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Honokiol Produces Anti-Neoplastic Effects on Melanoma Cells In Vitro

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Background: Melanoma continues to be a therapeutic challenge for the medical community owing to the scarcity of effective agents available to treat the disease. Honokiol, a traditional Chinese herb, has been proven to have anti-cancer effects in various cell types, therefore we hypothesized it may have similar cytotoxic capabilities against melanoma cells in vitro.

Methods: Two cell lines, SK-MEL2 and MeWo, were grown in culture and exposed to increasing doses of Honokiol. Cell proliferation, cytochrome *c* release into the cytosol, intra-cellular caspase activity, and mitochondrial depolarization were then evaluated after treatment with honokiol.

Results: Melanoma cells in culture underwent cell death, had increased cytosolic cytochrome *c*, showed greater caspase activity, and demonstrated increased mitochondrial depolarization after treatment when compared to controls.

Conclusions: It appears that honokiol is an effective inhibitor of cultured human melanoma cells.

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KEY WORDS: honokiol; melanoma; cancer; apoptosis; mitochondria; caspase

INTRODUCTION

Melanoma is the sixth leading cause of cancer-related death, with increasing annual incidences approaching 3% this century [1]. Despite advances in the surgical management of melanoma, its annual mortality remains unchanged, and patients with metastatic disease usually succumb. Disease stage, related to tumor thickness, ulceration, and nodal status at time of presentation continues to be the dominant factor in determining 5-year survival rates [2]. Interferon alfa-2b has been previously used as adjuvant therapy for melanoma [3,4]. Unfortunately, patients are unable to tolerate the multiple adverse effects of interferon, including neutropenia, hepatotoxicity, headache, fever, chills, and depression. It is painfully evident that other options for battling this malignancy are needed.

One such potential compound is honokiol, a derivative of *Magnolia officinalis*, a traditional Chinese herb with medicinal properties (Fig. 1). Honokiol is a biphenyl that has been shown to have anti-angiogenic, anxiolytic, anti-inflammatory, and anti-tumor properties [5,6]. The effect of honokiol on chronic lymphocytic leukemia, breast, lung, and prostate cancer cells has been studied and well-documented [7–11]. The goal of our study is to determine whether honokiol is able induce cell death in human melanoma cells and if so, to elucidate its anti-cancer mechanism of action.

MATERIALS AND METHODS

Drugs and Reagents

Honokiol powder (>98% purity) was purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in DMSO to create a stock solution of 37.6 mM and stored at −4°C. The irreversible pan-caspase inhibitor [Z-VAD (OMe)-FMK] was obtained from Calbiochem (La Jolla, CA), and dissolved in DMSO to create 50 mM stock solution. Cyclosporine A was purchased from Biomol International (Plymouth Meeting, PA) and dissolved in DMSO to create 20.8 mM stock solution. Thiazolyl blue tetrazolium bromide was purchased from Sigma (St. Louis, MO). The Cell Death Detection ELISA^{PLUS} was purchased from Roche (Mannhiem, Germany). The Apo-One® Homogenous Caspase 3/7 Assay was purchased from Promega (Madison, WI). The MitoProbe JC-1 Assay Kit was supplied by Molecular Probes, Inc. (Eugene, OR). The Cytochrome C Enzyme Immunometric Assay Kit was obtained from Assay Designs (Ann Arbor, MI). The Annexin V-FITC Apoptosis Detection Kit was from BioVision (Mountain View, CA).

Cell Culture

Human melanoma cell lines, SK-MEL2 and MeWo, were purchased from ATCC (Manassas, VA). Both cell lines were grown and maintained in MEM media with 10% fetal bovine serum and 1% penicillin. The MeWo cells used were wild type for both BRAF and NRAS mutations. The SK-MEL 2 cells used in the experiments were wild type for the BRAF mutation while possessing the Q61R variant of the NRAS mutation.

Cell Viability Assay

The SK-MEL2 and MeWo cells were plated 10^4 per well onto 96-well plates and allowed 24 hr to adhere. The cell lines were then treated with concentrations of honokiol ranging from 20 to 100 μM. Cells were treated with DMSO as the control. After 24, 48, and 72 hr, all media was aspirated from the wells and cell viability was assessed using the MTT assay. MTT is reduced to purple insoluble formazan in live cells. DMSO was used to solubilize the formazan and absorbance was read at 570 nm.

Portions of this work were presented in poster format at the New England Surgical Conference, September, 2009.

