Honokiol induces apoptosis and suppresses migration and invasion of ovarian carcinoma cells via AMPK/mTOR signaling pathway

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Abstract. Honokiol, a natural biphenolic compound, exerts anticancer effects through a variety of mechanisms on multiple types of cancer with relatively low toxicity. Adenosine 5'-phosphate-activated protein kinase (AMPK), an essential regulator of cellular homeostasis, may control cancer progression. The present study aimed to investigate whether the anticancer activities of honokiol in ovarian cancer cells were mediated through the activation of AMPK. Honokiol decreased cell viability of 2 ovarian cancer cell lines, with a half-maximal inhibitory concentration value of 48.71±11.31 µM for SKOV3 cells and 46.42±5.37 µM for Caov-3 cells. Honokiol induced apoptosis via activation of caspase-3, caspase-7 and caspase-9, and cleavage of poly-(adenosine 5'-diphosphate-ribose) polymerase. Apoptosis induced by honokiol was weakened by compound C, an AMPK inhibitor, suggesting that honokiol-induced apoptosis was dependent on the AMPK/mechanistic target of rapamycin signaling pathway. Additionally, honokiol inhibited the migration and invasion of ovarian cancer cells. The combined treatment of honokiol with compound C reversed the activities of honokiol in wound healing and Matrigel invasion assays. These results indicated that honokiol may have therapeutic potential in ovarian cancer by targeting AMPK activation.

Introduction

Ovarian cancer is one of the most common diseases in woman. The 5-year survival rates for patients with advanced ovarian cancer remain at 20-30% (1). Chemotherapy has been used to treat ovarian cancer for several decades. Alkylating agents were developed as single chemotherapy drugs during the 1970s (2). Cisplatin-based regimen was established as the standard first-line chemotherapy for patients with ovarian cancer in the late 1980s (3). Although cisplatin is a widely used and a highly active chemotherapeutic agent for ovarian cancer, its use has been limited due to its cumulative toxicities, in particular its nephrotoxicity (2). In addition, instances of intrinsic and acquired resistance to cisplatin have been observed in patients with ovarian cancer (2). Therefore, novel chemotherapeutic agents are urgently required to treat ovarian cancer, and to promote the effectiveness and decrease the side effects of cisplatin treatment.

Adenosine 5'-phosphate (AMP)-activated protein kinase (AMPK), a nutrient and energy sensor in mammalian cells, regulates glucose and lipid metabolism (4). AMPK is a heterotrimeric serine/threonine protein kinase that is composed of a catalytic \( \alpha \)-subunit and 2 regulatory subunits, the \( \beta \)- and \( \gamma \)-subunits. Under normal physiological conditions, AMPK protects cells against various metabolic stresses by maintaining homeostatic pools of the adenosine nucleotides [adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), and AMP] (4). However, the role of AMPK signaling in cancer has not yet been fully elucidated. Epidemiological investigations have suggested that treatment with metformin, a drug that activates AMPK, was associated with decreased incidence of diseases, including breast, lung, colon, prostate and pancreatic cancer (5-8). Experimental data have confirmed that metformin exerts an inhibitory effect on the growth of breast and pancreatic cancers (9,10). Clinical data have also confirmed that metformin may improve the overall survival of patients with diabetes and cancer either alone or in combination with chemotherapy (11,12). However, conventional AMPK activators including metformin may cause the toxic side effect of lactic acidosis with dazzling, muscle pain, tiredness and stomach pain (13). Therefore, the use of nontoxic and natural AMPK activators may be preferable to treat ovarian cancer and its chemoresistance.

The mammalian target of rapamycin (mTOR) serine/threonine kinase, a downstream effector of AMPK, exists in 2 biochemically distinct complexes, mTOR complex 1 and mTOR complex 2. mTOR, similarly to AMPK, serves critical roles not only in cell growth and cell proliferation but also...
in metabolism (14). AMPK phosphorylates and activates tuberous sclerosis 1 protein (TSC1)/tuberous sclerosis 2 protein (TSC2), thereby inhibiting mTOR. mTOR leads to inhibition of downstream targets p70S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), which are involved in cell growth primarily through the regulation of translation and protein synthesis (15). AMPK controls tumor development through the modulation of mTOR activity (16). Therefore, the AMPK/mTOR pathway is a promising target for cancer therapy.

