIGURE 1. ISO inhibited anchorage-independent growth of human bladder cancer T24T cells. A, the structure of ISO. B, representative images of colonies of T24T cells in soft agar assay without or with various concentrations of ISO. C, colonies were visualized under microscope and only colonies with over 32 cells were counted. Percentage of colony formation inhibition was expressed as relative to medium control in triplicate.

Western Blotting—Western blot assay was assessed as previously described in Ref. 19. Cells were plated in 6-well plates and cultured in normal 10% serum medium until 70–80% confluence. The culture medium was replaced and starved with 0.1% FBS media. After being cultured for 24 h, the cells were exposed to the indicated amount of ISO or indicated time with 60 µM ISO. The cells were washed once with ice-cold phosphate-buffered saline and collected with cell lysis buffer (10 mM Tris-HCl (pH 7.4), 1% SDS, and 1 mM Na3VO4). 80 µg of protein sample from the cell extracts were separated on SDS-PAGE, transferred, and probed with the indicated antibody. The protein band that was specifically bound to the primary antibody was detected using an alkaline phosphatase-linked secondary antibody and an ECF Western blotting system (Amersham Biosciences).

Flow Cytometry Assay—Flow cytometry assay was assessed as previously described in Ref. 20. After the time periods indicated, the ISO-treated and control cells were harvested and fixed in 75% ethanol. The fixed cells were stained in the buffer containing 0.1% Triton X-100, 0.2 mg/ml of RNase A, and 50 µg/ml of propidium iodide at 4 °C for 1 h and then examined by flow cytometry utilizing an EpicsXL flow cytometer (Beckman Coulter Inc., Miami, FL) on the FL3 channel, and the gate was set to exclude debris and cellular aggregates. 20,000 events were counted for each analysis, and three independent experiments for each group were conducted. The subdiploid DNA peak, immediately adjacent to the G0/G1 peak, represented apoptotic cells and was quantified by histogram analyses.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed using the EZ-CHIP kit (Millipore Technologies) according to the manufacturer’s instructions as described in our previous publication (21). Briefly, T24T cells were treated with 0.1% DMSO only or 60 µM ISO for 12 h. Then genomic DNA and the proteins were cross-linked with 1% formaldehyde. The cross-linked cells were pelleted, resuspended in lysis buffer, and sonicated to generate 200–500-bp chromatin DNA fragments. After centrifugation, the supernatants were diluted 10-fold and then incubated with anti-SP1 antibody or the control rabbit IgG at overnight at 4 °C. The immune complex was captured by protein G-agarose saturated with salmon sperm DNA, then eluted with the elution buffer. DNA-protein cross-linking was reversed by heating overnight at 65 °C. DNA was purified and subjected to PCR analysis. To specifically amplify the region containing the putative responsive elements on the human XIAP promoter, PCR was performed with the following pair of primers: 5′-TTTATCCTGATGACCTGTTG-3′ (from −214 to −187) and 5′-TTCTCTATGGATGTCTGCAGGT-3′ (from +39 to +60). The PCR products were separated on 2% agarose gels and stained with ethidium bromide, the images were then scanned with a UV light.

Statistical Methods—Student’s t test was utilized to determine the significance of differences between different groups. The differences were considered to be significant at p < 0.05.

RESULTS

ISO Inhibits Proliferation and Colony Formation of Bladder Cancer Cells—The chemical structure of ISO is 4-methoxyresveratrol (Fig. 1A), with a molecular weight of 258. We first examined the effects of cell proliferation and colony formation by ISO on bladder cancer cells. The T24T bladder cancer cell line was selected for further experiments due to its genetic background and strong tendency of migration. As shown in Fig. 1, B and C, ISO significantly inhibited anchorage-independent growth (colony formation) in a dose-dependent manner (p < 0.01). These data demonstrate the anti-cancer effects of ISO on human bladder cancer cells.

ISO Induces Apoptosis and Inhibits Cell Viability in Multiple Cancer Cell Lines—To assess the effect of ISO on cell viability, T24T was cultured with a range of ISO doses (20–60 µM) for 48 h. Cell viability of these cells was analyzed using the ATPase assay. A significant reduction of cell viability was observed in a dose-dependent manner (Fig. 2A). The IC50 of T24T cell lines was 55.2 ± 2.3 µM (n = 3).

