

Honokiol, a chemopreventive agent against skin cancer, induces cell cycle arrest and apoptosis in human epidermoid A431 cells

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Abstract

Honokiol is a plant lignan isolated from bark and seed cones of *Magnolia officinalis*. Recent studies from our laboratory indicated that honokiol pretreatment decreased ultraviolet B-induced skin cancer development in SKH-1 mice. The aim of the present investigation was to study the effects of honokiol on human epidermoid squamous carcinoma A431 cells and to elucidate possible mechanisms involved in preventing skin cancer. A431 cells were pretreated with different concentrations of honokiol for a specific time period and investigated for effects on apoptosis and cell cycle analysis. Treatment with honokiol significantly decreased cell viability and cell proliferation in a concentration- and time-dependent manner. Honokiol pretreatment at 50 $\mu\text{mol/L}$ concentration induced G0/G1 cell cycle arrest significantly ($P < 0.05$) and decreased the percentage of cells in the S and G2/M phase. Honokiol down-regulated the expression of cyclin D1, cyclin D2, Cdk2, Cdk4 and Cdk6 proteins and up-regulated the expression of Cdk's inhibitor proteins p21 and p27. Pretreatment of A431 cells with honokiol leads to induction of apoptosis and DNA fragmentation. These findings indicate that honokiol provides its effects in squamous carcinoma cells by inducing cell cycle arrest at G0/G1 phase and apoptosis.

Keywords: honokiol, apoptosis, cell cycle

Experimental Biology and Medicine 2011; 236: 1351–1359. DOI: 10.1258/ebm.2011.011030

Introduction

Honokiol, a naturally occurring biphenyl plant lignan, is isolated from bark and seed cones of plant *Magnolia officinalis*, and has been used in traditional Chinese and Japanese medicine.^{1–3} Honokiol exhibited potent anticancer activity *in vitro* and *in vivo* and was demonstrated to be a potential candidate for the treatment of colorectal carcinoma.^{4,5} Honokiol inhibited *in vitro* and *in vivo* growth of breast cancer through induction of apoptosis and cell cycle arrest.⁶ Honokiol caused G0/G1 phase cell cycle arrest in human prostate cancer cells in association with suppression of retinoblastoma protein level/phosphorylation and inhibition of E2F1 transcriptional activity.⁷ Honokiol potentiated apoptosis, suppressed osteoclastogenesis and inhibited invasion through modulation of the nuclear factor-kappa B (NF- κ B) activation pathway.⁸

Honokiol inhibited skin tumor promotion in a 7,12-dimethylbenz(α)anthracene-initiated and 12-O-tetradecanoyl phorbol-13-acetate-promoted skin carcinogenesis model.⁹ A recent study conducted in our laboratory showed chemopreventive effects of honokiol in ultraviolet B (UVB)-induced skin tumor development.¹⁰ In this study,

we found that topical application of honokiol significantly ($P < 0.05$) reduced tumor number and onset of tumorigenesis compared with a control in an UVB-initiated and UVB-promoted mouse skin model. Honokiol pretreatment was effective at very low concentrations (microgram per applications) in inhibiting UVB-induced skin tumorigenesis compared with other chemopreventive agents with comparable effects at milligrams per application.^{10–12} Our results indicated that chemopreventive effects were related to induction of apoptosis.¹⁰ Another study by Vaid *et al.*,¹³ confirmed our results on the chemopreventive effects of honokiol on UVB-induced skin cancer development. Although effects of honokiol on other cell lines have been investigated, the molecular mechanisms involved in anti-carcinogenic effects of honokiol against skin cancer are not well understood. Chemopreventive agents either cause induction of apoptosis and/or modulation of cell survival pathways. In order to elucidate the possible mechanism involved, in the present study we investigated the effects of honokiol on apoptosis and cell cycle of human epidermoid carcinoma A431 cells.

The results from this study demonstrated that in addition to induction of apoptosis,¹⁰ honokiol showed antiproliferative effects by causing cell cycle arrest at G0/G1 phase and provided insight into the mechanism by which cell cycle progression was inhibited by honokiol.

Materials and methods

Reagents

Honokiol (98% pure) was purchased from Nacal Tesque (Kyoto, Japan). Thiazolyl blue tetrazolium bromide (MTT) and other chemicals of analytical grade were purchased from Sigma Chemical Co (St Louis, MO, USA). A cell proliferation enzyme-linked immunosorbent assay (ELISA) kit and *in situ* cell death detection kit were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Vibrant Apoptosis Kit 2 and APO-bromodeoxyuridine (BrdU) terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit were purchased from Molecular Probes (Eugene, OR, USA). ECL Plus kit was purchased from Amersham Biosciences (Piscataway, NJ, USA). Primary antibodies such as cyclin D1, cyclin D2, cyclin E, cyclin B1, cyclin A, Cdc25C, p-Cdc25C, Cdk-2, Cdk-4, Cdk-6, Cdc25 p34, poly (ADP-ribose) polymerase (PARP), antimouse IgG horseradish peroxidase-linked and antirabbit IgG horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Kip1/p27 was purchased from BD-Pharmingen (San Diego, CA, USA) and anti-Cip1/p21 from Upstate Biotechnology (Lake Placid, NY, USA).

Cell culture

Human epidermoid squamous carcinoma A431 cells were purchased from American Type Culture Collection (Manassas, VA, USA). A431 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μ g/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere containing 95% air and 5% CO₂.

