Honokiol induces cytotoxic and cytostatic effects in malignant melanoma cancer cells

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Abstract

Background—Melanomas are aggressive neoplasms with limited therapeutic options. Therefore, developing new therapies with low toxicity is of utmost importance. Honokiol is a natural compound that has recently shown promise as an effective anti-cancer agent.

Methods—Honokiol effect on melanoma cancer cells was assessed *in vitro*. Proliferation and physiological changes were determined using hexoseaminidase assay and transmission electron microscopy. Protein expression was assessed by immunoblotting.

Results—Honokiol treatment inhibited cell proliferation and induced death. Electron microscopy demonstrated autophagosome formation. Reduced levels of cyclin D1 accompanied cell cycle arrest. Honokiol also decreased phosphorylation of AKT (known as Protein Kinase B) and mTOR (mammalian target of rapamycin), and inhibited γ-secretase activity by downregulating the expression of γ-secretase complex proteins especially APH-1 (anterior pharynx-defective 1).

Conclusions—Honokiol is highly effective in inhibiting melanoma cancer cells by attenuating AKT/mTOR and Notch signaling. These studies warrant further clinical evaluation for honokiol alone or with present chemotherapeutic regimens for treatment of melanomas.

Keywords

Melanoma; AKT; cyclinD1; cell cycle arrest; AKT; mTOR; γ-secretase

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INTRODUCTION

Malignant melanoma is an aggressive form of skin cancer with extremely poor survival and few therapeutic options. The incidence of melanoma in the United States has increased more rapidly in the past few decades than any other malignancy[1,2]. Approximately 40 to 60% of cutaneous melanomas may also carry B-Raf mutations that lead to the constitutive activation of downstream signaling cascade through the mitogen-activated protein kinase (MAPK) pathway[3,4]. Approximately 90% of these mutations result in the substitution of glutamic acid for valine at codon 600 (BRAF V600E) and renders B-RAF in constitutively active form that confers mitogenic responses for the growth of cancerous cells[5]. Finally, alterations of the chromosome 10q*24–26* region are present in 30–50% of melanoma cell lines and 5–20% of uncultured melanomas[6]. This region includes the lipid phosphatase PTEN (Phosphatase and tensin homologue deleted from chromosome 10), which functions by negatively regulating the phosphatidylinositol 3′-kinase (PI3K)/AKT pathway. Loss of PTEN function in melanoma needs further investigation[6]. The PI3K/AKT and Raf/MEK/ERK pathways promote tumorigenesis essentially by positively regulating cell survival, cell cycle progression[7,8,9], tumor angiogenesis[10,11,12], and tumor invasion[13,14,15]. All these events together activate a multitude of downstream signaling cascades including the Raf/MAPK kinase (MEK)/extracellular signal-regulated kinase (ERK) and PI3K/AKT pathways. B-RAF mutations activate the MAPK cascade and loss of PTEN activates PI3K; these two pathways are important mediators of melanoma downstream of Ras.

Aberrant activation of the Notch signaling has been also associated with the development of skin cancer. In melanomas, levels of Notch expression are associated with progression, and metastasis[16]. Notch signaling is initiated when a ligand such as Jagged interacts with the Notch transmembrane receptor, leading to translocation of NICD and binding of two cofactors to transactivate target genes, such as those in the hairy and enhancer of split (Hes), related with YRPW motif (Hey) and cyclin D1 proteins[16,17]. The importance of Ras-activation and the Notch pathways in melanoma suggests that they may be effective targets for prevention and treatment of melanomas. The real challenge in inhibiting these signaling cascades is that they regulate important growth and survival pathways shared by both neoplastic and normal cells. Therefore, it is of prime importance to develop a therapeutic strategy that able to exert the maximum effect on cancer cells with the minimum toxicity on normal cells.

Honokiol (Fig. 1A) is a biphenolic compound from *Magnolia officianalis* that is used in -traditional Chinese and Japanese medicine for the treatment of various ailments including ulcer, allergy, and bacterial infections. It is also used as a muscle relaxant and possesses antithrombotic activity[18]. Recent studies have shown that it has antitumor activity with low toxicity[18]. In this article, we investigated the effect of honokiol on melanoma cancer cells. This article focuses on defining nontoxic approaches toward eradicating melanoma cancer cells.