Herbal medicine has been widely used to treat illnesses for centuries. It is the most productive source of lead compounds for drug development (17). It includes various natural compounds with biological activities and therapeutic effectiveness, with minimum side effects (17). *Magnolia officinalis* is a species of *Magnolia*. Its roots and stem bark have been used for treating thrombotic stroke, gastrointestinal complaints, anxiety, nervous disturbance, allergic diseases and cancer (18). Studies have demonstrated that *Magnolia* extract is a safe medicine with low toxicity (18-20). Honokiol is a natural biphenolic compound derived from the bark of *Magnolia* trees with anti-oxidative, anti-inflammatory and anti-tumor properties (21). Several mechanisms involved in the anti-tumor activities of honokiol against leukemia, breast, pancreatic and prostate cancer, oral squamous cell carcinoma, and skin, gastric, bone and brain cancer have been suggested (22), including the induction of cell cycle arrest (23), apoptosis (24), autophagy (25), and anti-proliferative (26) and anti-invasive processes (27-29).

The present study evaluated the therapeutic potential of honokiol based on its anticancer properties, including its effects on apoptosis, migration and invasion in ovarian cancer cells. Additionally, the potential molecular mechanisms involved in its anticancer effects were explored.

**Materials and methods**

**Reagents.** Honokiol, compound C and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Dulbecco’s modified eagle’s medium (DMEM), McCoy’s 5A medium, fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc., (Waltham, MA, USA). RPMI-1640 Medium and Trypsin/EDTA were purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). The Cell Counting kit-8 was obtained from Dojindo Molecular Technologies, Inc., (Kumamoto, Japan). Rabbit polyclonal anti-human caspase-3 (cat. no. 9662), mouse monoclonal anti-human caspase-7 (cat. no. 9494), rabbit polyclonal anti-human caspase-9 (cat. no. 9502), rabbit polyclonal anti-human poly-(ADP-ribose) polymerase (PARP; cat. no. 9542), rabbit monoclonal anti-human phospho-AMPK (Thr172; cat. no. 2535), rabbit polyclonal anti-human AMPK (cat. no. 2532), rabbit polyclonal anti-human phospho-mTOR (Ser2448; cat. no. 2971), rabbit polyclonal anti-human mTOR (cat. no. 2972), rabbit polyclonal anti-human phospho-4EBP1 (Thr70; cat. no. 9455), rabbit polyclonal anti-human 4EBP1 (cat. no. 9452) and rabbit polyclonal anti-human β-actin (cat. no. 4967) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-mouse (cat. no. 7076) and anti-rabbit (cat. no. 7074; both 1:3,000) secondary antibodies were purchased from Cell Signaling Technology, Inc. Super Signal® West Pico Chemiluminescent substrate was purchased from Pierce; Thermo Fisher Scientific, Inc.

**Cell lines and culture.** Human ovary adenocarcinoma SKOV3, Caov-3 and NIH-3T3 cell lines were purchased from the Korean Cell Line Bank, Korean Cell Line Research Foundation (Seoul, Korea), and grown in McCoy’s 5A, DMEM and RPMI-1640 media, respectively, supplemented with 10% (v/v) FBS. Cells were maintained at 37°C in a humidified 5% CO₂-controlled incubator.

**Cell viability assay.** Cells were seeded at 5x10⁵ cells/ml in 96-well microplates and were cultured overnight to allow attachment. Honokiol (1-100 µM), compound C (20 µM), and AICAR (500 µM) were added to the medium. Following treatment, cell viability was assessed using the CCK-8 assay. CCK-8 (10 µl) solution was added, and cells were incubated for 3 h at 37°C. The optical density (OD) was assessed at 450 nm using a precision microplate reader (Molecular Devices, LLC., San Jose, CA, USA).

**Soft agar colony forming assay.** Cells (5x10⁵/ml) were suspended in growth medium (3 ml) containing 0.3% agar and 10% FBS. They were then applied to pre-solidified 0.6% agar (3 ml in FBS-free growth media) in 60 mm culture dishes. After 2-3 weeks of incubation at 37°C, colonies on soft agar were observed under a phase-contrast microscope (IX2-ILL100, Olympus Corporation, Tokyo, Japan) at a magnification of x40.