Honokiol solution

Honokiol was dissolved in dimethyl sulfoxide (DMSO) to make 0.05 mol/L stock solution, and then diluted in growth medium at different concentrations and used immediately. In all the assays, the final concentration of DMSO in growth medium was 0.4%.

MTT assay for cell viability

Cells (9000 cells/well) were plated in 96-well plates. After 24 h, cells were treated with different concentrations of honokiol (0, 25, 50 and 75 μ mol/L) for 12, 24 and 48 h using a control as cells treated with growth medium alone. At the end of each treatment, cells were incubated with 10% of MTT stock solution (5 mg/mL) for four hours. Thereafter, medium was aspirated and 150 μ L of DMSO was added to dissolve crystal dye formazan for one hour. Absorbance

was measured at 570 nm with absorbance at 650 nm to correct background for blank (media without cells) using a Spectra Max M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

BrdU assay for cell proliferation

BrdU incorporation assay was carried out using an ELISA kit (Roche Diagnostics GmbH) following the manufacturer's protocol. Briefly, 9000 cells/well were plated in a 96-well plate and treated with different concentrations of honokiol (0, 25, 50 and 75 μ mol/L) for 12, 24 and 48 h. At the end of each treatment period, cells were labeled with BrdU by incubating for three hours at 37°C. Then cells were fixed by using FixDenat solution for 30 min followed by incubating fixed cells with anti-BrdU-POD solution for 90 min. The cells were then rinsed with washing solution and incubated with substrate solution for 20 min. The reaction was stopped using 1 mol/L H₂SO₄ and absorbance of samples was measured using a microplate reader using 450 nm with absorbance at 690 nm as reference.

Apoptosis assay

Apoptosis was quantified by using Vibrant Apoptosis Kit 2 (Molecular Probes) as per the manufacturer's protocol. Briefly, A431 cells (2×10^5) were treated with honokiol (0, 50 and 75 μ mol/L) for 48 h. At the end of treatment, cells were washed with ice-cold phosphate-buffered saline and incubated with Annexin V labeled with Alexa 488 and propidium iodide (PI) for 15 min at room temperature. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) using CellQuest 3.3 software (Becton Dickinson, Franklin Lakes, NJ, USA). The early apoptotic cells were stained with Alexa 488 and give green fluorescence, represented in the lower-right (LR) quadrant. Late apoptotic cells were stained with both Alexa 488 and PI and give green and red fluorescence, represented in the upper-right (UR) quadrant.

TUNEL assay for DNA fragmentation

Apo-BrdU TUNEL assay kit (Molecular Probes) was used to quantify the amount of DNA fragmentation in A431 cells by using the manufacturer's protocol. Briefly, cells after being treated with honokiol (0, 50 and 75 μ mol/L) for 48 h, were harvested, collected and fixed by 1% (W/V) paraformaldehyde followed by 70% (V/V) ethanol. The fixed cells were labeled with BrdU followed by incubation with Alexa Fluor 488 labeled anti-BrdU for staining fragmented DNA in apoptotic cells. The stained cells were analyzed by flow cytometry. Positive and negative controls were run with each assay.

DNA analysis

Subconfluent A431 cells were treated with different concentrations of honokiol (0, 25, 50 and 75 μ mol/L) for 12, 24 and 48 h. After each treatment, cells were harvested and fixed in 70% ethanol in Dulbecco's phosphate-buffered saline. Fixed cells were treated with 100 μ L of RNase A (1 mg/mL) for

30 min at 37°C. After incubation, to each sample, 900 μ L of staining buffer and 20 μ L of PI were added and incubated for 30 min in dark. The samples were analyzed with BD FACScan™ flow cytometry (BD Biosciences, San Jose, CA, USA) using CellQuest Software (BD Biosciences).

Immunoblotting

For Western blot, 1.5×10^6 cells were plated in a 100-mm culture dish. Subconfluent A431 cells were treated with varying concentrations of honokiol (0, 25, 50 and 75 μ mol/L) for 12, 24 and 48 h. At the end of each treatment, cells were lysed and protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, IL, USA) with albumin as the standard.

The proteins (50 μ g) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking the membranes in 5% non-fat milk, membranes were probed with appropriate dilution of primary antibodies at 4°C overnight. The membranes were then incubated with horseradish peroxidase conjugated secondary antibody followed by detection by ECL plus detection system and were quantified by using UVP Biochem Gel Documentation system (UVP Inc, Upland, CA, USA). Equal protein loading is ensured by reprobing each membrane with β -actin antibody. Western blots were repeated for 3–5 samples. Representative bands from all samples are reported.

Statistical analysis

INSTAT software (GraphPad, San Diego, CA, USA) was used to analyze the data. Analysis of variance followed by Tukey's test was used. Significance in all experiments was considered at $P < 0.05$. All values are expressed as mean $\pm$ standard error.

Results

Honokiol treatment decreased cell viability in A431 cells

As this is the first time the anticarcinogenic effects of honokiol on A431 skin cancer cells were determined, first we conducted an MTT assay to determine the effects of honokiol on cell viability. Cells were treated with varying concentrations (0, 25, 50 and 100 μ mol/L) of honokiol for 12, 24, 48 and 72 h. Honokiol treatment did not show significant decrease in cell viability at 25 μ mol/L, but treatments of honokiol significantly decreased cell viability at 50–75 μ mol/L in a time-dependent manner. As shown in Figure 1a, after a 24-h treatment, honokiol 50 and 75 μ mol/L inhibited cell viability of A431 in 57.37–93.12% of cells. After a 48-h treatment, honokiol at 50–75 μ mol/L inhibited cell viability of A431 in 73.12–97.24% of cells. After 72-h treatment, honokiol at 50–75 μ mol/L inhibited cell viability of A431 in 84.51–100% of cells (Figure 1a).

