Introduction

Hypoxia is a common phenomenon occurring in the majority of human tumors (1). The microenvironment of tumors is unlike that of normal tissues because the proliferative status of the tumor cells and an irregular vascular supply result in the development of hypoxia (2, 3). The presence of hypoxia is significantly associated with aggressive tumor progression, resistance to chemotherapy and radiation, and poor prognosis (4). Tumor cells and tissues adapt to a hypoxic microenvironment through the activation of a number of hypoxia-related molecules and pathways, among which hypoxia-inducible factor 1 (HIF-1) is the most predominant one (5).

HIF-1 is overexpressed in many human cancers, and the levels of its activity in cells is correlated with tumorigenicity and angiogenesis (6, 7). HIF-1 is a heterodimeric transcriptional factor composed of α and β subunits (8, 9). The HIF-1α subunit is highly regulated by oxygen concentration and serves as a marker of hypoxia (10). In normoxic conditions, specific HIF-1α prolyl hydroxylase hydroxylates two proline residues (P402 and P564) within the oxygen-dependent degradation domain of HIF-1α, resulting in HIF-1α recognition by Von Hippel-Lindau (VHL) ubiquitin ligase complex that targets HIF-1α for degradation through the 26S proteasome pathway (11, 12). Similarly, an asparaginyl hydroxylase termed as factor inhibiting HIF-1 hydroxylates the asparagines residue 803 within the transactivation domain of HIF-1α, abolishing its interaction with the transcriptional co-activator p300/CBP (13). In contrast, hypoxia inhibits prolyl hydroxylase activity and consequently results in the accumulation of HIF-1α protein.

4′,6-Dihydroxy-4-methoxyisoaurone Inhibits the HIF-1α Pathway Through Inhibition of Akt/mTOR/p70S6K/4E-BP1 Phosphorylation

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Abstract. 4′,6-Dihydroxy-4-methoxyisoaurone (ISOA) is an isoaurone compound isolated from Trichosanthes kirilowii seeds, which was identified as an inhibitor of tumor growth. However, the mechanism by which ISOA inhibits hypoxia-inducible factor-1 (HIF-1)-mediated tumor growth is not fully understood. We here demonstrated the effect of ISOA on HIF-1 activation. ISOA showed a potent inhibitory activity against HIF-1 activation induced by hypoxia in various human cancer cell lines. This compound markedly decreased the hypoxia-induced accumulation of HIF-1α protein dose-dependently, whereas it did not affect the expressions of HIF-1β and topoisomerase-I (Topo-I). Further analysis revealed that the suppression of HIF-1α accumulation by ISOA was closely correlated with strong dephosphorylation of Akt, mammalian target of rapamycin (mTOR), and its effectors ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), a pathway known to regulate HIF-1α expression at the translational level. Furthermore, ISOA prevented hypoxia-induced expression of HIF-1 target genes and suppresses the invasiveness of tumor cells. Taken together, our results suggested that ISOA is an effective inhibitor of HIF-1 through targeting Akt/mTOR/p70S6K/4E-BP1 pathway, thereby, providing new perspectives into the mechanism of its anticancer activity.

Keywords: 4′,6-dihydroxy-4-methoxyisoaurone, HIF-1α, translation, antitumor

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Accumulated HIF-1α translocates to nuclei and dimerizes with HIF-1β to form a functional transcription factor capable of DNA binding at hypoxia response elements (HREs) and the transcriptional activation of target genes (15, 16). Although the oxygen-dependent regulation of degradation is the primary mechanism of HIF-1α accumulation, HIF-1α can also be regulated at the translational level (17). It has been shown that phosphorylation of phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway leads to HIF-1α protein synthesis (17). Activated mTOR phosphorylates p70S6 kinase (p70S6K), which in turn, phosphorylates the ribosomal protein S6 (RP-S6) and the eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP1) (18, 19). The phosphorylation of 4E-BP1 leads to the activation of eIF4E, initiating cap-dependent mRNA translation (15, 20).