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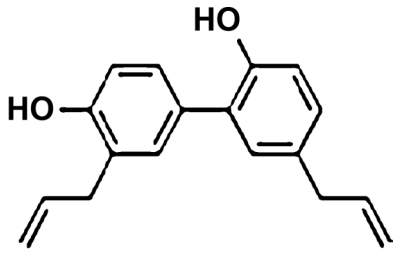

Fig. 1. Honokiol structure.

Cell Death Detection ELISA

Nucleosome accumulation in the cytosol of live cells was analyzed using the Cell Death Detection ELISA. Cells were plated 10^4 per well onto 96-well plates and allowed to adhere for 24 hr. SK-MEL2 was treated with 30 and 50 μM honokiol for 12 hr. MeWo cells were treated with 50 and 60 μM honokiol for 24 hr. The samples were then assessed for nucleosome accumulation in the cytosol of live cells, an indication of apoptosis, using the protocol provided by the manufacturer.

Apo-One® Caspase 3/7 Activity Assay

Caspase activity was analyzed using the Apo-One® Caspase 3/7 Activity Assay. Both cell lines were plated 10^4 per well on 96-well plates for 24 hr and allowed to adhere. After 24 hr, cells were treated with 40 μM honokiol for 5 or 12 hr. Cells treated with DMSO alone served as the control. Per the protocol of the Apo-One® Homogeneous Caspase-3/7 Assay, 100 μl of caspase reagent (caspase substrate + buffer) was added to each well and then mixed/agitated on a plate shaker. The cells were then incubated at room temperature for 30 min before caspase activity was assessed by measuring fluorescence at an excitation wavelength of 485 nm.

Caspase Inhibition Assay

The effect of caspase inhibition was assessed using the irreversible pan-caspase inhibitor Z-VAD (OMe)-FMK during the MTT cell viability assay to determine the degree of cellular rescue, if any. Both SK-MEL2 and MeWo cells were plated 10^4 per well on 96-well plates and allowed 24 hr to adhere. After 24 hr, the cells were then pre-treated for 2 hr with either media or 50 μM of the pan caspase inhibitor. After the 2-hr pre-treat, cells were then treated with DMSO or 30 μM honokiol (SK-MEL2) or 40 μM honokiol (MeWo) for an additional 24 hr. At the end of the treatment period, an MTT assay was performed to measure cell viability.

JC-1 Assay

The JC-1 assay measures mitochondrial depolarization. SK-MEL2 and MeWo cells were plated on six-well plates and allowed to adhere for 24 hr. Each cell line included a DMSO negative (baseline) control and a CCCP (a mitochondrial membrane potential disrupter) positive control. The cells were loaded with 2 μM JC-1 dye and incubated for 30 min at 37°C. The media was aspirated from the wells and media containing honokiol (30–80 μM) was added. The cells were incubated for an additional 30 min at 37°C. The samples were then washed and examined by flow cytometry. JC-1, a cationic dye, exhibits potential-dependent accumulation in mitochondria. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

Cyclosporine A

Cyclosporine A was used as to stabilize mitochondrial membranes. SK-MEL2 and MeWo cells were plated 10^4 per well on 96-well plates and allowed to adhere for 24 hr prior to treatment. After 24 hr, both cell lines were pre-treated for 2 hr with 25 μM Cyclosporine A or DMSO. After pre-treatment, the cells were treated with variable concentrations of honokiol, ranging from 30 to 60 μM and incubated for an additional 24 hr at 37°C. The cells were then assessed for viability using the MTT assay.

Cytochrome C Enzyme Immunometric Assay Kit

The cytochrome *c* EIA assay was used to determine if honokiol treatment resulted in the translocation of cytochrome *c* from the mitochondria to the cytosol. The assay was performed according to the kit instructions. Briefly, the SK-MEL2 and MeWo cells were plated in six-well plates and allowed to adhere for 24 hr. After 24 hr, the cells were treated with 20 or 30 μM honokiol for an additional 24 hr. At this time the cells were collected and the cell pellet was treated with digitonin cell permeabilization buffer. The cells were spun and the supernatant collected as the cytosolic fraction. The subsequent cell pellet was treated with RIPA cell lysis buffer, spun, and the supernatant was collected as the mitochondrial fraction. Results are expressed as picograms of cytochrome *c* per milligram of protein.