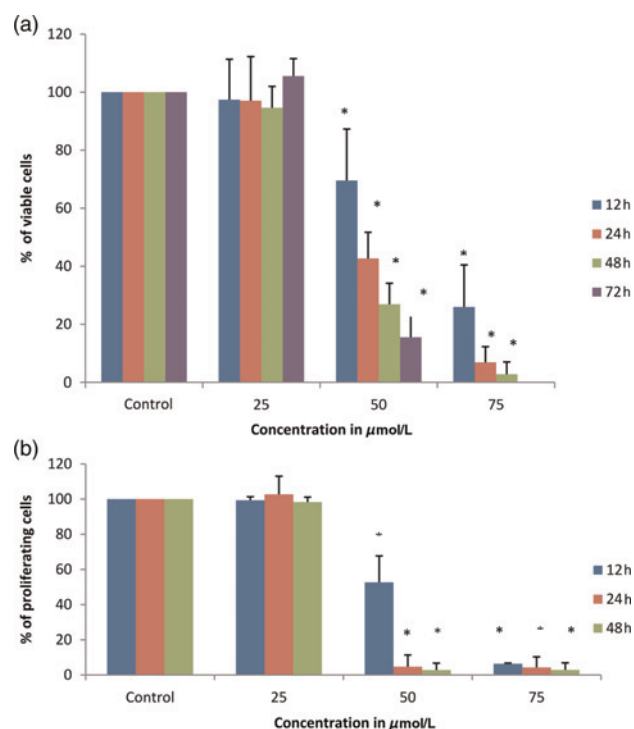


Figure 1 Effects of honokiol on cell viability (a) and cell proliferation (b) in A431 cells. A431 cells were treated with honokiol (0–75 μ mol/L) for 12, 24, 48 and 72 h. Thiazolyl blue tetrazolium bromide and bromodeoxyuridine assay were performed at the end of each time period as described in Materials and methods. Each value is given as mean $\pm$ SE from three experiments. *Significantly different $P < 0.05$ (A color version of this figure is available in the online journal)

Honokiol treatment inhibited cell proliferation in A431 cells

We investigated the effects of honokiol on cell proliferation in A431 cells by BrdU incorporation assay. For this purpose, cells were treated with varying concentration of honokiol (0, 25, 50, 75 μ mol/L) at 12, 24 and 48 h. Honokiol did not have any significant effect on cell proliferation at 25 μ mol/L. As shown in Figure 1b, for the 12-h treatment, honokiol at 50 and 75 μ mol/L inhibited cell proliferation by 47.3–93.65% as compared with control. For the 24-h treatment, honokiol at 50 and 75 μ mol/L resulted in 95–96% decrease of cell proliferation in A431 cells. For the 48-h treatment, honokiol at 50–75 μ mol/L inhibited 97% of cell proliferation as compared with control in A431 cells. Honokiol treatment significantly decreased the cell proliferation of human epidermoid carcinoma A431 cells, as measured by the BrdU incorporation assay (Figure 1b).

Overall, honokiol treatment inhibited the cell viability and cell proliferation of the A431 cell line in a concentration- and time-dependent manner. Therefore, further experiments were performed to investigate whether the decrease of cell viability in the A431 skin cancer cell line by honokiol treatment is related to the alteration in normal cell cycle distribution.

Honokiol induced G0/G1 phase cell cycle arrest in A431 cells

In order to understand the honokiol's mechanisms of inhibition of cell viability and cell proliferation, we investigated