Biochemists and biologists have been investigating a variety of purified compounds from herbs as possible sources of new anticancer drugs. As part of our continuing search for HIF-1α inhibitors from natural products, 4',6-Dihydroxy-4-methoxyisouroione (ISOA) was identified as an inhibitor of HIF-1α activation from Trichosanthes kirilowii seeds (21). We found that ISOA inhibited hypoxia-induced HIF-1α activation. This compound rapidly down-regulates not only HIF-1α by decreasing its protein expression without affecting mRNA levels, but also the expression of HIF target genes such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), which are essential for tumor growth. We here demonstrated that the suppression of HIF-1α accumulation by ISOA is associated with strong dephosphorylation of Akt, mTOR, and its effectors p70S6K and 4E-BP1 in the pathway known to regulate HIF-1α expression at the translational level.

Materials and Methods

Cell culture and reagents

HeLa, SK-Hep1 and Hep3B cells were grown in DMEM with penicillin (100 units/ml)-streptomycin (100 units/ml) (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA). All cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The hypoxic culture was kept in a gas-controlled chamber (Thermo Electron Corp., Marietta, OH, USA) maintained at 1% O₂, 94% N₂, and 5% CO₂ at 37°C. In some experiments cobalt chloride has been used to induce a hypoxia-mimicking condition. Cobalt chloride was reported as a widely used mimetic of hypoxia in a large range of cells; this molecule is known to inhibit proline hydroxylases leading to HIF-1α stabilization (22). ISOA was isolated from Trichosanthes kirilowii seeds, and its structure is shown in Fig. 1A. The purity of ISOA was more than 98% in high-performance liquid chromatography analysis.

Transfections and luciferase reporter assay

The ability of the compound to inhibit hypoxia-inducible factor was determined by HRE-dependent reporter assay as previously described (23). In brief, at 50%–80% confluence, HeLa cells were cotransfected with the vectors for pGL3-HRE-Luciferase plasmid containing six copies of HREs derived from the human VEGF gene and with pRL-CMV (Promega, Madison, WI, USA) using Lipofectamine™ 2000 reagent (Invitrogen). Following 24-h incubation, the cells were treated with various concentrations of ISOA and incubated for 16 h in hypoxia. Luciferase assay was performed using the Dual-luciferase reporter assay system according to the instructions of the manufacturer (Promega). Luciferase activity was determined in the Microlumat plus luminometer (EG&G Berthold, Bad Wildbad, Germany) by injecting 100 μl of assay buffer containing luciferin and measuring light emission for 10 s. Co-transfection with pRL-CMV (Promega), which expresses Renilla luciferase, was performed to enable normalization of data for transfection efficiency.

Measurement of in vitro invasion and cell viability

The ability of cells to invade through Matrigel-coated filters (invasion) was determined using a modified 24-well Boyden chamber (Corning Costar, Cambridge, MA, USA; 8-μm pore size) as previously described (24). HeLa cells were seeded at a density of 5 × 10⁴ cells in 100 μl DMEM containing 10% FBS in the upper compartment of the transwell. To determine the effect of ISOA, various concentrations of ISOA were added to the lower or upper compartment of transwell. After incubation for 24 h at 37°C in 5% CO₂, the cells that did not penetrate the filter were completely wiped out with a cotton swabs; and the cells that had migrated to the lower surface of the filter were fixed, stained, and counted in 5 randomly selected microscopic fields (100 ×) per filter. Cell viability was measured by a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich, St. Louis, MO, USA) (25). Briefly, untreated cells or treated cells with ISOA in a 96-well plate were incubated for 24 h followed by the addition of MTT to the cells. Optical densities were determined on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

Whole-cell extracts were obtained by lysing cells
in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethyl sulfonfonylfluoride) supplemented with the protease inhibitor cocktail (BD Biosciences, San Diego, CA, USA). HIF-1α protein was analyzed in nuclear extracts prepared from cells using NE-PER reagent (Pierce, Rockford, IL, USA), according to the instructions of the manufacturer. An aliquot of protein extracts were used to determine protein concentration by the Bradford method. Fifty micrograms of whole-cell extracts or 30 μg of nuclear extract protein per lane was separated by SDS-polyacrylamide gels, followed by transferring to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk and then incubated with the corresponding antibody. Antibody for HIF-1α was obtained from BD Biosciences (1:250, BD Biosciences). Antibodies for phospho (Ser473)-specific Akt, phospho (Ser2448)-specific mTOR, phospho (Thr389)-specific p70S6K, and phospho (Ser473)-specific Akt, phospho (Ser2448)-specific p70S6K, and phospho (Thr37/46)-specific 4E-BP1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for phospho (Thr389)-specific p70S6K, and phospho (Thr37/46)-specific 4E-BP1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for α-tubulin was from Sigma (St. Louis, MO, USA). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotec, Buckinghamshire, UK).

