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Growth Inhibitory Activity of Honokiol through Cell-cycle Arrest, Apoptosis and Suppression of Akt/mTOR Signaling in Human Hepatocellular Carcinoma Cells†

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Abstract – Honokiol, a naturally occurring neolignan mainly found in *Magnolia* species, has exhibited a potential anti-proliferative activity in human cancer cells. However, the growth inhibitory activity against hepatocellular carcinoma cells and the underlying molecular mechanisms has been poorly determined. The present study was designed to examine the anti-proliferative effect of honokiol in SK-HEP-1 human hepatocellular cancer cells. Honokiol exerted anti-proliferative activity with cell-cycle arrest at the G0/G1 phase and sequential induction of apoptotic cell death. The cell-cycle arrest was well correlated with the down-regulation of checkpoint proteins including cyclin D1, cyclin A, cyclin E, CDK4, PCNA, retinoblastoma protein (Rb), and c-Myc. The increase of sub-G1 peak by the higher concentration of honokiol (75 μM) was closely related to the induction of apoptosis, which was evidenced by decreased expression of Bcl-2, Bid, and caspase-9. Hohokiol was also found to attenuate the activation of signaling proteins in the Akt/mTOR and ERK pathways. These findings suggest that the anti-proliferative effect of honokiol was associated in part with the induction of cell-cycle arrest, apoptosis, and down-regulation of Akt/mTOR signaling pathways in human hepatocellular cancer cells.
Keywords – Honokiol, G0/G1 Cell-cycle arrest, Apoptosis, Akt/mTOR, SK-HEP-1 cells.

Introduction

Hepatocellular carcinoma is one of the most fatal cancers and ranked as the third cause of cancer-associated deaths in the world (Hussain *et al*., 2001; Bosch *et al*., 2004). Along with surgery and radiotherapy, chemotherapy is one of the most common strategy in hepatocellular cancer therapy, (Nagano, 2010). However, conventional anticancer drugs for hepatocellular carcinoma are typically non-selective cytotoxic molecules with significant systemic unexpected effects. Therefore, it is important to develop more safer and effective therapeutic agents for hepatocellular cancer cells.

Honokiol is a natural component mainly found in *Magnolia obovata* (family Magnoliaceae) (Cui *et al*., 2007; Han *et al*., 2007). In recent study, various biological activities of honokiol including anti-inflammation, anti-oxidant, neurotoxicity, angiopathy, thrombosis, anti-proliferative effects in various cancer cells have been reported (Cui *et al*., 2007; Fried and Arbiser, 2009; Shen *et al*., 2010; Tian *et al*., 2012; Zhang *et al*., 2013). In our recent study, we reported that the growth inhibition of breast cancer cells by honokiol was associated with the cell cycle arrest, apoptosis and down-regulation of c-Src/EGFR-mediated cell signaling pathway (Park *et al*., 2009). In addition, we also found that honokiol induced cell cycle arrest and apoptosis in human gastric cancer cells (Kang *et al*., 2012). Although the anti-proliferative activity of honokiol has been reported in various cancer cells, the underlying mechanisms of action remain to be clarified in hepatocellular cancer cells.

Herein, we report for the first time that honokiol exhibits anti-proliferative activity in human SK-HEP-1 human hepatocellular cancer cells, which was associated with G0/G1 cell-cycle arrest, apoptosis and the suppression of Akt/mTOR signaling pathways.

Experimental

Chemicals – Trichloroacetic acid (TCA), sulforhodamine B, propidium iodide, trypsin inhibitor, RNase A, and anti-β-actin primary antibody were purchased from Sigma (St.Louis, MO, USA). Rosewell Park Memorial Institute

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medium 1640 (RPMI 1640), fetal bovine serum (FBS), non-essential amino acid solution (10 mM, 100X), trypsin-EDTA solution (1X) and antibiotic-antimycotic solution (PSF) were from GIBCO-BRL (Grand Island, NY, USA). Rabbit polyclonal anti-CDK4, anti-cyclin A, anti-cyclin D1, anti-PCNA, anti-cMyc, anti-Bcl-2, anti-ERK, anti-phospho ERK antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased fromSanta Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-phospho-Rb (Ser 807/811), anti-Rb, anti-Bid, anti-caspase-9, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-mTOR (Ser2448), anti-mTOR antibody were obtained from Cell Signaling (Danvers, MA, USA). Mouse monoclonal anti-cyclin E was from BD Biosciences (San Diego, CA, USA). Honokiol isolated from the bark of *Magnolia obavata* was provided from Dr. KiHwan Bae (Chungnam National University, Korea).