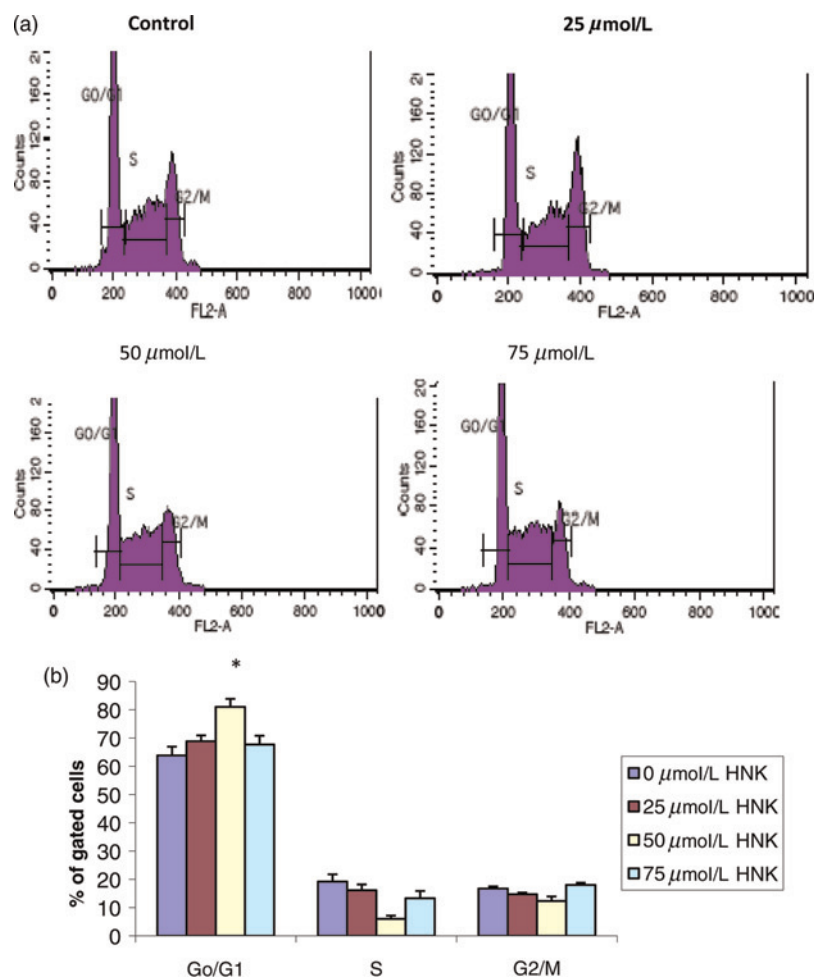


Figure 2 Effects of honokiol on cell cycle progression by flow cytometry in A431 cells. Cells were treated with varying concentrations of honokiol (0–75 $\mu\text{mol/L}$) for 24 h. At the end of each treatment cells were harvested, digested with RNase and stained with propidium iodide as described in Materials and methods and analyzed by flow cytometry. (a) Cell cycle distribution for 0, 25, 50 and 75 $\mu\text{mol/L}$ at 24 h. (b) Each point represents cell cycle distribution from three independent experiments. Data were summarized as mean $\pm$ SE. *Significantly different $P < 0.05$. HNK, honokiol (A color version of this figure is available in the online journal)

its effect on various phases of the cell cycle in A431 cells. Analysis of cell cycle phases was performed by flow cytometry after the treatment of cells with honokiol.

As shown in Figure 2, for A431 cells, honokiol at 25 $\mu\text{mol/L}$ produced relatively small changes in DNA distribution between all phases of the cell cycle. However, at the 24-h treatment, honokiol at 50 $\mu\text{mol/L}$ resulted in a significant ($P < 0.05$) higher number of A431 cells in the G0/G1 phase as measured by the distribution of DNA content compared with the control. The increase in cell population in the G0/G1 phase caused by honokiol was associated with a corresponding shift in the population of cells mainly in the G2/M phase. The effects of honokiol on cell cycle arrest at the G0/G1 phase appear to be permanent.

Honokiol changed expressions of proteins involved in the G0/G1 phase transition in A431 cells

Cell cycle progression and arrest processes are dependent on the levels of cyclins, cyclin-dependent kinases (Cdks) and their inhibitors, and thus we have investigated the changes in the expression of cell cycle protein in A431 cells after honokiol treatment. Marked changes in the expression of

proteins that are known to play a role in G0/G1 phase progression were observed in the presence of honokiol in A431 cells. As shown in Figure 3, cyclin D1, a protein involved in both G0/G1 phase was down-regulated starting at 12 h at 75 $\mu\text{mol/L}$, and at 24–48 h time point with 50–75 $\mu\text{mol/L}$ concentrations. The expression of cyclin D2 was found to be decreased at 12, 24 and 48 h with 50–75 $\mu\text{mol/L}$ of honokiol treatment. Next, cyclin E, a protein that associates with Cdk2, which is a necessary step for the progression from G1 to S phase was investigated. The level of cyclin E protein was decreased as early as 12-h treatment at 75 $\mu\text{mol/L}$; at 24 and 48 h, just the 75 $\mu\text{mol/L}$ concentration affected the expression of this protein.

Cdks are proteins involved in the cell cycle; we evaluated Cdk2 protein (involved in G1–S–G2 phases) and Cdk4 and Cdk6 proteins (involved in G1 phase). As presented in Figure 3, honokiol caused a decrease in Cdk2 expression at 24 and 48 h at 50–75 $\mu\text{mol/L}$. Cdk4 expressions were inhibited by honokiol at 24 and 48 h at 75 $\mu\text{mol/L}$; the expression of Cdk6 was also decreased by honokiol treatment at 24–48 h at 50–75 $\mu\text{mol/L}$. Then, we assessed the expression of cell cycle inhibitor proteins p21 and p27. Honokiol treatment to A431 cells significantly increased expression of p27 at 12,