**Immunofluorescence assay**

HeLa cells were seeded into 24-well plates at 1 × 10^4 cells/well. Twenty-four hours later, cells were treated with ISOA (10 μM) and incubated for 12 h under hypoxia. Cells treated with DMSO and under normoxic or hypoxic conditions were used as negative and positive controls, respectively. After treatment, cells were rinsed once in PBS, fixed in fresh 4% paraformaldehyde for 30 min at room temperature, and permeabilized with 0.2% Triton X-100. Cells were blocked with 5% BSA in PBS for 30 min and incubated overnight with the HIF-1α antibody at 4°C, followed by incubation with Alexa fluor® 488 goat anti-mouse IgG (H + L) for 30 min at room temperature and then with DAPI (4',6-diamidino-2-phenylindole) for 30 min before observation. The HIF-1α protein appeared green under fluorescence microscopy and the nuclei appeared blue. The green and blue images were merged using Image J software to produce cyan fluorescence in areas of co-localization.

**VEGF ELISA**

HeLa cells were plated in a 96-well plate at a density of 1 × 10^5 cells per well and treated with various concentrations of ISOA for 16 h under normoxic or hypoxic conditions. The VEGF levels in the culture supernatant were determined by ELISA using the Duo-Set ELISA development kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer’s instructions.

**RT-PCR analysis**

Total RNA from HeLa cells was obtained using RNA Mini kit (Qiagen, Valencia, CA, USA). Total RNA (2 μg) was used to perform reverse transcription-PCR (RT-PCR) using a RT-PCR kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The PCR primers for VEGF were 5’-GCTCTACCTCCAC CATGCCAA-3’ (sense) and 5’-TGGAAGATGTCCTC CAGGGTC-3’ (antisense); for EPO, 5’-CACTTCCCG CAACCTTTCGG-3’ (sense) and 5’-GTCAAGGCTT GCCACCTAAG-3’ (antisense); for HIF-1α and topoisomerase-I (Topo-I) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for HIF-1α and topoisomerase-I (Topo-I) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for α-tubulin was from Sigma (St. Louis, MO, USA). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotec, Buckinghamshire, UK).

**Statistical analyses**

All values are expressed as mean ± S.D. A comparison of the results was performed with one-way ANOVA and Tukey’s multiple comparison tests (Graphpad Software, Inc., San Diego, CA, USA). Statistically significant differences between groups were defined as P-values less than 0.05.

**Results**

**Identification of ISOA as a HIF-1α pathway inhibitor from a cell-based screening assay**

To investigate whether ISOA inhibited HIF-1α transcriptional activation, we transfected HeLa cells with a luciferase reporter gene driven by 6 specific HRE. A substantial increase of luciferase activity was observed in cells cultured in hypoxic conditions, whereas ISOA dose-dependently inhibited hypoxia-induced luciferase activity (Fig. 1B). Given that the inhibition of HIF-1α transcriptional activation might be correlated with ISOA-induced cytotoxicity, parallel studies of cell viability were performed (Fig. 1C). After the HeLa cells were treated with ISOA (up to 10 μM) for 24 h, no significant alteration of cell viability was observed relative to the untreated control group.

**ISOA decreases HIF-1α protein levels in a dose-dependent manner**

To explore the underlying mechanism of ISOA activity,
HIF-1α levels, ISOA had almost no effect on the levels of HIF-1β and Topo-I proteins. The effect of ISOA on HIF-1α protein levels was not restricted to human cervical carcinoma HeLa cells, and comparable IC_{50} values were also observed in human hepatocellular carcinoma Hep3B (IC_{50} = 2.90 ± 0.04 μM) and SK-Hep1 cells (IC_{50} = 2.76 ± 0.01 μM) (Fig. 2: C, D, and E). We next performed an immunofluorescence assay to evaluate the effect of ISOA on HIF-1α expression in HeLa cells. Following 12-h treatment, ISOA (10 μM) exerted almost complete inhibition of HIF-1α protein levels in cell nuclei induced by hypoxia in HeLa cells (Fig. 2F).

