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Effect of Isoimperatorin on the Proliferation of Prostate Cancer Cell Line DU145 Cells

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Abstract – We isolated a coumarin compound, isoimperatorin ($C_{16}H_{14}O_4$ mw: 270) from *Angelica koreana* through silica gel column chromatography, and characterized it by NMR. Here, for the first time we observed that isoimperatorin (25, 50 and 100 μM) treatment for 24-72h inhibited growth and induced death in human prostate carcinoma DU145 cells. Further, in mechanistic investigation, isoimperatorin-induced cell growth inhibition was associated with a strong increase in G1 arrest in cell cycle progression, which started at 24h of the treatment. These findings suggest a novel anticancer efficacy of isoimperatorin mediated via induction of G1 arrest against hormone refractory human prostate carcinoma DU145 cells.

Key words □ isoimperatorin, *Angelica koreana*, prostate cancer (PCA), DU145 cells

INTRODUCTION

Prostate Cancer (PCA) is the most prevalent male malignancy as well as the second leading cause of cancer-related deaths in American men (Jemal *et al.*, 2003). More than 70% of all prostate cancer cases are frequently diagnosed in men over age 65 in United States and worldwide (Bosland *et al.*, 2002). The induction of human PCA has been viewed as a multistage process, involving progression from small, latent carcinomas of low histological grade to large, metastatic carcinomas of higher grade (Godley *et al.*, 1996). It is becoming clear that in the genesis of PCA, a variety of pathogenic pathways exist. Among the widely accepted risk factor for PCA are age, race, ethnicity, dietary habits, and androgen secretion and metabolism (Ross *et al.*, 1994). At present, there is no effect therapy available for the treatment of androgen-independent stage of prostate cancer, which usually arises after hormonal deprivation/ablation therapy (Feldman *et al.*, 2001). Nowadays numbers of prostate cancer and colon cancer cases are increasing in Korea.

Angelica koreana (Umbelliferae) has been traditionally used in oriental medicine as an analgesic. It's also used for treating headache, perspiration and edema (Chi *et al.*, 1970). Isolated isoimperatorin, imperatorin, oxypeucedanin etc. has been reported to have various biological activities including antimutagenesis (Cai *et al.*, 1997; Wall, 1988), uterus contraction, increase of blood pressure (Chi *et al.*, 1970) antioxidative activity and anticancer effect (Oh *et al.*, 2002; Fujioka, *et al.*, 1999). A certain Umbelliferae plant-*Angelica gigas* had been reported to have antiprostate cancer effect (Yim *et al.*, 2005).

Angelica gigas was isolated from pyranocoumarin group compounds, decursin and decursinol. The pyranocoumarin group compounds are highly effective to exhibit antiprostate cancer activity but the furanocoumarin compound does not. We used the roots of *Angelica koreana* to isolate furanocoumarin compound, isoimperatorin (Fig. 1), and for the first time, we tested its efficacy against human prostate carcinoma cell line. We investigated the efficiency in inhibiting growth and death, arrest in cell cycle progression and western blotting in prostate carcinoma cell line DU145 cells.

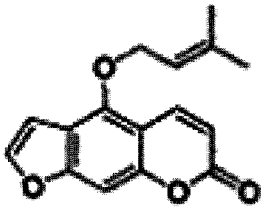

Fig. 1. The structure of isoimperatorin.

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MATERIALS AND METHODS

Plant material, isolation and characterization of isoimperatorin

The roots of *Angelica koreana* (family Umbelliferae) were verified by Professor Sookyeon Lee. The Extraction and fractionation of air-dried powdered root was done as reported (Cho *et al.*, 1998). Silica gel column chromatography was used to isolate isoimperatorin (Cho *et al.*, 1998). This compound was characterized by NMR (Bruker AVANCE 400 NMR spectrometer). The purified furanocoumarin compound, isoimperatorin ($C_{16}H_{14}O_4$) with a molecular weight of 270, was dissolved in DMSO as stock solutions, and used directly for cell culture treatments.

Cell line and reagent

Human prostate carcinoma cell line DU145 was obtained from American Type Culture Collection (Manassas, VA). Prostate cancer cell was cultured in RPMI-1640 with 10% fetal bovine serum (Hyclon, Logan, UT) under standard culture conditions (37°C, 95% humidified air and 5% CO_2). RPMI 1640 and other cell culture materials were from Life Technologies, Inc. (Gaithersburg, MD). Anti-Cip1/p21 antibody was from Calbiochem (Cambridge, MA), and anti-Kip1/p27 antibody was obtained from Neomarkers, Inc. (Fremont, CA). Antibodies to cyclin-dependent kinase (CDK); CDK2, CDK4, CDK6, cyclin D1 and cyclin E were from Sants Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell growth and death assays

Cells were plated at 5,000 cells/cm^2 density in 60mm plates under the standard culture conditions detailed above. After 24 hours, cells were fed with fresh medium and treated with dimethyl sulfoxide (DMSO) alone (control) or 25, 50 and 100 μM/L doses of isoimperatorin in DMSO. The final concentration of DMSO in the culture media was 0.1%, which did not exhibit any effect on the assay systems (Choi *et al.*, 2000). After the desired treatment time, cells were collected by a brief trypsinization, and counted in duplicate with a hemocytometer using Trypan blue dye to score dead cells.