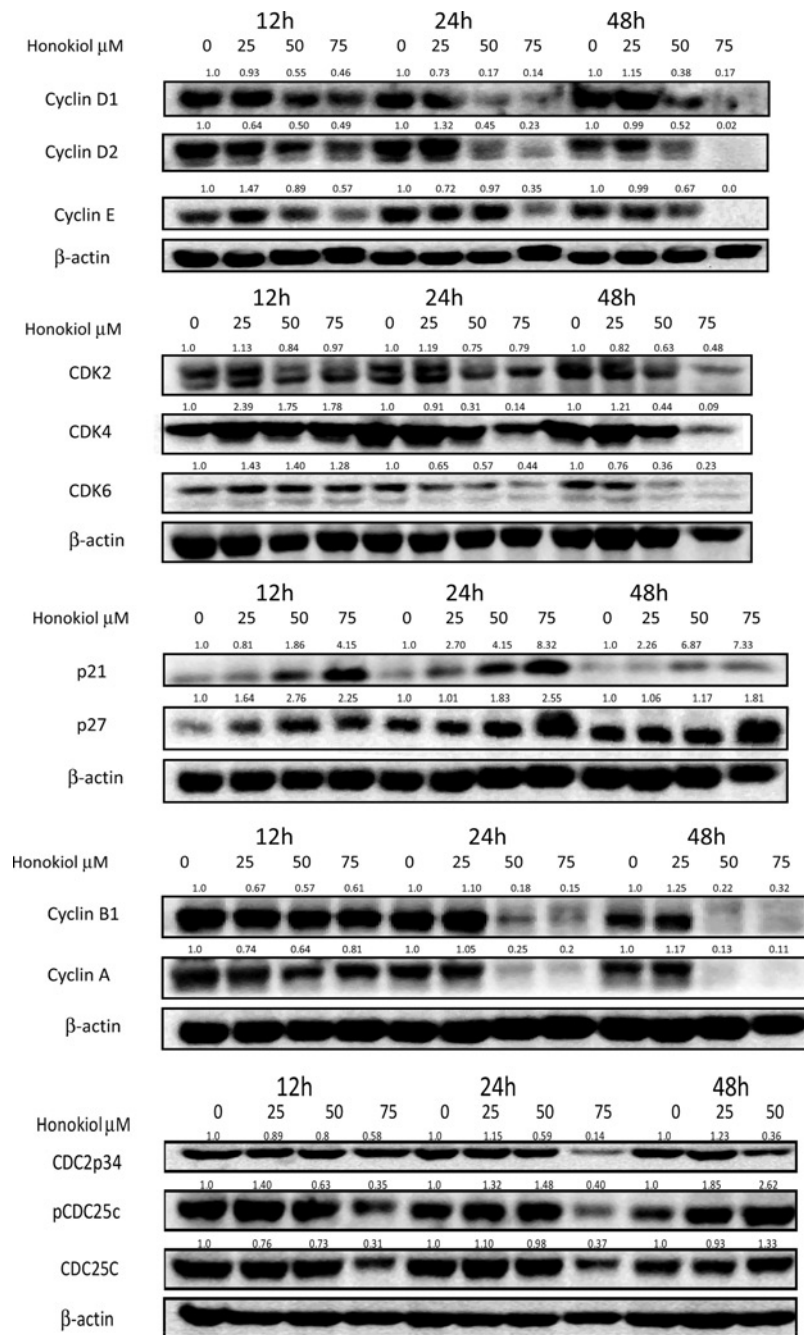


Figure 3 Effects of honokiol on expression of cell cycle regulatory proteins in G0/G1 phase and G2/M in A431 cells. Cells were treated with varying concentrations of honokiol (0, 25, 50 and 75 $\mu\text{mol/L}$) for 12, 24 and 48 h and then harvested and thereafter cell lysates were prepared. Total cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blot analysis. Membranes were probed with different primary antibodies in milk overnight followed by appropriate secondary antibody and ECL detection. β -Actin was used to verify equal loading of samples. The number above the bands indicates the ratio compared with the control

24 and 48 h, whereas p21 expression was increased at 12 and 24 h in a concentration-dependent manner (Figure 3). The level of p21 expression in the presence of honokiol was lower at all concentrations at all time points when compared with the expression of p27. Honokiol may have differential effects on the expression of p21 and p27. Furthermore, decreased expression of p21 could also be attributed to its rapid turnover on its dissociation from cyclin-Cdk complex. Honokiol changed the expressions of proteins involved in the G2/M phase transition in A431 cells. As

shown in Figure 3, cyclin A, a protein involved in both S and G2 phase was down-regulated at the 24-h time point with 50–75 $\mu\text{mol/L}$ doses. The expression of Cdk2, which associates with cyclin A for cell cycle progression, was found to be decreased at 24 h with 50 $\mu\text{mol/L}$ of honokiol treatment. Next, cyclin B1, a protein involved in M phase was investigated; in the presence of honokiol, the level of cyclin B1 protein was decreased at 24-h treatment at 50–75 $\mu\text{mol/L}$ (Figure 3). Although expression of cyclin B1 was decreased after honokiol treatment, no effects on

mitosis were observed. Cyclin B1 activates Cdc2. Therefore, we determined the impact of honokiol on the expressions of Cdc2. As presented in Figure 3, honokiol at 75 $\mu\text{mol/L}$ inhibited the expression of Cdc2 for 24–48-h treatment. Cdk2s are maintained in an inactive state through the phosphorylation of Thr 14 and Tyr 15. The rate-limiting step in the activation of Cdk2s is dephosphorylation of these residues by Cdc25 phosphatases.¹⁴ There are three mammalian cdc25 homologs: Cdc25A, Cdc25B and Cdc25C.¹⁴ In order to better understand the effects of honokiol on Cdk2s, we further determined the impact of honokiol on the expressions of Cdc25A, Cdc25B and Cdc25C in A431 cells. Of all the Cdc25 phosphatases tested, significant changes in the expression pattern

were observed only in Cdc25C with honokiol. As presented in Figure 3, honokiol decreased the Cdc25C expression at 75 $\mu\text{mol/L}$ at all time points. Cdc2 can be activated through the dephosphorylation of Thr 14 and Tyr 15 by phosphorylating Cdc25C to p-Cdc25C at Ser 216.¹⁴ The expressions of p-Cdc25C were decreased by honokiol treatment at 12–24 h and 75 $\mu\text{mol/L}$ of honokiol.