**Western blot analysis.** Cells were harvested using Trypsin-EDTA, and washed twice in cold PBS. Cells were lysed with lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.5% NP-40, 1 mM PI, 1 mM DTT, 1 mM PMSF) and placed on ice for 1 h. The supernatant was obtained by centrifugation at 13,000 x g for 10 min at 4°C. A Pierce BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to measure protein concentrations. Equal amounts of protein (50 µg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were then blocked with 5% skim milk in PBS containing 0.05% Tween-20 (PBST) for 1 h at 25°C to prevent nonspecific antibody binding, then incubated with caspase-3, caspase-7, caspase-9, PARP, AMPK, phospho-AMPK, mTOR, phospho-mTOR, 4EBP1, phospho-4EBP1 and β-actin primary antibodies (1:1,000) overnight at 4°C. Subsequent to washing with PBST, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (both 1:3,000) at room temperature for 2 h and visualized with enhanced chemiluminescence using Super Signal® West Pico Chemiluminescent substrate. Band intensity was quantified by densitometry using ImageJ software [version 1.52; National Institutes of Health (NIH), Bethesda, MD, USA] and was normalized to loading controls.

**Annexin V-PI double staining assay.** Cells were cultured at a density of 1x10⁵/ml and treated with honokiol and compound C
Wound healing assay. Cells were seeded into 6-well plates and incubated in serum-free medium for 18 h. The cellular monolayer was wounded with a 10 µl-pipette tip and washed with serum-free media to remove cells detached from the plates. These cells were incubated in the presence and absence of honokiol for 48-72 h in growth medium containing 10% FBS. The medium was then replaced with PBS and images of the cells were captured using a phase-contrast microscope at a magnification of x40. Results were quantified using ImageJ software (version 1.52).

Matrigel invasion assay. BD Biocoat Transwell Invasion Chambers were used to perform cell invasion assays. Each insert was equipped with a 6.4 mm diameter PET porous membrane (pore size=8 µm) coated for 6 h at 37°C with Matrigel Matrix (BD Biosciences). Cells (2.5x10⁴) were suspended in serum-free medium (300 µl) with or without drugs. Cells were placed in the upper chamber while medium (500 µl) containing 10% FBS was added to the lower chamber as a chemoattractant. Following incubation for 24 h at 37°C, non-invading cells were removed from the upper surface of the membrane, while invading cells on the lower surface of the membrane were stained with 0.1% hematoxylin for 30 min at room temperature. The membranes were then removed and light microscopy was used to count invading cells at a magnification of x40. Results were normalized to control cells, and the relative invasion is expressed as mean ± standard deviation (SD) of migrating cells compared with the control cells.

Statistical analysis. Statistical analyses were performed using IBM® SPSS® Statistics v.24.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance followed by a Tukey's post-hoc test was used for calculating significance between different groups. Values are presented as mean ± SD of 3 independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of honokiol on ovarian cancer cell proliferation and colony formation. To evaluate the therapeutic potential of honokiol for ovarian cancer treatment, the human ovarian cancer SKOV3 and Caov-3 cell lines were cultured with increasing concentrations of honokiol (1-100 µM) for 24 h. Cell viability (%) was then determined by CCK-8 assay. Honokiol significantly inhibited the growth of ovarian cancer cells in a dose-1(1-100 µM) and time-(6-24 h) dependent manner (Fig. 1A and B). Honokiol induced a dose-dependent decrease in the growth of ovarian cancer cells, with a half-maximal inhibitory concentration (IC₅₀) values of 48.71±11.31 µM for SKOV3 cells and 46.42±5.37 µM for Caov-3 cells after 24 h of treatment. Honokiol also exhibited low toxicity in the non-cancer NIH-3T3 fibroblast cell line. Subsequently, the effect of honokiol on cell colony formation was investigated. Consistent with its cancer cell toxicity, honokiol inhibited the colony formation property of SKOV3 and Caov-3 cells in a dose-dependent manner (Fig. 1C).

Honokiol activates AMPK in ovarian cancer cells. To additionally examine the mechanism by which honokiol induced growth inhibition of ovarian cancer cells, AMPK signaling in SKOV3 and Caov-3 cells was studied. Honokiol dose-dependently induced the phosphorylation of the Thr172 subunit of AMPK in ovarian cancer cells (Fig. 2A). Honokiol-induced AMPK activation was associated with a decreased level of phosphorylation of mTOR (Fig. 2A). To additionally confirm these changes, the AMPK inhibitor compound C was used in combination with honokiol. Compound C is a potent, selective and reversible ATP-competitive inhibitor of AMPK (inhibitor constant=109 nM in the presence of 5 µM ATP and absence of AMP). The results indicated that compound C attenuated honokiol-induced AMPK activation (Fig. 2B), and rescued ovarian cancer cells from cell growth inhibition induced by honokiol (Fig. 2C). Conversely, treatment with AMPK activator AICAR in combination with honokiol markedly induced AMPK activation (Fig. 2B) and ovarian cancer cell death (Fig. 2C). These results indicated that AMPK regulation modulated honokiol-induced cell death.