Cell culture – Human hepatocellular carcinoma SK-HEP-1 cells, obtained from the Korean Cell Line Bank (Seoul, Korea), were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. Cells were maintained at 37 °C in humidified atmosphere with 5% CO_2.

Evaluation of growth inhibitory potential – SK-HEP-1 cells (5×10^4 cells/mL) were treated with various concentrations of test compound for 3 days. After treatment, cells were fixed with 10% TCA solution, and cell proliferation was determined with sulforhodamine B (SRB) protein staining method (Jo *et al*., 2012). The result was expressed as a percentage, relative to solvent-treated control incubations, and the IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration).

Analysis of cell cycle dynamics by flow cytometry – Cell cycle analysis by flow cytometry was performed as previously described (Kang *et al*., 2009). Briefly, SK-HEP-1 cells were plated at a density of 1×10^6 cells per 100-mm culture dish and incubated for 24 h. Fresh media containing test samples were added to culture dishes. After 24 h, the cells were harvested (trypsinization and centrifugation), fixed with 70% ethanol, and incubated with a staining solution containing 0.2% NP-40, RNase A (30 μg/mL), and propidium iodide (50 μg/mL) in phosphate-citrate buffer (pH 7.2). Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer. At least 20,000 cells were used for each analysis, and results were displayed as histograms of DNA content. The distribution of cells in each phase of cell cycle was calculated using ModFit LT2.0 program.

Evaluation of the protein expression by Western blot – SK-HEP-1 human hepatocellular carcinoma cells were exposed with various concentrations of honokiol for 24 h. After incubation, cells were lysed and protein concentrations were determined by BCA method. Each protein (40 μg) was subjected to SDS-PAGE. Proteins were transferred onto PVDF membranes by electroblotting, and membranes were treated for 1 h with blocking buffer [5% non-fat dry milk in phosphate-buffered saline-0.1% Tween 20 (PBST)]. Membranes were then incubated with indicated antibodies (mouse anti-β-actin, diluted 1 : 2,000; other antibodies, diluted 1 : 1,000 in PBST) overnight at 4 °C, washed three times for 5 min with PBST. After washing, membranes were incubated with HRP-conjugated anti-mouse IgG diluted 1 : 2,000 in PBST for 2 h at room temperature, washed three times for 5 min with PBST, and visualized by HRP-chemiluminescent detection kit (Lab Frontier, Seoul, Korea) using LAS-3000 Imager (Fuji Film Corp., Japan) (Kang *et al*., 2012).

Statistical analysis – Data were presented as means ± SE for the indicated number of independently performed experiments. Statistical significance ($p < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with Dunnett's t-tests.

Results and Discussion

To determine the effects of honokiol on the growth of human hepatocellular cancer cells, its growth inhibitory potential was evaluated by a colorimetric sulforhodamine B (SRB) protein dye staining method. As shown Fig. 1, honokiol exhibited potent growth inhibition of SK-HEP-1 human hepatocellular cancer cells with the IC_{50} value of 44.1 μM. In particular, cells exposed to the highest concentration (100 μM) exerted remarkable decrease in cell survival. Morphological changes by treatment with honokiol were also examined using phase-contrast microscopy. Following treatment with honokiol (25, 50, and 75 μM), the number of cells were decreased compared to control cells, and especially, floating cells were observed when treated with 75 μM indicating that cell death was induced. As shown in Fig. 2, cells treated with honokiol exhibited morphological changes with distinct rounded shapes and detachment in a time- and concentration-dependent manner when compared to vehicle-treated control cells.

Cell proliferation is generally controlled by the progression of three distinctive phases (G0/G1, S, and G2/

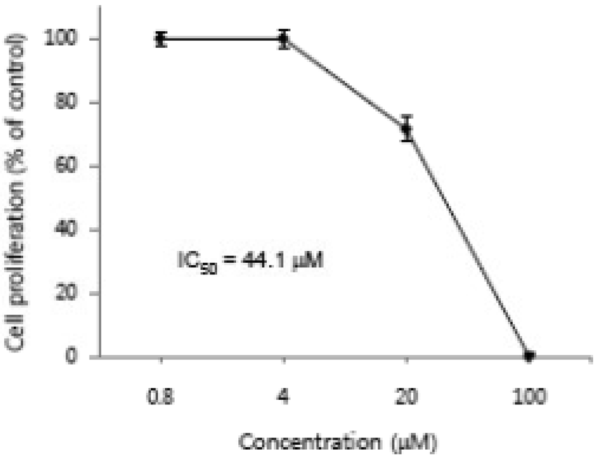

Fig. 1. Effect of honokiol on the proliferation of SK-HEP-1 human hepatocellular cancer cells. SK-HEP-1 cells were plated at 10,000 cells in 96-well plate in RPMI supplemented with 10% FBS, and incubated with the test compound as the indicated concentrations for 3 days. Anti-proliferative effect was determined using SRB assay. The values of % cell growth are calculated by the mean absorbance of samples/absorbance of vehicle-treated control. Data are represented as the means ± S.E. (n = 3).

