Honokiol causes G_0-G_1 phase cell cycle arrest in human prostate cancer cells in association with suppression of retinoblastoma protein level/phosphorylation and inhibition of E2F1 transcriptional activity

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Abstract

The present study was undertaken to gain insights into the mechanism of cell cycle arrest caused by honokiol, a constituent of oriental herb *Magnolia officinalis*. The honokiol treatment decreased the viability of PC-3 and LNCaP human prostate cancer cells in a concentration- and time-dependent manner, which correlated with G_0-G_1 phase cell cycle arrest. The honokiol-mediated cell cycle arrest was associated with a decrease in protein levels of cyclin D1, cyclin-dependent kinase 4 (Cdk4), Cdk6, and/or cyclin E and suppression of complex formation between cyclin D1 and Cdk4 as revealed by immunoprecipitation using anti–cyclin D1 antibody followed by immunoblotting for Cdk4 protein. The honokiol-treated PC-3 and LNCaP cells exhibited a marked decrease in the levels of total and phosphorylated retinoblastoma protein (Rb), which correlated with the suppression of transcriptional activity of E2F1. Exposure of PC-3 and LNCaP cells to honokiol resulted in the induction of p21 (PC-3 and LNCaP) and p53 protein expression (LNCaP). However, small interfering RNA (siRNA)–mediated knockdown of either p21 (PC-3 and LNCaP) or p53 (LNCaP) protein failed to confer any protection against honokiol-induced cell cycle arrest. The honokiol treatment caused the generation of reactive oxygen species (ROS), and the cell cycle arrest caused by honokiol was partially but significantly attenuated in the presence of antioxidant *N*-acetylcysteine. In conclusion, the present study reveals that the honokiol-mediated G_0-G_1 phase cell cycle arrest in human prostate cancer cells is associated with the suppression of protein level/phosphorylation of Rb leading to inhibition of transcriptional activity of E2F1. [Mol Cancer Ther 2007; 6(10):2686–95]

Introduction

The oriental medicinal herb *Magnolia officinalis/grandiflora* and its bioactive constituent honokiol exhibit a variety of biological effects, including antimicrobial, antithrombotic, and anxiolytic effects (1–3). The root and stem bark of this plant has been used in Chinese and Japanese medicine for the treatment of various ailments, including thrombotic stroke, gastrointestinal problems, and anxiety (4). Recent studies have provided experimental evidence to document anticancer effects of honokiol (5–14). For example, the ethanol extract of *Magnolia obovata* and honokiol inhibited the migration of HT-1080 human fibrosarcoma cells (6). Honokiol treatment caused apoptotic cell death in CH27 human squamous lung cancer cell line in association with the up-regulation of Bad and down-regulation of Bcl-xL protein expression, release of cytochrome *c* from the mitochondria to the cytosol, and activation of caspases (7). In addition, honokiol exhibited potent antiproliferative and antiangiogenic activity against transformed angiosarcoma cell line SVR (8). The honokiol treatment inhibited transplanted SVR angiosarcoma growth in nude mice (8). Mobilization of free calcium to the cytosol through a phospholipase C–mediated pathway in rat cortical neurons as well as neuroblastoma SH-SY5Y cells upon treatment with honokiol has also been described (9). The honokiol displayed anticancer activity against human colorectal carcinoma cell line RKO *in vitro* and *in vivo* and prolonged life span of tumor-bearing nude mice (10). The honokiol-mediated reversal of resistance to conventional anticancer drugs through the induction of caspase-dependent and caspase-independent apoptosis has been reported in human multiple myeloma cells (12). The honokiol treatment was found to increase 1,25-dihydroxyvitamin D_3– and retinoic acid–induced differentiation in leukemia cells (14).

More recently, honokiol was shown to inhibit tumor necrosis factor-α–stimulated nuclear factor-κB (NF-κB) activation in cancer cells (15). In a separate study, honokiol treatment potentiated apoptosis, suppressed osteoclastogenesis, and inhibited invasion through the modulation of NF-κB (16). The NF-κB is a transcription factor involved in the regulation of various genes, including inflammatory cytokines, chemokines, cell adhesion molecules, growth factors, and IFNs (17). The NF-κB regulates the gene expression of a number of antiapoptotic proteins, including

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cIAP1, cIAP2, Bfl-1/A1, and survivin (18–21). Interestingly, NF-κB is constitutively activated in a variety of hematologic and solid tumor cells, including prostate cancer cells (22–25).