Annexin V/Propidium Iodide

Cells were assessed for the translocation of membrane phosphoatidylserine from the inner face of the plasma membrane to the cell surface using Annexin V-FITC apoptosis detection kit. This translocation of phosphoatidylserine is considered a feature of live cells undergoing apoptosis. SK-MEL2 and MeWo were set up in six-well plates and allowed to adhere for 24 hr. The cells were then treated with DMSO or 30 μM honokiol and incubated for an additional 24 hr. The cells were then stained with Annexin V and propidium iodide and run on a flow cytometer according to the product insert.

Statistical Analysis

One-way ANOVA with a Bonferroni post-test were used to evaluate the significance of the results.

RESULTS

Cell Viability After Treatment With Honokiol

The MTT cell viability assay demonstrates that honokiol decreases cell viability in a dose- and time-dependent manner in both cell lines (Fig. 2). All time points and concentrations had statistically significant results ($P < 0.001$) except for 20 μM honokiol with MeWo at 24 hr. This concentration initially had a proliferative effect which then decreased over time. The anti-proliferative effect was most robust in the SK-MEL2 cells where after 24 hr cell viability decreased to 22% of control in the presence of 40 μM of honokiol. The remainder of SK-MEL2 treatment doses and time points resulted in cellular survival rates ranging from 80% to 6% of control. The MeWo cells had similar results with respect to time and dose response with 56% of cells remaining viable after 24 hr in the presence of 40 μM of honokiol. Similar to the SK-MEL2 cells, MeWo cell viability ranged from 75% to 8% of control as both time and the concentration of honokiol increased.

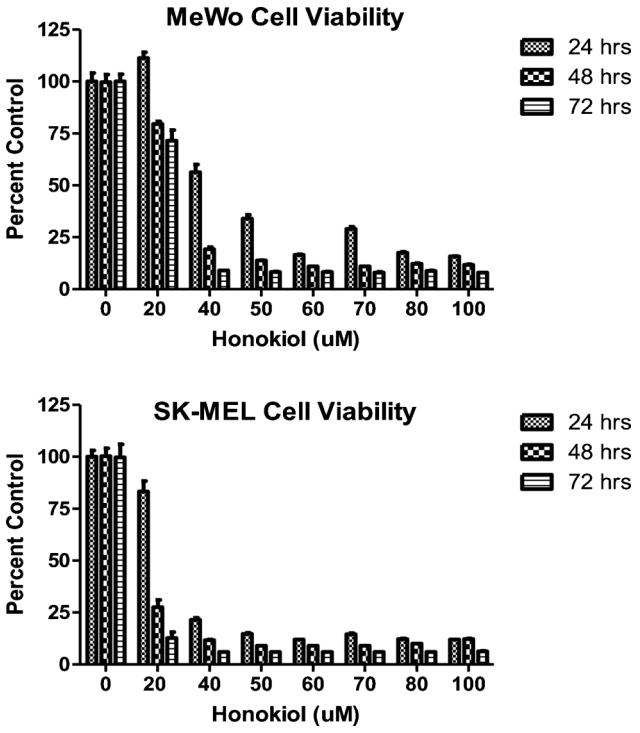

Fig. 2. MTT proliferation assay.

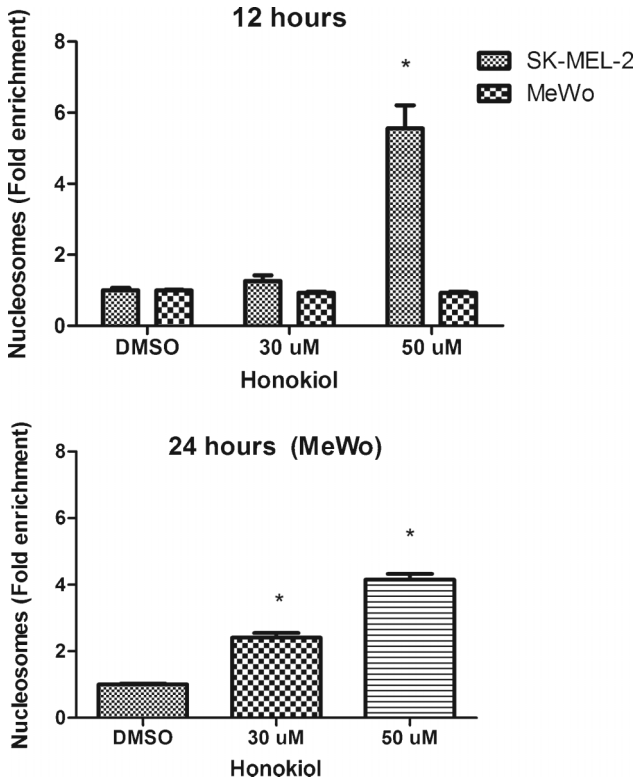

Fig. 3. Cell death ELISA.