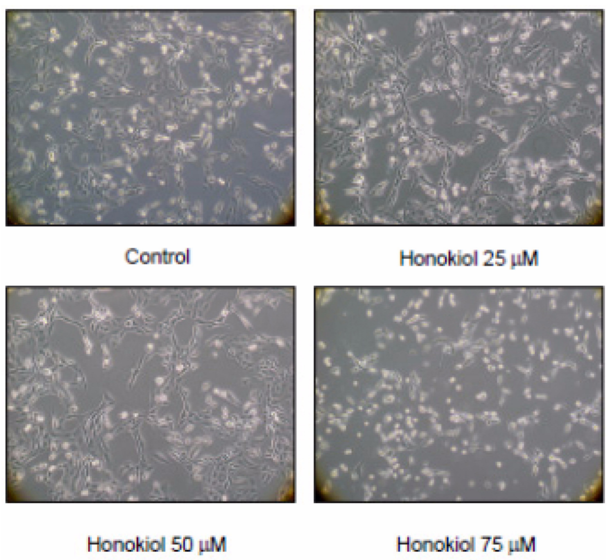

Fig. 2. Morphological changes mediated by honokiol in SK-HEP-1 cells. Cells were treated with or without various concentrations of honokiol for 24 h. Cells were photographed by inverted microscopy (magnification 100×).

M) of the cell cycle, and cell-cycle arrest is considered one of the most common causes of the inhibition of cell proliferation (Kastan and Bartek, 2004). To investigate whether honokiol could affect the cell cycle regulation, flow cytometric analysis was performed after staining cells with propidium iodide. As shown in Fig. 3, treatment of honokiol resulted in a considerable accumulation of cells

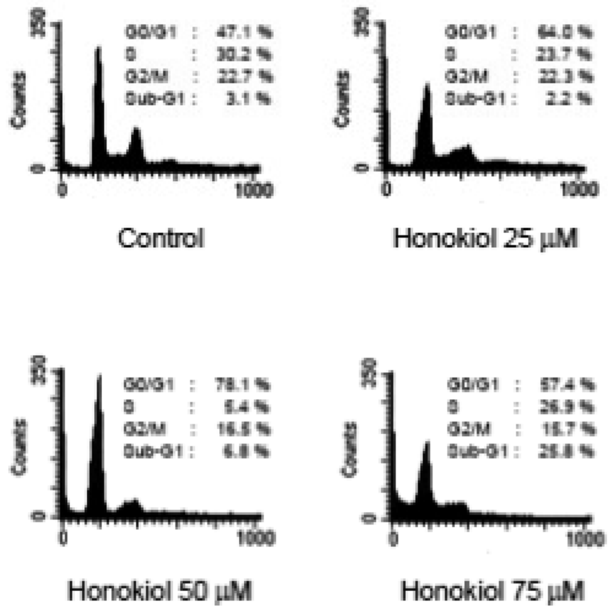

Fig. 3. Effect of honokiol on the regulation of cell cycle distribution in SK-HEP-1 cells. Cells were seeded at 1 × 10^6 cells in 100 mm dish in RPMI supplemented with 10% FBS, and then treated with various concentration of honokiol for 24 h. The cell cycle distribution was analyzed by flow cytometry as described in Materials and Methods.

in the G0/G1 phase (25 µM; 64.0%, 50 µM; 78.1%) by up to 31% increase in comparison with the distribution of control cells. However, the treatment of higher concentration of honokiol (75 µM) subsequently increased the sub-G1 phase, indicative of apoptotic peaks. These data show that honokiol induces cell- cycle arrest in the G0/G1 phases at 25 and 50 µM concentration and induction of cell death at 75 µM, a relatively high concentration.