**ISOA did not change HIF-1α mRNA levels**

To determine whether the inhibition of HIF-1α expression inhibition by ISOA was a downstream effect from decreased HIF-1α gene transcription or HIF-1α mRNA stability, we analyzed HIF-1α mRNA levels by RT-PCR. ISOA did not change HIF-1α mRNA levels under either normoxia or hypoxia during 12 h of treatment (Fig. 3A). This suggests that ISOA-mediated decrease of HIF-1α expression is not regulated at the transcription level.

**ISOA decreases expression of HIF-1α target genes and suppresses the invasiveness of tumor cells**

The expression of VEGF and EPO, which are involved in tumor cell proliferation, angiogenesis, invasion, and metastasis, is known to be regulated by HIF-1α (6, 15). We therefore examined whether ISOA can suppress the expression of these genes. VEGF and EPO mRNA levels were measured by RT-PCR analysis in HeLa cells. Treatment of the cells with ISOA resulted in a dose-dependent inhibition of VEGF and EPO mRNA expression (Fig. 3A). The concentrations to inhibit the expression of HIF-1α target genes were comparable with those of HIF-1α protein accumulation. This result led us to measure the VEGF protein concentration in the culture supernatant by ELISA. Consistently, the hypoxic induction of secreted VEGF protein was dose-dependently inhibited by ISOA (Fig. 3B). Reduced expression of VEGF and EPO might be responsible for diminished invasion of tumor cells in ISOA treatment. Therefore, whether ISOA modulates invasion activity was examined in vitro with a Matrigel invasion assay. HeLa cells were seeded in the top chamber of a Matrigel invasion chamber and were incubated with various concentrations of ISOA for 24 h. The result showed that ISOA significantly decreased invasiveness compared to the vehicle control, accounting for the anti-invasive activity of ISOA (Fig. 4).
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Fig. 2. Effect of ISOA on the expression of HIF-1α protein. A, C, and D) HeLa, SK-Hep1 and Hep3B cells were incubated under normoxia, or hypoxia for 12 h, in the absence or presence of the indicated concentration of ISOA. Whole-cell lysates for HIF-1β and nuclear extract for HIF-1α were analyzed by western blotting. The same blot was reprobed with an anti-Topo-I antibody as a loading control. B) HeLa cells were pretreated with the indicated concentrations of ISOA for 30 min and then treated with CoCl₂ (200 μM). After 12 h incubation, the whole-cell lysates for HIF-1β and nuclear extract for HIF-1α were analyzed by western blotting. The same blot was reprobed with an anti-Topo-I antibody as a loading control. E) Data presented in A – D are representative of 3 independent experiments. The HIF-1α bands were digitized and expressed as a ratio of Topo-I. The HIF-1α protein level was inhibited by ISOA with IC₅₀ values of 2.95 ± 0.01 μM for A, 2.72 ± 0.02 μM for B, 2.76 ± 0.01 μM for C, and 2.90 ± 0.04 μM for D. F) HeLa cells were cultured in chamber slides under normoxic or hypoxic conditions and treated with or without ISOA (10 μM) for 12 h. After fixation, the slides were stained with anti-HIF-1α (1:100) antibody and Alexa fluor® 488 goat anti-mouse IgG (H + L) and examined by fluorescence microscopy. DAPI staining shows the location and size of nuclei. Left column, DAPI; Middle column, HIF-1α; Right column, Merge; magnification = 40 ×.
Downregulation of the Akt/mTOR/p70S6K/4E-BP1 signaling pathway by ISOA correlates with inhibition of HIF-1α synthesis

Because PI3K-Akt-mTOR-p70S6K-4E-BP1 pathways have been implicated in the regulation of HIF-1α protein synthesis at the translational level (10, 17, 26), we tested whether ISOA affects the phosphorylation of these translation initiation factors. As shown in Fig. 5, ISOA dose-dependently inhibited the expression of phospho-Akt, phospho-mTOR, phospho-p70S6K, and phospho-4E-BP1 induced by hypoxia. These results are comparable with the downregulation of HIF-1α protein expression by ISOA (Fig. 2). Taken all together, these results indicated that ISOA could inhibit HIF-1α protein expression through the suppression of Akt/mTOR/p70S6K/4E-BP1 signaling pathways.