To determine whether ISO could inhibit cell viability due to apoptosis, the DNA cycle of T24T cells were examined. The T24T bladder cells were treated with ISO in concentrations of 0, 20, 40, and 60 µM for 48 h. High-resolution flow cytometric analysis of propidium iodide-stained nuclei revealed a substantial increase in sub-G1 DNA content (apoptotic peak) at 48 h compared with cells treated with 0.1% DMSO (v/v) as a negative control (Fig. 2B).

PARP cleavage is often associated with apoptosis and has served as one of the hallmarks of apoptosis and caspase activation. Therefore, we determined whether induction of T24T cell apoptosis by ISO was mediated through activation of CASPASE-3 and cleavage of PARP proteins. Treatment of
T24T with ISO for 24 h resulted in a dose-dependent increased cleavage of CASPASE-3 and PARP proteins compared with the DMSO-treated cells (Fig. 2C). Because the IC_{50} of the T24T cell line was 55.2 ± 2.3 μM, 60 μM ISO was used in the following experiments. The ISO-induced activation of CASPASE-3 and PARP were also determined and confirmed in a time-dependent manner from 3 to 36 h, with pre-treatment with ISO (Fig. 2D). Additionally, we determined the obvious cleavage of PARP in a dose-dependent manner with pre-treatment with ISO for 24 h in the other 3 cancer cell lines, RT112 bladder cancer (Fig. 2E), UMUC3 bladder cancer cell (Fig. 2F), and HCT116 colon cancer cell (Fig. 2G). These results indicated reproducible apoptotic effects of ISO on different human cancer cell lines.

**ISO Down-regulates XIAP Protein Level in a Dose- and Time-dependent Manner**—Having established that ISO could induce the apoptosis of T24T and other cancer cell lines in a dose- and time-dependent manner, we next focused on the molecular mechanisms of apoptosis induced by ISO. It has been reported that ISO exhibits anti-inflammation, antioxidant effects through suppressing the ERKs, p38, PKCα, NF-κB, and the PI3K/AKT pathway (4). The activation and protein expression levels of those proteins upon ISO treatment were evaluated in T24T cells. As shown as Fig. 3A, there were no significant changes observed, indicating that these pathways may not be implicated in the anti-cancer activity of this ISO compound.

IAPs family and BCL-2 family members are known to be regulators of CYTOCHROME c releasing from mitochondria during apoptosis. In addition to the BCL-2 family, IAP family proteins also regulate CASPASE activity, thus affecting apoptosis. Therefore, the expression of three IAP family proteins, XIAP, cIAP-1, and cIAP-2, were determined. ISO treatment down-regulated the XIAP protein level in dose- and time-dependent manners. However, it did not significantly affect cIAP-1 and cIAP-2 proteins, nor did it have a significant effect on BCL-2 family proteins, such as Bax, BCL-2, and BCL-XL (Fig. 3A). To elucidate the mechanism leading to XIAP down-regulation upon ISO treatment, the N terminally HA-tagged XIAP were transfected into RT112 and HCT116 XIAP^−/− cells, respectively. The pEBB empty vector was transfected at the same time as the control. As shown as Fig. 4A, the exogenous HA-tagged XIAP inhibited the endogenous XIAP expression in RT112 (HA-XIAP) cells. Meanwhile, the results also showed that treatment with ISO did not affect the reconstituted expression of HA-tagged XIAP in RT112 (HA-XIAP) (Fig. 4A) and HCT116 XIAP^−/− (HA-XIAP) cells (Fig. 4B), whereas it attenuated endogenous XIAP expression in the RT112 (vector) and HCT116 (vector) cells. These results strongly excluded the possibility that ISO regulated XIAP protein expression at the degradation level.