MATERIALS AND METHODS

Cell line and reagents

B16-F10 cells (American Type Culture Collection) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Sigma-Aldrich) and antibiotic solution (Mediatech Inc.) at 37° C in a humidified atmosphere containing 5% CO_2. Cells used in this study were within 20 passages after receipt or resuscitation (approximately 3 months of noncontinuous culturing). Growth medium was changed after every 3 day and cells were split (1:3) when they reached 80% confluence. For honokiol treatment, stock

solution of honokiol was prepared in DMSO, stored at − 20°C in aliquots, and diluted with fresh medium immediately before use. Honokiol was purchased from Sigma Aldrich.

Cell Viability Assay

Assessment of cell proliferation after honokiol treatment was performed by Hexoseaminidase assay[19]. Cells were plated in 96 well plate, grown over night and treated next day with increasing concentrations of honokiol (0–50 μM) for 24, 48 and 72h. Cell growth was calculated as percent viability = [(A/B)x100], where A and B are the absorbance of treated and control cells, respectively.

Immunoblot Analysis

Cell lysates after honokiol treatment were extracted and quantitated by using Pierce BCA protein assay kit (Thermo Scientific, IL, USA). Cell lysates were subjected to SDS-PAGE and blotted onto nitro cellulose membrane (GE Healthcare) and proteins of interest were detected by using enhanced chemiluminescence system (GE Healthcare). Antibodies for immunoblotting were purchased from Cell Signaling Technology Inc. USA, GenScript Inc. USA, and Santa Cruz Biotechnology Inc. USA. For equal loading of protein in each well, each blot were normalized with their respective internal controls (β-actin or β-tubulin, total AKT and mTOR).

Statistical analysis

All experiments were performed in triplicates. Significant differences were analyzed using student's t test. Data were considered to be statistically significant if $p < 0.05$. Data was expressed as mean ± SD between triplicate experiments performed thrice.

RESULTS

Honokiol affect viability of B16-F10 melanoma cells

To study the effect of honokiol on melanoma cell growth, B16-F10 melanoma cells were treated with increasing concentrations of honokiol (0–50 μM) for 24, 48 and 72h and performed cell proliferation by using Hexoseaminidase assay. Honokiol treatment significantly decreased cell proliferation of melanoma cells in dose and time dependent manner with respect to their respective controls ($p < 0.05$). Decrease in cell proliferation was observed within 24 h of Honokiol treatment which was further suppressed over the next 72 h in a dose dependent manner respectively (Fig. 1B). We further, studied the morphological changes in B16-F10 by transmission electron microscopy. Honokiol treatment increased the autophagosomes like vacuoles formation in B16-F10 cells (Fig. 1C). All together, these findings indicate that honokiol showed cytotoxic effects on melanoma cancer cells.

Honokiol down regulates the expression of Cyclin D1 in melanoma cells

Honokiol effectively attenuated the levels of cyclin D1 protein in B16-F10 cells in time and dose dependent manner (Fig. 2A) and thus resulted in cell cycle arrest of melanoma cancer cells. Honokiol attenuated cyclin D1 levels in B16-F10 cells further strengthen the fact that significant decrease in cell growth of B16-F10 cells by honokiol was partly due to inhibition of cell proliferation and induction cell death and partly due to cell cycle arrest. Altogether, this data demonstrated that honokiol has both cytostatic and cytotoxic properties against melanoma cancer cells.

Signaling affected by honokiol in melanoma cells

Most of the chemotherapeutic agents against melanoma are partly or fully designed for targeting of malfunctioned Ras and Raf signaling molecules in melanoma cancer cells.

Therefore we further tried to explore the pathways involved in honokiol induced cell death and growth inhibition of melanoma cells. We observed that honokiol significantly affected the Ras and Raf signaling in melanoma cells by dephosphorylating the constitutively phosphorylated AKT and mTOR protein in B16-F10 cells in dose and time dependent manner (Fig. 2B and 2C). This substantiate the fact that honokiol is inhibiting the Ras and Raf signaling in melanoma cells and thus induced the cell death, cell cycle arrest and inhibited the growth of aggressive melanoma cells. In addition to conventional melanoma signaling, honokiol was also found to inhibit cancer stem cells related signaling i.e. Notch signaling. Honokiol significantly attenuated the levels of APH1 in B16-F10 cells in time dependent manner (Fig. 2D). This protein is the part of the γ-secretase enzyme complex. Gamma-secretase is the key enzyme involved in cleavage of Notch intracellular domain (NICD) and this cleaved notch get translocated to nucleus for transcriptional up-regulation of downstream client genes specially cyclin D1.