Honokiol induces apoptosis via AMPK activation in ovarian cancer cells. To examine whether the induction of apoptosis contributed to the honokiol-mediated decrease in cell viability of ovarian cancer cells, Annexin V-FITC/PI staining was performed to analyze the population of apoptotic cells. Treatment with honokiol for 24 h increased the population of apoptotic cells. However, compound C treatment with honokiol decreased Annexin V-PI-positive cell numbers (Fig. 3A). The mechanism involved in apoptotic cell death induced by honokiol treatment was then investigated. SKOV3 and Caov-3 cells were treated with honokiol and compound C for 24 h. Western blot analysis was used to analyze the activation of caspase-3, -7, -9, and cleavage of PARP. Following treatment with honokiol, activation of caspase-3, -7, and -9 and increased cleavage of PARP were observed. However, treatment with compound C in combination with honokiol attenuated the activation of caspase-3, -7 and -9, and increased cleavage of PARP induced by honokiol alone (Fig. 3B). Cell cycle analysis by flow cytometry also revealed an accumulation of sub-G₀/G₁ cells following honokiol treatment, while treatment with compound C decreased the proportion of sub-G₀/G₁ cells (Fig. 3C). These results demonstrate that honokiol-induced apoptosis was involved in its AMPK-mediated anticancer activity.

Honokiol inhibits migration and invasion of ovarian cancer cells. To determine whether honokiol impaired the migration and invasion of ovarian cancer cells, SKOV3 and Caov-3 were subjected to honokiol treatment. Wound healing assays indicated that honokiol treatment significantly inhibited the migration distance between the leading edge of cells. However,
compound C reversed this activity (Fig. 4A). The Matrigel invasion assays demonstrated that treatment with 50 µM honokiol resulted in a 66.9 and 80.7% decrease in migration of SKOV3 and Caov-3 cells, respectively, compared with the untreated cells. The decrease in Matrigel invasion induced by honokiol was significantly reversed by compound C treatment (Fig. 4B).
These results suggested that honokiol-induced AMPK activation inhibited the migration and invasion of ovarian cancer cells. Based on the results of the present study, the potential biological activities of honokiol are illustrated in Fig. 5.

Discussion

Honokiol has generated increasing interest in cancer studies, due to its multi-functional effects, including its anticancer, anti-angiogenesis and anti-migration properties, which have been demonstrated in vitro and in vivo using preclinical models (30). Previous studies have demonstrated that honokiol may induce growth inhibition and apoptosis in various types of cancer, including lung, breast, colon and prostate cancer in vitro and in vivo (31-34). The present study demonstrated that honokiol induced cytotoxicity and inhibited proliferation in the ovarian cancer SKOV3 and Caov-3 cell lines, whereas the normal NIH-3T3 cell line exhibited low cytotoxicity. These results are consistent with a previous study that revealed that the IC50 values of honokiol at 24 h for SKOV3, Coc 1, Angelen and A2780 cells were 16.7, 19.6, 16.4, and 14.9 µg/ml, respectively (35).
Figure 3. Honokiol induces apoptosis in ovarian cancer cells. (A) Cells were pre-treated with compound C for 2 hours prior to treatment with honokiol (50 µM). An Annexin V-FITC/PI double staining flow cytometry assay was used to measure apoptotic cells. (A-a) Control treatment in SKOV3 cells. (A-b) Control treatment in the Caov-3 cells. (A-c) Honokiol treatment (50 µM) in SKOV3 cells. (A-d) Honokiol treatment (50 µM) in Caov-3 cells. (A-e) Compound C (20 µM) treatment in SKOV3 cells. (A-f) Honokiol (50 µM) + Compound C (20 µM) treatment in SKOV3 cells. Data in the graph are representative of 3 independent experiments. The percentages of apoptotic cells are presented as mean ± standard deviation. *P<0.05 vs. single honokiol treatment.

(B) Cells were treated with compound C (20 µM) prior to stimulation by honokiol (50 µM) for 24 hours. Western blot analysis determined the caspase-3, caspase-7, caspase-9 and PARP protein expression levels. β-actin was used as an internal control. *P<0.05 vs. honokiol-only treatment.