Cell Death ELISA

The Cell Death ELISA kit enables the user to determine the amount of cellular accumulation of nucleosomes in the cytoplasm of live cells. The enrichment of nucleosomes in the cytoplasm of the apoptotic cell is due to the fact that during apoptosis DNA degradation occurs several hours before plasma membrane breakdown. Our examination of the lysates of SK-MEL2 and MeWo cells treated with increasing concentrations of honokiol demonstrated a greater degree of nucleosome accumulation with higher doses of the investigational compound. The SK-MEL2 lysate had a 1.26- and 5.56-fold increase in accumulated nucleosomes over control when treated for 12 hr with 30 and 50 μM of honokiol, respectively. The MeWo cell lysate had a 4- and 5.9-fold increase in accumulated nucleosomes over control when treated for 24 hr with 50 and 60 μM of honokiol, respectively. The difference in the treatment times was a reflection of the sensitivity of each cell line to honokiol (Fig. 3).

Caspase Activity

We confirmed an increase in caspase 3/7 activity in honokiol-treated melanoma cells. In the SK-MEL2 cells, there was a statistically significant 2.5-fold increase in caspase activity compared to control after treatment with 40 μM honokiol for 12 hr. In the MeWo cells, there was also a statistically significant 1.7-fold increase in caspase activity after 12 hr of treatment with 40 μM honokiol compared to the DMSO control (Fig. 4).

Caspase Inhibition Assay

A pan-caspase inhibitor [Z-VD (OMe)-FMK] was used in the MTT cell viability assay with SK-MEL2 and MeWo treated with 30 or 40 μM honokiol, respectively. Neither cell line underwent "rescue" when a caspase inhibitor was present (Fig. 5). In fact, in the MeWo cell line the use of the pan-caspase inhibitor resulted in a slight *decrease* in cellular survival.

JC-1 Assay

Mitochondrial depolarization, implicating a role of the mitochondria in honokiol induced cell death, was found to increase in a dose-dependent manner. In SK-MEL2 cells, 50%, 52%, and 74% of mitochondria had become depolarized when exposed to 30, 40, and 50 μM of honokiol, respectively. The MeWo cells had 15%, 40%, and 76% mitochondrial depolarization in response to 40, 60, and 80 μM of honokiol (Table I). SK-MEL2 cells respond more vigorously to honokiol, requiring less of the compound for depolarization, but also have a higher background level (DMSO treated) of mitochondrial depolarization.

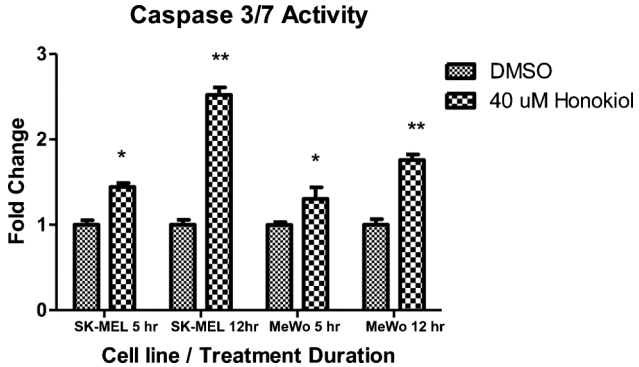

Fig. 4. Caspase activity.

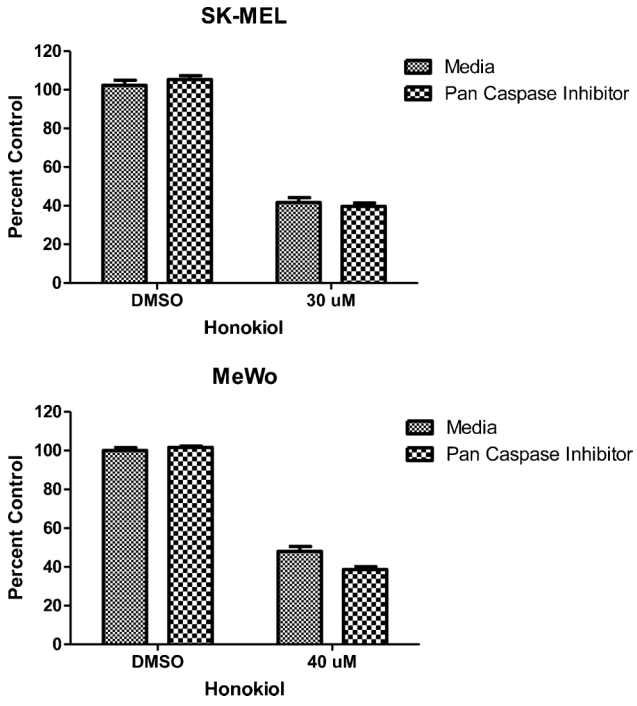

Fig. 5. Caspase inhibition.