Honokiol-induced apoptosis in A431 cells

To investigate whether cell death caused by honokiol is an apoptotic response, we next treated cells with varying concentrations of honokiol (50, 75 $\mu\text{mol/L}$) for 48 h, followed

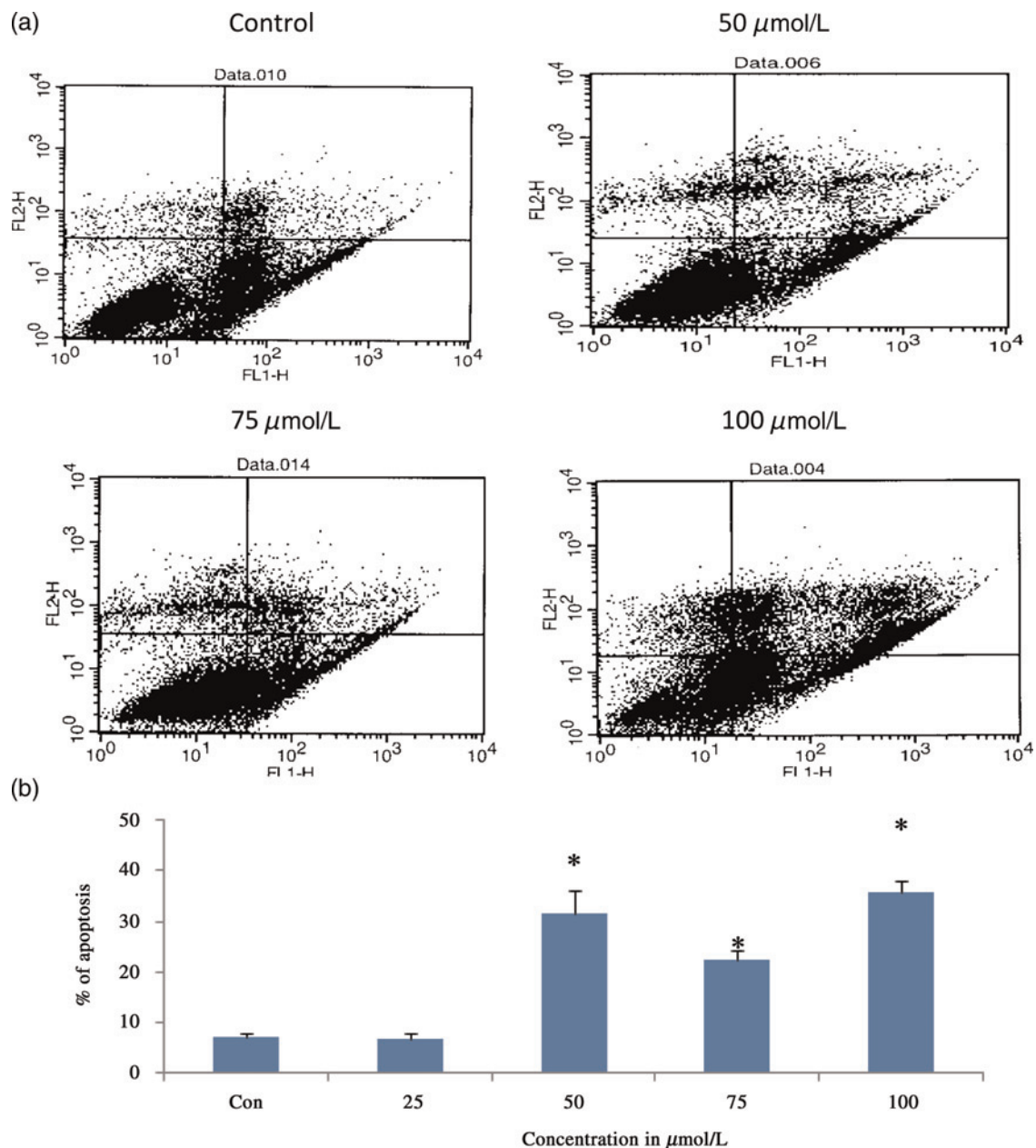


Figure 4 Effects of honokiol on apoptosis by AnnexinV/PI staining. (a) A431 cells were treated with (0, 50, 75 and 100 $\mu\text{mol/L}$) honokiol for 48 h. Cells were harvested and were stained by using Vibrant Assay Kit as described under Materials and methods and apoptosis was determined by using flow cytometry. Lower quadrant represents percentage of early apoptotic cells. Upper-right quadrant indicates percentage of late apoptotic cells. (b) Data represent mean $\pm$ SE of three observations. *Significantly different $P < 0.05$ (A color version of this figure is available in the online journal)

by Annexin V/PI staining using Vibrant Apoptosis kit and were analyzed through FACS. As shown in Figure 4, early apoptotic cells are represented in the LR quadrant and late apoptotic cells in the UR quadrant. The results showed that compared with the DMSO-treated control showing 2% of apoptotic cells, honokiol treatment (100 $\mu\text{mol/L}$) resulted in 26% of apoptosis at 48 h. These results suggest that honokiol treatment induced apoptosis significantly ($P < 0.05$) in a concentration-dependent manner.

Honokiol induces DNA fragmentation in apoptotic cells

Since Annexin V/PI staining detects early stage of apoptosis, in order to investigate effects of honokiol on DNA

fragmentation, which is a hallmark of apoptosis that commits cells to die, the TUNEL assay was performed. Similar to the apoptotic assay, cells were treated with varying concentration of honokiol (50 and 75 $\mu\text{mol/L}$) for 48 h. As shown in Figure 5a, M1 is used to indicate DNA-fragmented cells. Compared with the DMSO-treated control, showing 0.8% of DNA fragmentation, honokiol-treated A431 cells at 50 and 75 $\mu\text{mol/L}$ resulted in 1.17% and 21% of DNA fragmentation. The results suggest that 50 $\mu\text{mol/L}$ did not induce DNA fragmentation, whereas 75–100 $\mu\text{mol/L}$ concentration significantly increased DNA fragmentation (Figure 5b).