Cell cycle analysis

Prostate cancer cell DU145 was grown in 10% serum condition in their respective culture mediums as mentioned above. At 30% confluence, cells were treated with DMSO control or 25, 50, and 100 μM/L isoimperatorin and at the end of desired treatment time; cells were collected and processed for cell cycle analysis. Briefly, 0.5×10^5cells were suspended in 0.5 mL of saponin/propidium iodide solution [0.3% saponin (w/v), 25 μg/mL propidium iodide (w/v), 0.1 mM EDTA, and 10 μg/mL RNase (w/v) in PBS], and incubated overnight at 4°C in the dark. Cell cycle distribution was then analyzed by flow cytometry.

Effect of isoimperatorin on cell cycle regulatory molecules in DU145 cells

DU145 cells were grown in RPMI 1640 medium and treated with DMSO control or 25, 50, and 100 μM/L doses of isoimperatorin for the desired times. Equal volumes of DMSO (0.1% v/v) were present in each treatment. Following isoimperatorin treatments, cell lysates were prepared in nondenaturing lysis buffer [10 mmol Tris-HCl (pH 7.4), 150mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM sodium orthovanadate, 0.5% NP-40, and 5 units/mL aprotinin]. For lysate preparation, the medium was aspirated and cells were washed twice with ice-cold PBS followed by incubation in lysis buffer for 20 minutes. Then, cells were scraped and kept on ice for an additional 30 minutes, and finally cell lysates were cleared by centrifugation at 4°C for 30 minutes in a tabletop centrifuge. Protein concentration in lysates was determined using Bio-Rad detergent-compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA) by Lowry's method.

For Western blotting, 80 μg of protein lysate per sample was denatured with 2X SDS-PAGE sample buffer and subjected to SDS-PAGE on 12% or 16% tris-glycine gel. The separated proteins were transferred on to nitrocellulose membrane followed by blocking of membrane with 5% nonfat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature or overnight at 4°C. Membranes were probed for the protein levels of Cip1/p21, Kip1/p27, CDK2, CDK4, CDK6, cyclin D1, and cyclin E using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody, and visualized by enhanced chemiluminescence (Amersham) detection system. Membranes were stripped and reproved with ß-actin primary antibody as a protein loading control.

Statistical analysis

The data were analyzed using the Jandel Scientific Sigma Stat 2.03 software. Student's *t* test was employed to assess the statistical significance of difference between control and

isoimperatorin-treated groups. A statistically significant difference was considered to be present at $p< 0.05$.

RESULTS

NMR and Mass characterization of isoimperatorin

Isoimperatorin: colorless needle crystal form. mp: 109-110 °C, ^{1}H NMR (400MHz, $CDCl_3$): 1.60(1H, s, H-4"), 1.70(1H, s, H-5"), 4.85(2H, d, J=7.0Hz), 5.43(1H, t, J=7.0Hz), 6.15(1H, d, J=9.8Hz), 6.85(1H, dd, J=2.4Hz and J=1.0Hz, H-3'), 6.95(1H, br s, H-8), 7.45(1H, d, J=2.4Hz, H-2'), 7.95(1H, dd, J=9.8Hz and J=0.5Hz, H-4)

Effects of isoimperatorin on growth and death of DU145 cells

To assess the biological activity of isoimperatorin in terms of cell growth and death, DU145 cells were treated with 25, 50, and 100 μM/L doses of isoimperatorin for 24, 48 and 72hours (Fig. 2). Isoimperatorin (25-100 μM/L) showed a strong dose- and time-dependent inhibition of cell growth, accounting for 20% to 37% (p<0.001), 29.5% to 50.5% (p<0.001) and 55.5% to 64.6% (p<0.001), growth inhibition after 24, 48 and 72 hours of treatment, respectively (Fig. 1). Furthermore, we observed that isoimperatorin have cytotoxic effect on DU145 cells, in which similar treatment (25-100 μM/L for 24, 48 and 72, hours) with isoimperatorin caused 11.8% to 23.9% (p<0.001) cell death versus 8.9% to 11.4% in controls. Overall, these results suggested growth inhibitory and cytotoxic effects of isoimperatorin human prostate carcinoma DU145 cells.