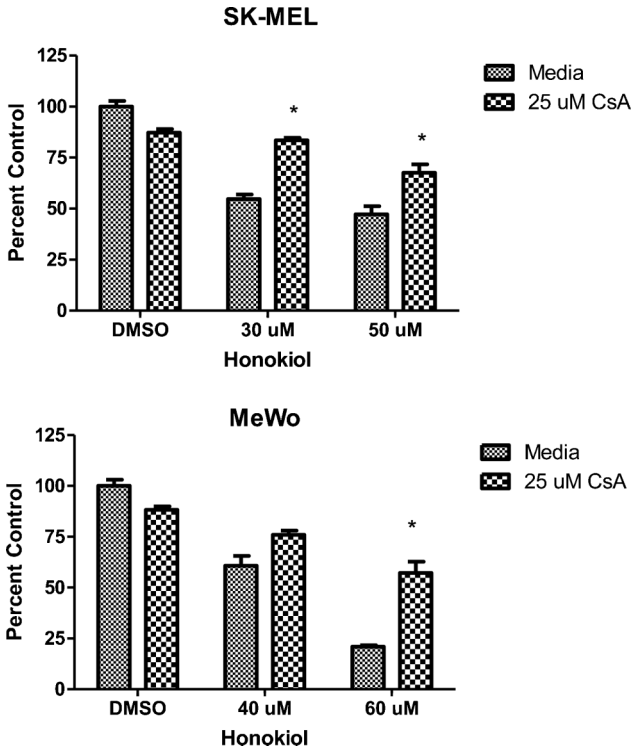

Fig. 6. Cyclosporine A.

Cyclosporin A

To evaluate if the mitochondrial membrane was a potential source of the cellular death signal in cells treated with honokiol, cyclosporine A was used to stabilize mitochondrial membranes in culture prior to treatment. SK-MEL2 cells, after cyclosporine A pre-treatment, demonstrated a 23% and 26% increase in cellular survival over controls, with 30 and 50 μM of honokiol, respectively. MeWo cells treated with the same cyclosporine A pre-treatment followed by 40 and 60 μM of Honokiol resulted in a 15% and 36% increase in cellular survival over controls, respectively. All the results except MeWo at 40 μM honokiol were statistically significant ($P < 0.05$) (Fig. 6).

TABLE I. JC 1 Assay (Mitochondrial Depolarization)

	Healthy mito	Depolarized mito	Red/green ratio
	Red %	Green %	
JC-1 staining			
SK-MEL			
DMSO	80	20	4.000
30 μM HNK	50	50	1.000
50 μM HNK	48	52	0.923
70 μM HNK	26	74	0.351
CCP (pos, control)	30	70	0.429
MeWo			
Celtic	90	10	9.000
40 μM HNK	85	15	5.667
60 μM HNK	60	40	1.500
80 μM HNK	85	21	0.316
CCCP (pos. control)	37	63	0.587

Cytochrome C

Cytochrome *c* release from the mitochondrial membrane to the cytosol is a feature of apoptosis. SK-MEL2 and MeWo cells both showed a significant increase in cytosolic cytochrome *c* over background. Interestingly, SK-MEL2 exhibited a much higher baseline (DMSO treated) level of cytosolic cytochrome *c* (Fig. 7).

Annexin V/Propidium Iodide

Annexin V binds phosphoatidylserine translocated to the outside of the cell during apoptosis. Propidium iodide binds DNA, and in this assay only labels dead cells. Annexin V/PI staining revealed no honokiol induced Annexin V binding to live cells above background (DMSO) in SK-MEL2. However, the PI positive (dead cell) fraction increased from 9% to 60%. In MeWo there was a slight increase in

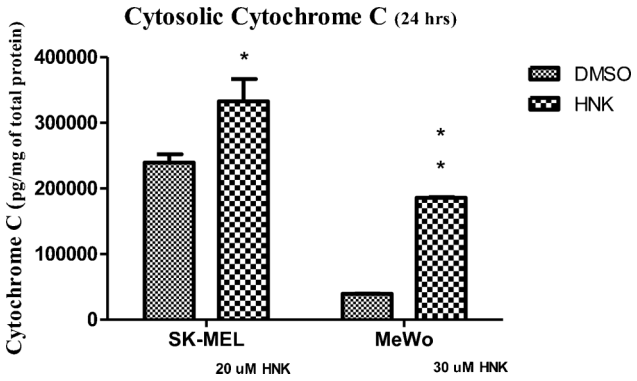

Fig. 7. Cytosolic cytochrome *c*.