By Western blotting analysis, we observed that honokiol treatment to A431 cells increased the expression of PARP,

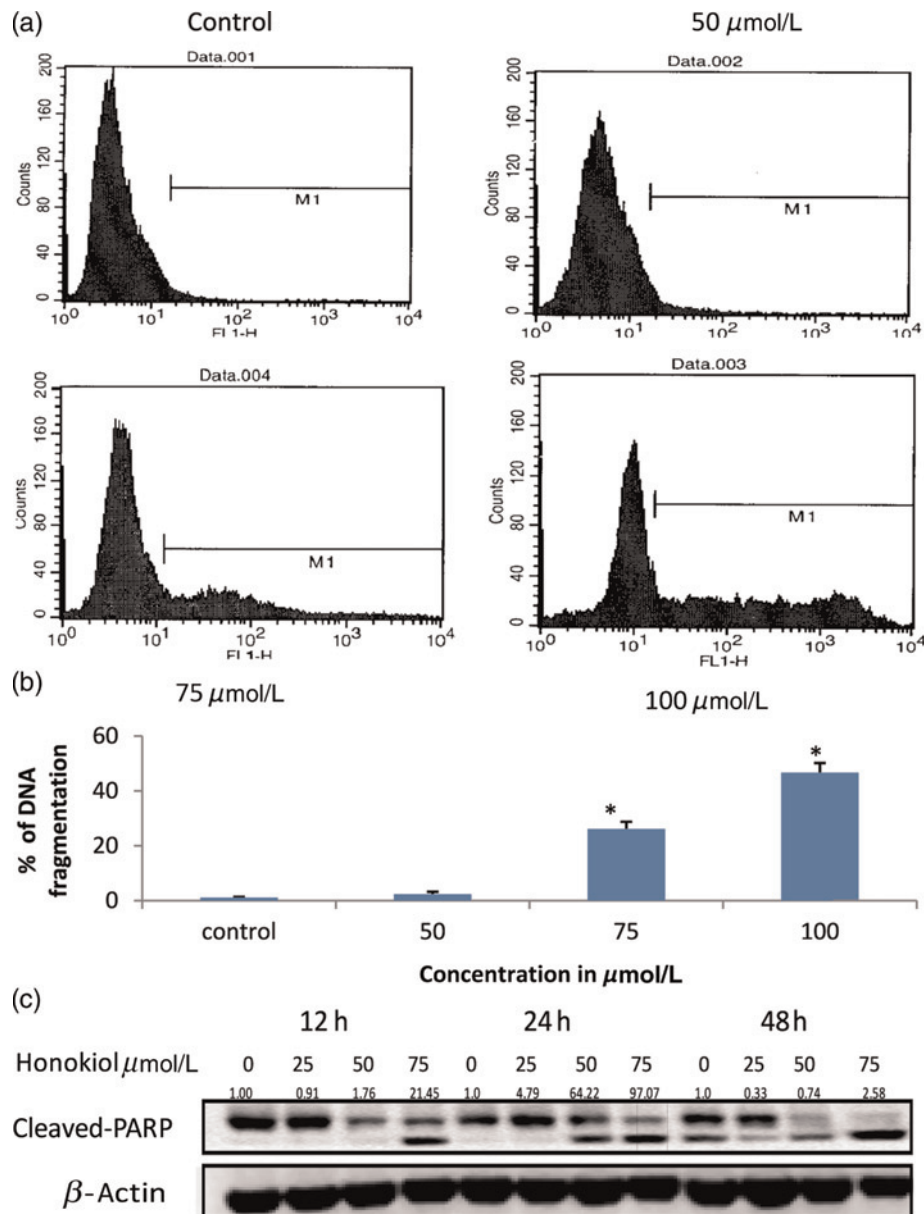


Figure 5 Effects of honokiol on DNA fragmentation in A431 cells by TUNEL assay. Cells were treated with honokiol (0, 50, 75 and 100 $\mu\text{mol/L}$) and harvested after 48 h. (a) The marker M1 in the histograms represents apoptotic cells with fragmented DNA. (b) Percent of DNA fragmented cells by flow cytometry in control and honokiol-treated cells. Each datum represents mean $\pm$ SE. *Significantly different $P < 0.05$. (c) Effects of honokiol on apoptotic protein in A431 cells. The number above the bands indicates the ratio to the control. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (A color version of this figure is available in the online journal)

a protein involved in DNA fragmentation, and thereby induced apoptosis (Figure 5c).

Discussion

In our previous studies, honokiol, a naturally occurring plant lignan, has shown to have chemopreventive effects against UVB-induced skin tumor development in SKH-1 mice.¹⁰ However, its molecular mechanism involved in chemoprevention has not yet been studied. The present study was designed to understand the mechanism of honokiol involved in its anticarcinogenic effects. Inhibition of cell proliferation and induction of apoptosis are two key events that modulate chemopreventive effects of any agent.^{15,16} In the present study, we investigated the effects of honokiol on inhibiting cell growth and inducing apoptosis. For this purpose, we used human epidermoid squamous A431 cells as an *in vitro* model. Our data showed that honokiol significantly inhibits cell viability and proliferation of A431 cells in a concentration- and time-dependent manner, which suggests that honokiol can have chemotherapeutic effects against non-melanoma squamous cell skin cancer.