Discussion

HIF-1 plays a central role in tumor progression and angiogenesis in vivo. Oncogenic activation (e.g., Ha-ras, myc, or src) or loss of tumor suppressor function (e.g., p53, PTEN, or VHL) is associated with HIF-1α-mediated tumor progression (27). Exposure to a variety of growth factors has also been shown to increase HIF-1 activity in normoxic and hypoxic conditions. HIF-1α is overexpressed in many human cancers and has been associated with tumor aggressiveness, vascularity, treatment failure, and mortality (28, 29). In addition, tumor growth and angiogenesis in xenograft tumors also depends on HIF-1 activity and the expression level of HIF-1α (7). Due to the potential role of HIF-1 as a target for cancer therapy, the development of small-molecule HIF-1 inhibitors represents a major challenge in the field of cancer treatment. In an effort to search for new anticancer natural products, ISOA, a rare natural 2(3H)-benzofuranones was identified as a potent HIF-1 and NF-κB inhibitor from a MeOH extract of the seed of T. kirilowii (21). So far, only a few synthetic isoaurones have been reported to
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show strong cytotoxicity, inhibition of topoisomerases I and II, and a weak cell cycle arrest at the G2/M phase (30). In this study, we have shown that ISOA inhibited HIF-1 activity and VEGF production in human cancer cell lines. ISOA was also found to downregulate the Akt/mTOR/p70S6K/4E-BP1 pathways, uncovering a novel mechanism for its anticancer activity.

VEGF and EPO are hypoxia-inducible neuroprotective cytokines with their gene transcription mainly mediated by HIF-1 (31). VEGF is an endothelial specific mitogen that increases peripheral oxygen delivery by stimulating angiogenesis, involving endothelial cell migration, proliferation, and differentiation, as well as extracellular matrix proteolysis. Following hypoxia, VEGF protein expression increases because HIF-1α bound to the hypoxic response element in its 5′ flanking region for the transcriptional activation (32). In adults, systemic EPO is mainly produced by the kidneys and in small amounts by the liver, and is essential for maintenance of tissue oxygen homeostasis by stimulating red blood cell production (33). Because they can be induced by HIF-1, whether ISOA could suppress EPO and VEGF transcription in the HeLa cells was further examined. As expected, ISOA decreased the VEGF and EPO mRNA levels.

In mammalian cells, the PI3K-Akt-mTOR pathway plays key roles in cell metabolism, nutrition regulation, protein synthesis, and tumorigenic processes (34). mTOR is one of the downstream targets of PI3K/Akt in regulating protein synthesis (35). mTOR carries out its functions through two different complexes: mTORC1 and mTORC2 (36, 37). mTORC1 regulates protein synthesis through its downstream targets: 4E-BP1 and p70S6K. 4E-BP1 is a negative factor for protein translation initiation as it binds tightly and blocks the function of eIF4E. The phosphorylation of 4E-BP1 by the mTORC1 complex inactivates it, freeing eIF4E to carry out its normal function in translation initiation (38). mTORC1 can also phosphorylate p70S6K, which stimulates the p70S6K ribosomal protein and other components of the translational machinery, including eIF4B and eEF2K (18). Moreover, it has been reported that 4E-BP1 and p70S6K play essential roles in HIF-1α translation (15).

In the present study, we have shown that treatment of HeLa cells with ISOA suppressed the phosphorylation of Akt, the phosphorylation of mTOR, in addition to their effectors p70S6K and 4E-BP1, which paralleled with the loss of HIF-1α expression. Therefore, given the key role of these pathways in the regulation of HIF-1α expression, our results strongly suggested that ISOA-induced suppression of Akt/mTOR/p70S6K/4E-BP1 pathways might be involved in the inhibition of HIF-1α expression.

In summary, this study shows, for the first time, that ISOA inhibits the Akt/mTOR/p70S6K/4E-BP1 signaling pathways and HIF-1 activity in HeLa cells. Thus, we have elucidated an important mechanism of the anticancer activity of ISOA, related to cell invasion and angiogenesis, which are essential for the adaptation of cancer cells to microenvironmental hypoxia and hence for tumor progression. This mechanism may in part explain the
anticancer effect of ISOA and provide a rationale for the development of ISOA as an anticancer drug.

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Conflicts of Interest

The authors declare that there are no financial conflicts of interest in regard to this work.

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