**ISO Inhibits XIAP Transcription, but It Does Not Affect XIAP mRNA Stability**—To clarify the underlying mechanisms of ISO down-regulation of XIAP protein expression, we examined mRNA levels of XIAP in comparison to CIAP-1 and CIAP-2. Consistent with the results obtained at protein levels, ISO treatment led to marked reductions of XIAP mRNA in dose- and time-dependent manners, whereas it did not affect the CIAP-1.
that XIAP was not down-regulated at mRNA stability by ISO using actinomycin D. The result showed that ISO did not affect the XIAP mRNA level in actinomycin D-treated T24T cells, whereas it reduced XIAP mRNA expression in T24T cells in the absence of actinomycin D treatment (Fig. 4E), suggesting that ISO did not regulate XIAP mRNA stability.

We further compared the endogenous XIAP mRNA and exogenous HA-XIAP mRNA levels in both RT112 (HA-XIAP) and HCT116 XIAP−/− (HA-XIAP) cells. The results indicated that the exogenous HA-XIAP mRNA showed no observable changes after ISO treatment, whereas the endogenous XIAP mRNA was remarkably down-regulated in RT112 (Vector) and HCT116 (Vector) cells upon ISO treatment (Fig. 4, F and G). These results indicate that ISO regulated XIAP protein expression at the transcriptional level, rather than mRNA stability. This notion was fully supported by the results obtained from T24T stably transfected with the XIAP promoter-driven luciferase reporter. Treatment of T24T XIAP promoter-luciferase cells with ISO resulted in inhibition of XIAP promoter transcription activity in a time-dependent manner (Fig. 4H).

ISO Treatment Suppresses Transcription Factor SP1 Expression, Transactivation, and Specific Binding to XIAP Promoter—To identify the transcription factor responsible for ISO down-regulation of XIAP transcription, TFANSFAC® Transcription Factor Binding Sites Software (Biological Database, Wolfenbüttel, Germany) was used for bioinformatics analysis of the XIAP promoter region. The results revealed that the promoter region of the human XIAP gene contains the putative DNA-binding site of cellular oncogene Fos (c-FOS), c-JUN, FOS-related antigen-1 (FRA-1), heat shock factor-1 (HSF-1), Jun-D, nuclear factor of activated T cells (NF-AT), nuclear factor κB (NF-κB), and specific proteins 1 (SP1) (Fig. 5A). We next examined changes in the nuclear translocation of related transcription factors upon ISO treatment for 12 h. As shown in Fig. 5B, inhibition of SP1 protein expression clearly occurred in the nuclear protein extract pretreated with 60 μM ISO for 12 h. In contrast, there was no significant suppression of the transcription factors of c-FOS, c-JUN, FRA-1, HSF-1, JUN-D, NF-AT, and NF-κB.

To further confirm the mechanism of down-regulation of SP1 by ISO, we tested the effects of ISO on the protein and mRNA levels of SP1 in T24T cells. The results indicated that the and CIAP-2 mRNA levels (Fig. 4, C and D). The results indicated that ISO treatment attenuated XIAP expression at the mRNA level. We further carried out experiments to confirm
SPI1 mRNA level was not reduced even though its protein level was profoundly down-regulated upon ISO treatment (Fig. 5, C and D), suggesting that ISO regulates SPI1 protein expression at levels of either protein translation and/or degradation.

To confirm that down-regulation of XIAP by ISO was mediated by SPI1, we tested the effects of ISO on SPI1 transactivation in T24T cells stably transfected with SPI1-luciferase reporter containing three SPI1 consensus binding sites. The results, as expected, showed that ISO treatment significantly blocked SPI1 transactivation in a time-dependent manner in T24T cells (Fig. 5E).

To study whether down-regulation of the SPI1 level by ISO is associated with SPI1 sites in the XIAP promoter in vitro, we performed a CHIP assay with T24T cells followed by PCR with primers specifically targeting the SPI1 binding region from −214 to +60 in the XIAP promoter. As shown in Fig. 5F, compared with the DMSO control, ISO treatment could suppress the binding of SPI1 to the XIAP promoter region between −214 and +60 (Fig. 5F). Considering that there are two SPI1 binding sites between −214 and +60 (−144 and −25) and that the previous report (11) showed that XIAP promoter activity is significantly decreased by double mutation of two SPI1 sites at −144 and −25, we suggest that ISO down-regulation of XIAP transcription is mediated by its targeting and inhibiting of SPI1 expression, transactivation, and specific binding to SPI1 binding sites, particularly the two SPI1 binding sites at −144 and −25 in the XIAP promoter region as shown in Fig. 5G.