DISCUSSION

Honokiol, because of its diverse anti-oxidative, anti-inflammatory, anti-tumor, and antimicrobial pharmacological properties, is a putative candidate for both therapeutic interventions and chemoprevention. Previous studies have shown efficacy of honokiol against several types of cancer like pancreatic, colon and breast without any toxicity[20]. Unlike other polyphenolic agents, which have been hindered by poor absorption and rapid excretion, honokiol exhibits a significant systemic levels in blood in preclinical models, and it can also cross the blood brain barrier[20]. We have demonstrated honokiol's effect on inducing B16-F10 melanoma cancer cells cell death studied by using hexoseaminidase assay and by electron microscopy. Honokiol began to induce these changes within 24 h of treatment and the effect remained at 48 and 72 h of treatment. Honokiol mediated cell death was partly due to the formation of autophagosomes like vacuoles in B16-F10 cells, as indicated by arrow in electron micrograph. Additional studies are required to confirm the types of vacuoles in B16-F10 cells after honokiol treatment. Subsequently we demonstrated that honokiol also exert cytostatic effects by significantly down regulating the cyclin D1 levels in B16-F10 cell in dose and time dependent manner. Cyclin D1, often found to be overexpressed in human melanomas, is an important positive regulator of the G1-S cell cycle transition through its binding and activation of CDK4/6 kinases. Activation of CDKs further inactivate retinoblastoma protein and blocks its growth-inhibitory activity, promotes release of bound E2F which finally leads to cell cycle progression[21,22]. Recent studies also reported the honokiol induced G0/G1 cell cycle arrest in colon and lung cancer cells[18,23]. Further studies are required to study the transition of the cells through the various phases of the cell cycle are along with the checkpoint-related markers to confirm this phenomenon.

We further explored the honokiol attenuated molecular pathways involved in cell death and cystatic effects in B16-F10 cells. Melanomas often consist of mutant BRAF or N-RAS, and consequently increased ERK-MAPK and AKT activities[24]. Preclinical studies have demonstrated a close and complex interconnection of the RAS-RAF-MEK-ERK and the PI3K-AKT-mTOR signaling pathways which are involved in growth, progression and drug resistance of melanomas. AKT has a central role in regulating apoptosis and tumor regression[25]. In BRAF V600E mutant cells, AKT activation was required for initiation of melanoma, demonstrating the interdependence of these two pathways in melanoma. Our data reveal that honokiol significantly inhibited the AKT and mTOR phosphorylation in dose and time dependent in B16-F10 melanoma cells. This strengthen the facts that honokiol act on PI3K-AKT-mTOR signaling pathway. In this connection, others have shown that topical treatment of melanomas with specific PI3K inhibitor (Ly294002) results in significant reduction of TPras transgenic melanoma growth in severe combined immunodeficient (SCID) mice[26]. Additionally, inhibition of either the Raf/MEK/ERK

pathway by the MEK1/2-specific inhibitor U0126 or PI3K leads to a reduction in tumor invasion and angiogenesis[26,27,28].

Role of Notch expression has been reported in progression and metastasis of melanomas[16]. Notch signaling is 10- to 30-fold higher in the stem cells when compared with other cell types[29]. In this study, we have determined that honokiol inhibited the Notch signaling by inhibiting the essential members of the γ-secretase complex, the critical enzyme that cleaves and releases the NICD from the membrane. Honokiol decreased the APH1 protein levels in dose dependent manner. Therefore, honokiol–mediated inhibition of melanoma cancer cell growth is also partly mediated via inactivation of Notch-1 signaling cascade. Recent study suggested the role of Notch1 in prevention of cancer cells from cytotoxic insults. This was substantiated by ectopic expression of the Notch intracellular domain partially rescuing the colon and breast cancer cells from cytotoxic compound's effect[18,30]. One more recent study also showed that honokiol arrests cell cycle, induces apoptosis, and potentiates the cytotoxic effect of gemcitabine by affecting NF-κB[31,32]. Recent studies proposed that honokiol alone or in combination of radio- or chemotherapy resulted in a growth inhibition of colon, pancreatic and glioma *in vitro* and *in vivo*[18,32,33]. In addition, the honokiol significantly suppressed Notch-1 activation. Taken together, these data suggest that the honokiol to target cancer stem cells is an attractive novel potential agent for the treatment and prevention of various cancers. Further studies are warranted to show the efficacy of the honokiol in the clinical setting. It would also be interesting to determine whether there are other clients for the γ-secretase complex and the role of these client proteins in cancer stem cell biogenesis.