TABLE II. Annexin V/Propidium Iodide Staining

	Annexin V single positive	Propidium iodide positive
SK-MEL		
DMSO	9%	9%
30 μM honokiol	9%	60%
MeWo		
DMSO	3%	3%
30 μM honokiol	5%	43%

Annexin V binding from 3% to 5% and also an increase in the dead cell population from 3% to 43% (Table II).

DISCUSSION

The anti-cancer effects of honokiol are well documented and include such varying cell types as squamous cell lung cancer, colorectal carcinoma, and breast cancer. Our current study is the first to evaluate its possible role against melanoma. Our results show that two different melanoma cell lines, MeWo and SK-MEL2, are susceptible to the anti-proliferative effects of honokiol. The exact mechanism of action by which honokiol exerts its anti-neoplastic actions against cancerous cell lines remained unknown, but a mitochondrial source of cell death is suspected.

Our cell viability data is clear: honokiol exerts a potent anti-neoplastic action with higher concentrations trending towards more inhibition. The largest impact upon cellular proliferation was seen in the lower doses of honokiol, 30–50 μM concentrations. Generally, the SK-MEL2 cell line was more sensitive to honokiol and therefore the concentrations for many experiments were adjusted accordingly.

Results of the Cell Death ELISA demonstrate a DNA damaging effect of honokiol on these melanoma cell lines. The accumulated nucleosomes in the cytosol of honokiol-treated cells demonstrate the DNA damage (DNA laddering) that occurs with apoptosis. The caspase activity data reveals increased activity of caspases in both cell lines. The increased activity of caspases within treated cells is indicative of changes that herald imminent cellular death. However, the presence of increased caspase activity only identifies a cell with dramatic intra-cellular responses to noxious insults. Honokiol places melanoma cells at a greater risk for death. Therefore, inhibition of some portion of the caspase cascade could have a reparative or rescue effect on the melanoma cells after an initial injury by a pro-apoptotic agent. However, our cell lines showed no increase in cell viability when treated with a pan-caspase inhibitor. From these results, it appears that honokiol exerts its effects on SK-MEL2 and MeWo melanoma cell lines through caspase-independent mechanisms.

Cytochrome *c* translocation from the mitochondria to the cytosol of the cell is also a feature of apoptosis. Cytochrome *c* translocation activates downstream caspases resulting in nuclear apoptosis. Both SK-MEL2 and MeWo exhibited increased cytosolic cytochrome *c* in response to honokiol treatment. Interestingly, SK-MEL2 had a much higher background (DMSO treated) cytosolic cytochrome *c*. This cell line, which was more sensitive to honokiol than MeWo, had higher background levels of mitochondrial depolarization, cytosolic cytochrome *c*, and Annexin V staining.

Mitochondria have been recently implicated as a target for chemotherapeutic agents due to their role in apoptotic pathways mediated, in part, through the release of intra-mitochondrial substrates including reactive oxygen species following their membrane depolarization [12]. Our data suggests honokiol may target the mitochondria, evidenced by an increased fraction of depolarized mitochondria in the JC-1 assay. The increased cell viability after pre-treatment with cyclosporine A, a mitochondrial membrane stabilizer, adds further credence to this mechanism of action. Cellular mitochondrial depolarization was definitively found in both cell lines after only 30 min of treatment. The dose-dependent manner in which honokiol causes mitochondrial depolarization was also evident.

Our research demonstrates that the anti-tumor effect of honokiol extends to two melanoma cell lines—SK-MEL2 and MeWo. These results, combined with previous studies, suggest a potential therapeutic role for honokiol in the treatment of melanoma. Further in vitro studies are warranted to elucidate the precise mechanism of action of this promising anti-cancer compound.

CONCLUSIONS

The Chinese herb honokiol is becoming a compound of interest in the fight against melanoma. While its mechanism of action is still unclear, its effectiveness in reducing the viability of numerous cancers in vitro makes it an ideal candidate for further study.

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