**ISO Down-regulation of XIAP Expression Is Responsible for Its Induction of Apoptotic Responses and Inhibition of Anchorage-independent Growth of RT112 and HCT116 Cells**—The above results demonstrated that ISO treatment dramatically attenuated bladder cancer cell anchorage-independent growth in soft agar assay, a tumor inhibitory activity associated with the down-regulation of XIAP expression and increased cell apoptotic responses. To test the contribution of XIAP down-regulation to the induction of apoptotic responses by ISO, we evaluated the effect of ectopic HA-XIAP expression in both cancer cell line RT112 and HCT116 cells. The results showed that the ISO-induced apoptotic responses were blocked by ectopic expression of HA-XIAP by stable transfection of exogenous HA-tagged XIAP in RT112 (HA-XIAP) (Fig. 6, A and B) and HCT116 XIAP−/− (HA-XIAP) cells (Fig. 6, C and D). These results demonstrated that XIAP down-regulation by ISO was responsible for its induction of apoptotic responses. Considering the importance of the cell apoptotic effect on anti-cancer activity, we anticipated that XIAP down-regulation was further responsible for ISO inhibition of cancer cell anchorage-independent growth in soft agar assay. To test this notion, the effects of ISO on cancer cell anchorage-independent growth was compared between RT112 and HCT116 cells with or with-
out ectopic expression of exogenous HA-tagged XIAP protein. As shown in Fig. 7, the inhibition of anchorage-independent growth by ISO treatment in RT112 and HCT116 cells was almost completely reversed in ectopic introduction of exogenous HA-XIAP. The results are fully consistent with HA-XIAP inhibition of ISO-induced apoptosis in both cell lines, further supporting our conclusion that ISO down-regulation of XIAP expression is a major mechanism responsible for its induction of apoptotic response and inhibition of cancer cell anchorage-independent growth, as well as anti-cancer effect.

DISCUSSION

The present study explores the pro-apoptotic and anti-cancer effects of ISO in human bladder cancer RT112, UMUC3, and T24T cell lines and the colon cancer HCT116 cell line. The data reported here supports the notion that ISO cannot only inhibit anchorage-independent cell growth of cancer cell lines, but also down-regulates XIAP expression and induces apoptosis for the same cancer cells. The down-regulation of XIAP by ISO is caused by direct inhibition of the expression and binding activity of nuclear transcription factor SP1 with XIAP promoter SP1 binding sites, leading to the inhibition of XIAP gene transcription. Because ISO does not show any inhibition of exogenous HA-XIAP expression, and ectopic expression of exogenous HA-XIAP in both RT112 and HCT116 cells attenuates ISO induction of apoptosis, as well as inhibiting anchorage-independent cell growth of cancer cell lines, we conclude that the ability of ISO to down-regulate XIAP accounts for its effects on its pro-apoptotic and anti-cancer effect on multiple cancer cell lines.

ISO was isolated from G. cleistostachyum growing in Yun-Nan province, the southwest part of China. It belongs to a group of naturally occurring polyhydroxy stilbenes. Several studies have demonstrated that ISO has many biological effects through an antioxidant mechanism involving the inhibition of phosphorylation of PKC, ERK1/2, JNK, and P38, further leading to direct or indirect inhibition of NF-κB and AP-1 activation (3, 4). There is an accumulation of epidemiologic data supporting the fact that chronic inflammatory diseases are frequently associated with increased risk of cancers (22–24). However, few studies have explored the effects and molecular mechanisms of anti-proliferative and pro-apoptotic by ISO on tumor cells.