Induction of G1 arrest in cell cycle progression of the DU145 cells by isoimperatorin

Inhibition of deregulated cell cycle progression in cancer cell is an effective strategy to halt tumor growth (Singh *et al.*, 2002; Grana *et al.*, 1995). We observed a growth inhibitory effect of isoimperatorin. We analyzed its possible inhibitory effect on cell cycle progression following 25, 50, and 100 μM/L doses of isoimperatorin treatment for 24 to 72 hours. The results showed that isoimperatorin-treated DU145 cells led to a marked accumulation of cells in the G1 phase arrest (Fig. 3).

Effect of isoimperatorin on cell regulatory molecules in DU145 cells

Defects in the regulation of cell cycle progression are thought to be one of the most common features of transformed cells (Hunter *et al.*, 1994). As shown in Fig. 4, the treatment of DU145 cells in culture with isoimperatorin resulted in a significant up-regulation of CDKI Cip1/p21 protein levels compared to the control group. The effect of isoimperatorin was both dose- and time-dependent. Maximum up-regulation was observed at 24h after treatment, because no significant increase was evident after 48hrs (data not shown). Similar to Cip1/p21, the treatment of DU145 cells with isoimperatorin also resulted in a significant up-regulation of protein levels in another CDKI, Kip1/p27, which was also dose-dependent on isoimperatorin as well as the time of treatment. When the effect of isoimperatorin on the expression of CDKs was assessed, as shown in Fig. 4 isoimperatorin treatment of DU145 cells resulted in dose-and time-dependent decrease in the protein levels of CDK4. However, no change in the levels of CDK2 and CDK6 was observed following isoimperatorin treatment up to a concentration of 100iM/L. We assessed the effect of isoimperatorin on the protein levels of cyclins associated with CDK2 (cyclin E) and with CDK4 and CDK6 (cyclin D1). As shown in Fig. 4, the treat-

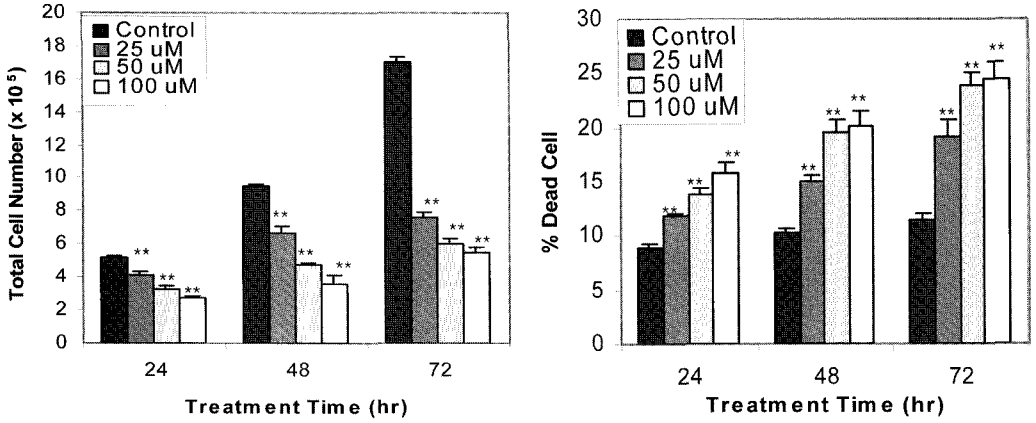

Fig. 2. Effects of isoimperatorin on (A) cell growth, (B) death in DU145 cells. **, p<0.001 vs. control.

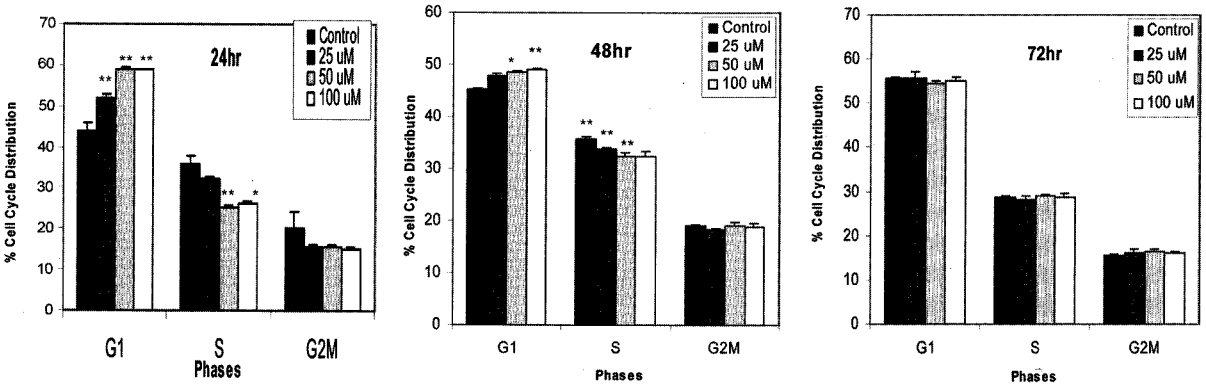

Fig. 3. Effects of isoimperatorin oncell cycle progression in DU145 cells. *p<0.01, **p<0.001 vs. control.