(C) Flow cytometry analysis examined the effects of honokiol (50 µM) and compound C (20 µM) on cell cycle distribution. (C-a) Control treatment in SKOV3 cells. (C-b) Honokiol treatment (50 µM) in SKOV3 cells. (C-c) Compound C (20 µM) treatment in SKOV3 cells. (C-d) Honokiol (50 µM) + Compound C (20 µM) treatment in SKOV3 cells. Results are representative of 3 independent experiments. *P<0.05 vs. honokiol-only treatment. FITC, fluorescein isothiocyanate; PI, propidium iodide; PARP, poly-(adenosine 5'-diphosphate-ribose) polymerase.
Previous studies have suggested that the upstream kinases of AMPK, including liver kinase B1 (LKB1), are frequently mutated and deleted in human cancer (36-38). Genetic deletion of the LKB1 gene results in a loss of AMPK activity that represents a common event in cancer cell growth (39). Being directly activated by the tumor suppressor LKB1, AMPK regulates...
the activation of 2 other tumor suppressors, TSC1 and TSC2, which are critical regulators of mTOR (40). AMPK-initiated mTOR inhibition suppresses downstream effectors p70S6K and 4EBP1, regulating transcription, translation, protein stability, mRNA turnover and cell size (40,41). Previous studies have demonstrated that several AMPK activators, mTOR inhibitors and their combination, including metformin, AICAR or rapamycin, may suppress cancer cell growth (42-47). Therefore, AMPK is an essential target for cancer therapy.

Honokiol targets multiple signaling pathways including epidermal growth factor receptor, nuclear factor kappa-light-chain-enhancer of activated B cells B, signal transducer and activator of transcription 3, and mTOR, which serve essential roles in cancer initiation and progression (48). Previous data have suggested that honokiol affects melanoma and breast cancer cell growth by targeting AMPK signaling (28,49). However, whether AMPK targeting via honokiol is the cause of its anticancer effects in ovarian cancer is unclear. In the present study, activation of AMPK in honokiol-treated ovarian cancer cells was observed, which may have contributed to the cell death pathway.

As honokiol has demonstrated inhibitory effects on the viability of human ovarian cancer cells, the present study examined whether it modulated cell cycle progression and induced apoptotic cell death in the same manner as AMPK activation. The results indicated that honokiol may lead to the caspase-dependent apoptotic death of ovarian cancer cells, causing an increase in the sub-G₁ population of apoptotic cells.
Induction of apoptosis was indicated by the elevated expression of apoptotic markers, including activation of caspase-3, caspase-7 and caspase-9, and cleavage of PARP. These characteristics of apoptosis were inhibited by compound C, a pharmacological inhibitor of AMPK. Previous studies have suggested that AMPK activation may induce apoptosis and cell cycle arrest in various types of cancer, including breast, colon and oral cancer (50-52). Therefore, honokiol may induce apoptosis in ovarian cancer cells through AMPK activation.

Previous studies have revealed that the activation of AMPK by metformin not only inhibits cell proliferation, but also decelerates cell migration (53). Honokiol suppresses metastasis by inhibiting cell migration in neuroblastoma (25), and breast (29), and renal cancer (54). However, to the best of our knowledge, honokiol-induced metastatic activity on ovarian cancer has never been investigated. To address the functional role of honokiol in ovarian cancer metastasis, the present study examined the anti-metastatic effect of honokiol on ovarian cancer cells. It was identified that AMPK activation by honokiol treatment suppressed cell migration and the invasive properties of ovarian cancer cells. These results suggested that honokiol may be a potential therapeutic target for treating metastatic ovarian cancer.

In summary, honokiol, a natural compound, exhibited anticancer activities against the ovarian cancer SKOV3 and Caov-3 cell lines. Honokiol significantly suppressed cell proliferation and induced apoptosis of ovarian cancer cells by activating AMPK. Honokiol also inhibited their metastatic and invasive activities, potentially through AMPK activation. Although honokiol has been implicated in AMPK signaling in other cancer types, to the best of our knowledge, this is the first study of the role of AMPK in ovarian cancer. The results indicated that honokiol has potential clinical application for preventing and treating ovarian cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JSL designed the study and prepared the manuscript. JBP, MSL and EYC performed experiments and analyzed the data. JYS and YBK were involved in the study conception and design, and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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