Further study was designed to examine the effect of honokiol on the expression of cell cycle regulatory proteins associated with G0/G1 cell-cycle arrest by Western blot analysis. Honokiol suppressd the protein expression of checkpoint proteins such as cyclin D1, CDK4 and PCNA, and also down-regulated the expression of biomarkers such as cyclin A and E in the transition of G1 to S phase in a concentration-dependent manner (Fig. 4A). Since these proteins are known to form active complexes of cyclin-CDK, and mediate the inactivation of Rb tumor suppressor protein, the level of the phosphorylation of Rb was determined (Schwarts and Shah, 2005). As a result, both the phosphorylated- and total Rb were suppressed by honokiol. In addition, the expression of c-Myc, an oncoprotein that causes the cell cycle progression (Pelengaris *et al*., 2002), was markedly down-regulted in cells treated with honokiol compared with control cells. To further study the apoptotic

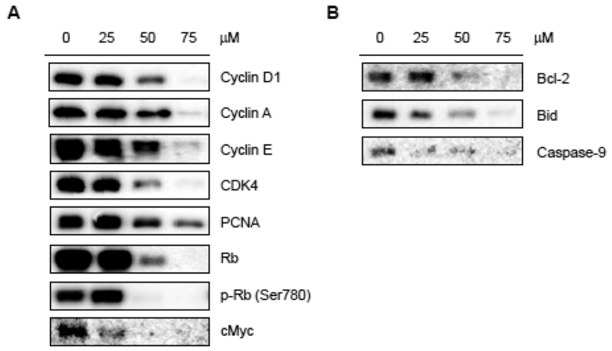

Fig. 4. Effect of honokiol on the expression of cell cycle (A) and apoptosis (B)-associated proteins in SK-HEP-1 cells. Cells were treated with the indicated concentrations of honokiol for 24 h. The expression level of proteins was analyzed by Western blot.

mechanisms underlying the cytotoxic activities of honokiol, the effects of honokiol on the expression of Bcl-2, Bid and procaspase-9 were examined. As shown in Fig 4B, honokiol suppressed the expression of Bcl-2 and Bid, antiapoptotic proteins, and caspase-9, and these events subsequently affect to the induction of apoptosis.

Further study was conducted to determine whether the anti-proliferative effect of honokiol was associated with the regulation of the cell signaling transduction pathway. The Akt and mammalian target of rapamycin (mTOR) has been known to be one of the main signaling pathways to regulate cell proliferation, growth, differentiation, and survival (Wullschleger *et al*., 2006; Alexander *et al*., 2010). The abnormal activation of these pathways is frequently found in various cancer cells including hepatocellular carcinoma (Kudo, 2011; Zhou *et al*., 2011). Therefore, targeting Akt/mTOR signaling patrhway is considered a promising approach for cancer management (Wullschleger *et al*., 2006). To investigate whether honokiol is able to affect the regulation of Akt/mTOR signaling pathway, the expression levels of the phosphorylated form of Akt and mTOR were evaluated. As shown in Fig. 5, honokiol remarkably suppressed the expression of phosphorylated Akt and mTOR. In addition, honokiol down-regulated the expression of phosphorylated ERK, which is considered one of key mechanisms for promoting cell cycle progression and cell proliferation. Therefore, the down-regulation of Akt/mTOR and ERK signaling might be in part associated with the anti-proliferative effect of honokiol in SK-HEP-1 cells.

In conclusion, the present study revealed that honokiol is a potent inhibitor of the growth of human hepatocellular cancer cells, and the growth inhibition is correlated with cell-cycle arrest and induction of apoptosis. The growth inhibition is also associated with the modulation of signal

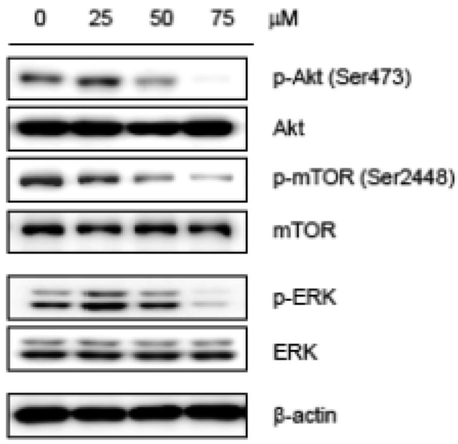

Fig. 5. Effects of honokiol on the expression of signal transduction proteins. SK-HEP-1 cells were treated for 24 h with various concentration of honokiol and the protein expressions were measured by Western blot analysis.

transduction pathway in the Akt/mTOR signaling. The results demonstrate that honokiol might have a therapeutic potential in the management of human hepatocellular cancer cells.

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