Acknowledgments

Abbreviations

AKT	known as Protein Kinase B
APH-1	Anterior pharynx-defective 1
BCA	Bicinchoninic acid
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
ERK	Extracellular signal-regulated kinases
mTOR	Mammalian target of rapamycin
PTEN	Phosphatase and tensin homologue deleted from chromosome 10
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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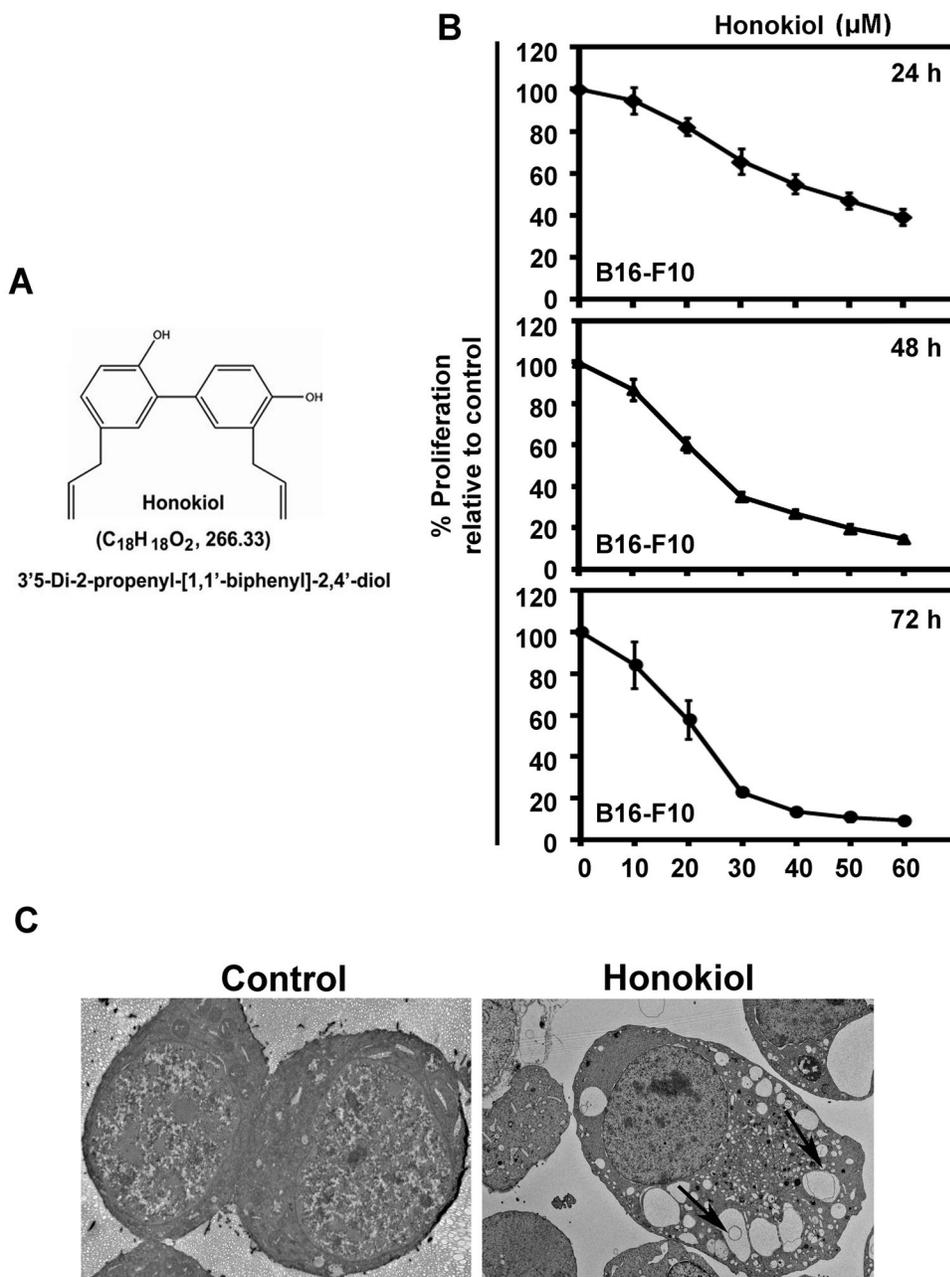
Figure 1.
Effect of Honokiol on melanoma cancer cells. A) Chemical structure of honokiol. B) B16-F10 cells were incubated with increasing doses of honokiol (0–50 µM) at different time intervals (24, 48 and 72h) to determine the cell proliferation of B16-F10 cells. The honokiol treatment resulted in a significant dose and time-dependent decrease in cell proliferation compared to their respective controls ($P < 0.05$). C) Transmission electron micrograph showing honokiol induced vacuoles formation in B16-F10 cells at 24 h of honokiol (30 µM) treatment. All images were taken at 2000 magnification.