To halt the growth of cancer, control of cell cycle progression in cancer cells is one of the effective strategies as in most cases cell cycle regulators are deregulated.^{17,18} Cyclins and cyclin-dependent kinases (Cdks) complexes are regulators of cell cycle progression where cyclins are regulatory units and Cdks are catalytic units.^{19,20} For sequential progression in cell cycle from G1/S transition, cyclin D1-Cdk4 and cyclin E-Cdk2 complexes act predominately.²¹ Similarly, cyclin B1/Cdc2 (Cdk1) is a key regulator during G2/M transition and is regulated by phosphorylation. Activation of cyclin B1/Cdc2 is mediated through dephosphorylation of inhibitory sites of Cdc2 by the phosphatase, CDc25C.²² CDK inhibitory proteins, p21WAF1 (p21) and p27KIP (p27), negatively regulate kinase activity of the cyclins/Cdks complexes which are up-regulated in cancer cells in response to antiproliferative signals.²³

Our *in vitro* data demonstrate that honokiol treatment to A431 cells induces G1 phase cell cycle arrest. In addition to this, we found that honokiol treatment decreased expression of cyclin D1, D2, E and Cdk2, Cdk4, Cdk6 at 24 and 48 h in a concentration-dependent manner, which are promoters in cell cycle progression in G1 phase, suggesting the disruption of uncontrolled cell cycle progression in A431 cells. Further, we observed that honokiol treatment significantly increased the expression of cyclin-dependent kinase inhibitors (Cdkis) p21 and p27 at 12, 24 and 48 h. We observed that honokiol decreased cell proliferation by up-regulating the expression of (Cdkis) together with a decrease in the expression of cyclins and Cdks and could be an effective chemotherapeutic agent for skin cancer. These effects of honokiol are similar to those observed by Hahm *et al.*⁷ in a human prostate cancer cell line and by our group in melanoma cell lines (UACC-62, SKMel-2, unpublished data). Ahn *et al.*²⁴ has reported the antiproliferative, proapoptotic, anti-invasive, antiosteoclastogenic, antiangiogenic and antimetastatic effects of honokiol mediated through the suppression of the NF- κ B-regulated

gene pathway. Previous studies²⁵ have demonstrated the inhibition of epidermal growth factor receptor signaling, including downstream inhibition of mitogen-activated protein kinase, Akt, signal transducer and activator of transcription 3 (STAT 3) and expression of STAT3 target genes BCL-X(L) and cyclin D1 in head and neck squamous cell carcinoma by honokiol treatment. Thus, the observed effects of honokiol are not specific for only skin cancer cell lines. Honokiol may be effective in preventing and treating a variety of cancers by a similar mechanism.

The cell cycle arrest in cancer cells in G1 phase allows cells either to undergo repair mechanisms or apoptosis. In most cases of malignancies, cancer cells do not respond to cytotoxic effects of chemotherapeutic agents by becoming resistant to cancer. As a result we examined the effects of honokiol on apoptosis.^{26–29} Apoptosis plays an important role in homeostasis. A fundamental event in apoptosis is the activation of caspases. Activated caspases can break PARP as one of the apoptotic markers. Death receptor pathway (extrinsic pathway) and mitochondrial pathway (intrinsic pathway) are two major pathways of apoptosis. In the extrinsic pathway, when death ligand binds to death receptor, this activates caspase-8 which can active caspase-3. In the intrinsic pathway, disruption of mitochondrial potential leads to the release of cytochrome C in the cytosol which interacts with Apaf-1 and forms apoptosomes. This can now recruit procaspase-9 and activate it to caspase-9. Activated caspase-9 can activate caspase-3 leading to the activation of PARP, resulting in apoptosis.^{30,31}

Our previous studies have indicated the induction of caspases-8, 9, 3 and PARP by honokiol leading to apoptosis.¹⁰ Our data showed that honokiol treatment for 48 h (50–100 μ mol/L) induced apoptotic cell death (Figure 4). By employing different approaches such as Annexin V/PI staining and TUNEL (DNA fragmentation) assay, we demonstrated that honokiol induced apoptosis in A431 cells in a concentration-dependent manner. Furthermore, this apoptotic effect was confirmed by the induction of PARP in a concentration-dependent manner (Figure 5).

Overall, the present study demonstrates that honokiol treatment inhibited cell growth of human epidermoid A431 cells by causing G0/G1 cell cycle arrest by down-regulation of cyclin-Cdk complex and up-regulation of Cdkis, and leads to induction of apoptosis by intrinsic and extrinsic pathways. This study reports for the first time the effects of honokiol on cell cycle progression and apoptosis in human epidermoid A431 cells. Further studies on the effects of honokiol on other signaling pathways are required to investigate the detailed mechanism(s) of action of honokiol.

Author contributions: CC performed the MTT assay, apoptosis assay, TUNEL assay and wrote the manuscript; RG performed the BrdU assay and cell cycle assays including Western blots for various proteins; RSK and AY helped with flow cytometric analysis; GC assisted in analyzing the cell cycle data; HF identified the honokiol for the studies; and CD conceived and executed the idea overall and wrote the final draft of the manuscript. CC and RG contributed equally to this work.

ACKNOWLEDGEMENTS

This study was supported by the Department of Pharmaceutical Sciences Graduate Program and Translational Cancer Research Center funded by the State of South Dakota.

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(Received January 28, 2011, Accepted June 11, 2011)