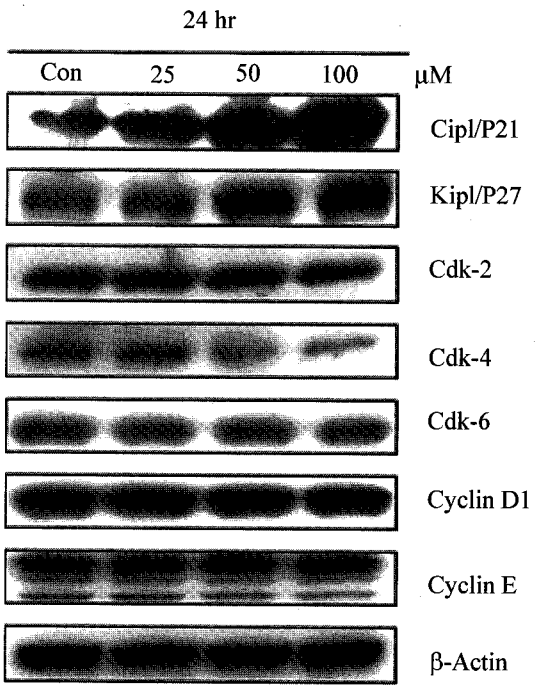

Fig. 4. Effects of isoimperatorin on G1 cell cycle regulators, CDK-associated and cyclin-associated kinase activities in DU145 cells.

ment of DU145 cells with isoimperatorin did not result in any alterations in the expression of cyclin D1 and cyclin E, respectively. Based on our findings we observed a significant increase in the expression of CDKIs Cip1/p21 and Kip1/p27, which bind to and inactivate CDKs (Morgan, 1995) of DU145 cells right after isoimperatorin treatment. Then, we assessed whether an up-regulation of CDKIs exerted any effects on the kinase activity of CDKs and associated cyclins in isoimperatorin treated DU145 cells.

DISCUSSIONS

The results of this study demonstrate that isoimperatorin treatment of prostate carcinoma cell line DU145 causes significant growth inhibition and that effect is related to G1 phase arrest, furthermore, confirmed cell cycle regulatory molecules in DU145 cells.

CDKs, CDKIs, and cyclins play essential roles in regulation of cell cycle progression (Grana *et al.*, 1995). CDKIs are tumor suppressor proteins that down-regulate the cell cycle progression by binding with active CDK-cyclin complexes and thereby inhibiting their kinase activities (Grana *et al.*, 1995; Hunter *et al.*, 1994 and Morgan, 1995). The important CDKIs include Cip1/p21, a universal inhibitor of CDKs whose expression is mainly regulated by the p53 tumor suppressor protein (Xinong *et al.*, 1993); and Kip1/p27 that is also up-regulated in response to antiproliferative signals (Toyoshima *et al.*, 1994; Polyak *et al.*, 1944). The increased expression of G1 cyclins in cancer cells provide them an uncontrolled growth advantage because most of these cells either lack CDKI or possess nonfunctional CDKI or have low expression of CDKI (Hunter *et al.*, 1994). Consistent with these reports, cell cycle analysis data showed that isoimperatorin caused a strong G1 arrest in cell cycle progression of DU145 cells. Further, mechanistic investigation showed that isoimperatorin-induced G1 arrest is mainly mediated via an up-regulation of Cip1/p21 and Kip1/p27 at initial treatment time. Concomitant with CDKIs induction, we also observed a decrease in kinase activity-associated with CDKs and cyclins. However, when cell cycle phase distributions are compared with alterations in cell cycle regulatory molecules, an increase in CDKIs as well as a decrease in CDK 4 could be attributed to the one of the major cause of isoimper-

atorin-induced G1 arrest and cell growth inhibition at later treatment times. The increased expression of CDKIs by isoimperatorin could have relevance for PCA, as a decreased Kip1/p27 expression in prostatic carcinomas has been associated with aggressive phenotype and poor prognosis and failure of irradiation response in PCA patient has been linked to the loss of Cip1/p21 function (Cheng *et al.*, 2000). DU145 cells lack functional p53, therefore, our data suggest CDKIs up-regulation by isoimperatorin involves p53-independent pathway.

In conclusion, our present findings showing the *in vitro* anticancer efficacy of isoimperatorin, G1 cell cycle arrest effect and their mechanistic is up-regulates Cip1/p21 and Kip1/p27 levels and inhibits CDK4 on the growth of prostate carcinoma DU145 cells.

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