XIAP is a member of the IAP family and plays a key role in cancer cell survival, metastasis, and proliferation (25). There is growing evidence showing the correlation between XIAP overexpression and malignant cancer aggression (4), drug resistance (26), and poor clinical prognosis in many malignancies (27). Poorly differentiated carcinomas also display significantly higher levels of XIAP expression than well differentiated carcinomas (28). Thus, down-regulation of XIAP expression and inhibition of XIAP function attract a great deal of attention in
the field of cancer therapy. There is abundant research focusing on XIAP depletion or knockdown in cancer cells leading to reduction in cell migration and invasion (7, 29). Molecular approaches for targeting XIAP expression include phosphorothioate antisense oligonucleotide (AEG-35156)-targeted XIAP mRNA (30), small interfering RNA (siRNA) (31), and a small molecule inhibitor of XIAP (32). However, safety is the major limitation of the aforementioned approaches, along with other concerns such as poor stability, membrane penetration, and transfection efficiency (33). Therefore, identifying and exploring an effective anti-cancer drug that can down-regulate XIAP expression is of high significance. Our current study showed that treatment of various cancer cells with ISO could down-regulate XIAP expression, and that this down-regulation of XIAP expression is crucial for ISO induction of apoptosis and inhibition of anchorage-independent growth of various human cancer cells. The functional significance of endogenous XIAP down-regulation in apoptosis and cancer cell anchorage-independent growth was confirmed by the fact that ISO-induced apoptosis and inhibition of anchorage-independent growth was reversed by introduction of HA-tagged XIAP expression in RT112 and HCT116 cancer cells. Interestingly, as shown as Fig. 4A, the exogenous XIAP expression significantly inhibited the endogenous XIAP expression in RT112 cells. We speculated that constitutive ectopic expression of exogenous HA-XIAP might lead its expressed cells to reduce endogenous XIAP expression to overcome the impact of exogenous XIAP on cell function.

SP1 is an important transcription factor that is involved in the regulation of many gene expressions and cellular functions (34–36). Our results demonstrate that ISO down-regulation of XIAP expression is mediated by its inhibition of transcription factor SP1 expression. The SP1 mRNA level was not reduced even though its protein level was profoundly down-regulated upon ISO treatment (Fig. 5, C and D), suggesting that ISO regulates SP1 protein expression at levels of either protein translation and/or degradation. Further elucidation of this issue is one of the major future directions for the ISO anticancer project.

Our further results demonstrated that ISO could down-regulate transcription factor SP1 transactivation and binding activity to the XIAP promoter region, without effects on the previously reported antioxidation signal pathway, including PKC, ERK1/2, JNK, NF-κB, and P38 (3, 4). Our results are con-
Down-regulating XIAP by Isorhapontigenin in Human Cancer Cells

consistent with a previous report showing that two putative SP1 binding sites (−144 and −25 bp) with the 5′-untranslated region were involved in the SP1-mediated regulation of XIAP expression (11). Based on our results from the ChIP assay, SP1 appears to be a major participant in transcription factor binding to the GC-box site of the XIAP promoter and is critically involved in down-regulating XIAP transcription upon ISO treatment. Collectively, our results demonstrate that XIAP is an important target gene of ISO, and that it is regulated through the inhibition of SP1 expression, transactivation, and binding activity with the XIAP promoter following ISO treatment.

In summary, the present study discloses a novel function of ISO as a pro-apoptosis and anti-cancer compound through down-regulation of XIAP. The down-regulation of XIAP by ISO is mediated by inhibition of SP1 expression, transactivation, and the binding activity of SP1 to the XIAP promoter, as proposed in Fig. 5G. Based on our current data, we conclude that SP1 suppression is a major transcription factor responsible for down-regulation of XIAP expression and apoptosis. Further elucidation of the molecular mechanisms underlying of SP1 down-regulation is one of our major directions of future studies on the ISO anti-cancer effect.

The novel biological effect of ISO may contribute to the potential utilization of ISO as a new treatment strategy against malignant cancer cells, with the highly expressed XIAP gene, such as pancreatic cancer (37), gastric cancer (38), colon cancer (39), bladder cancer (40), and lung adenocarcinoma (31), although additional in vitro and in vivo studies are required. These current studies provide important insights into the understanding of the molecular mechanism(s) that are responsible for the anti-cancer effect of ISO, as well as contributing valuable information that can be used in the designing and synthesis of other new conformation-constrained derivatives for the treatment of cancers with XIAP overexpression.

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