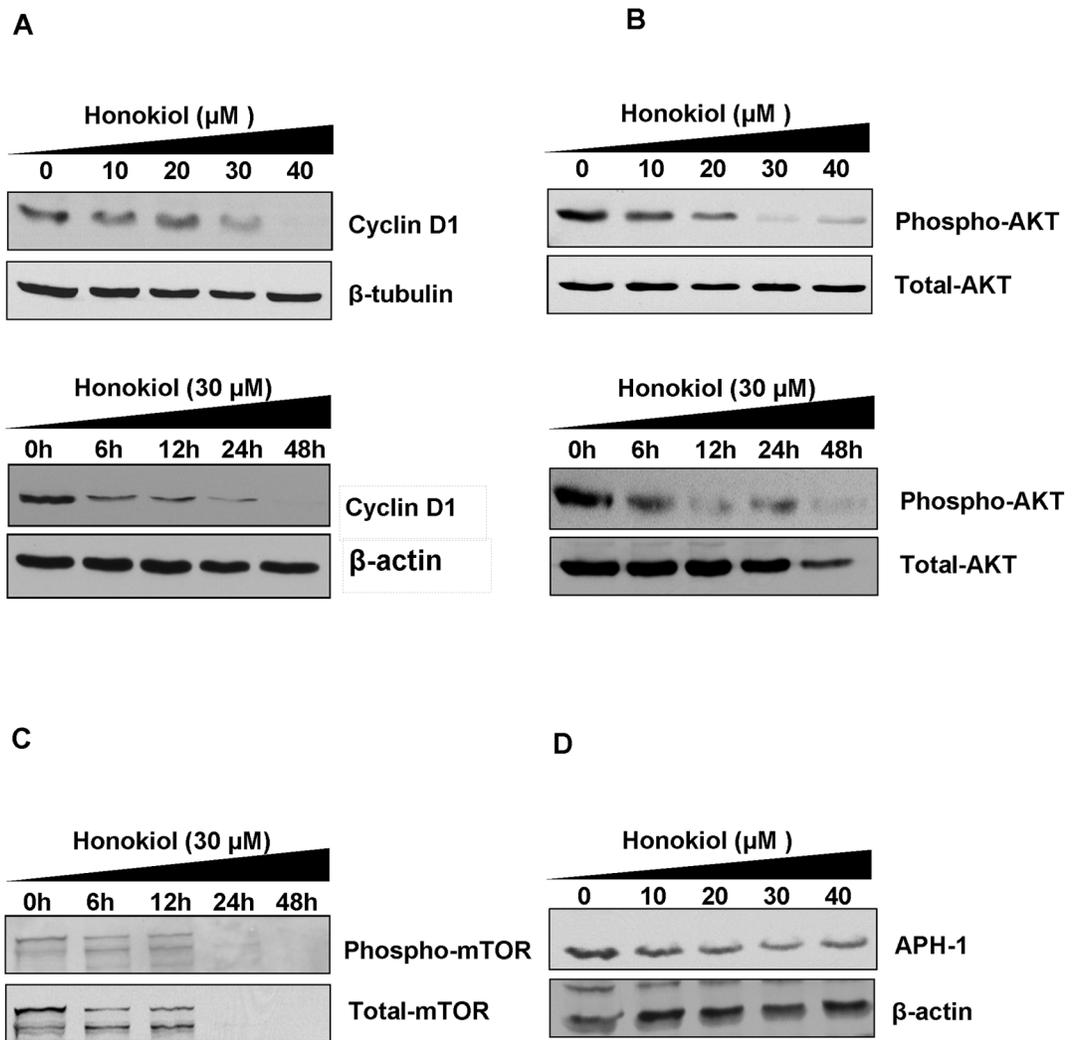
Figure 2.
Honokiol attenuates AKT/mTOR and Notch signaling in B16-F10 melanoma cells. A) Immunoblotting was done for cyclin D1 protein levels in B16-F10 cells. Cells were treated with increasing concentrations of honokiol (0–40 μM) for 24 h of time period to study the dose course effect of honkiol. For time course effects, cells were treated with 30 μM of honokiol for 12, 24 and 48 h. After honokiol treatment, protein lysates were prepared and analyzed by Immunoblotting for Cyclin D1 protein. B) and C) Honokiol affects AKT/mTOR signaling. Immunoblot analyses of lysates after honokiol treatment showed significant reduction in phosphorylation of AKT and mTOR phosphorylation and hence their activities in dose and time dependent manner. D) Honokiol affects Notch signaling. Honokiol inhibits Notch signaling by inhibiting the γ-secretase complex proteins. Honokiol showed significant reduction in the expression of APH1.