Because honokiol treatment suppresses NF-κB activation (15, 16), we hypothesized that this phytochemical might inhibit growth of human prostate cancer cells. In the present study, we tested this hypothesis using PC-3 (an androgen-independent cell line lacking functional p53) and LNCaP (an androgen-responsive cell line with wild-type p53) human prostate cancer cell lines as a model. Here, we show that honokiol treatment decreases viability of both cell lines in association with G_0-G_1 phase cell cycle arrest. The honokiol-induced cell cycle arrest correlates with the suppression of retinoblastoma protein (Rb) level and its phosphorylation leading to the inhibition of transcriptional activity of E2F1. We show further that the honokiol-induced cell cycle arrest in human prostate cancer cells is accompanied by the generation of reactive oxygen species (ROS), and the cell cycle arrest caused by honokiol is partially but significantly attenuated in the presence of antioxidant *N*-acetylcysteine (NAC).

Materials and Methods

Reagents

Honokiol was supplied by LKT Laboratories as a white powder of ~99.6% purity. Stock solution of honokiol (final concentration, 50 mmol/L) was prepared in DMSO, stored at −20°C, and diluted with fresh complete medium immediately before use. An equal volume of DMSO (final concentration, <0.1%) was added to the controls. RPMI 1640 was from Mediatech, Inc.; F-12 K medium, trypsin-EDTA solution, antibiotic mixture, sodium pyruvate, HEPES buffer, and fetal bovine serum were from Life Technologies; OligofectAMINE was from Invitrogen; FuGENE 6 was from Roche; 5 (and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate succinimidyl ester (DCFDA) was from Molecular Probes; RNase A was from Promega; protease inhibitor cocktail was from BD Biosciences PharMingen; and phosphatase inhibitors, NAC and propidium iodide, were from Sigma. The antibodies against cyclin D1, cyclin E, E2F1, and cyclin-dependent kinase 2 (Cdk2) were from Santa Cruz Biotechnology. The antibodies against Cdk4 and p21 were from BD Biosciences PharMingen. The antibodies against Cdk6, phospho-(Ser^{15})-p53, total Rb, phospho-(Ser$^{807/811}$)-Rb were from Cell Signaling Technology. Total anti-p53 antibody and MG132 were from Calbiochem. Protein A/G plus-Agarose immunoprecipitation reagent was from Santa Cruz Biotechnology. A control nonspecific siRNA (UUCUCCGAACGUGUCACGUdTdT) was from Qiagen. The p21- and p53-targeted siRNA previously validated in our laboratory (26, 27) were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. The sequences of the p21- and p53-targeted siRNA are not revealed by the manufacturer. The dual-Luciferase Reporter Assay kit and pRL-CMV were from Promega.

Cell Culture and Cell Viability Assay

Monolayer cultures of PC-3 and LNCaP cells were maintained as described previously (26–28). Each cell line was maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. The effect of honokiol on cell viability was determined by trypan blue dye exclusion assay essentially as described previously (28).

Analysis of Cell Cycle Distribution

The effect of honokiol treatment on cell cycle progression was determined by flow cytometry following staining with propidium iodide as described previously (29). In brief, desired cells (1×10^6) were treated with honokiol or DMSO, and both floating and attached cells were collected. The cells were fixed in 70% ethanol overnight at 4°C. The cells were then stained with propidium iodide, and the cell cycle distribution was determined using a Coulter Epics XL flow cytometer.

Immunoblotting

Lysates from control and honokiol-treated cells were prepared as described previously (28, 29). Nuclear extracts from control and honokiol-treated cells for immunoblotting of E2F1 were prepared according to the method of Schreiber et al. (30) with some modifications. Briefly, cells were treated with honokiol, harvested, resuspended in a solution containing 10 mmol/L HEPES (pH, 7.9), 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.07% NP40, protease inhibitor, and phosphatase inhibitors and vortexed briefly. After 15 min incubation on ice, the extract was centrifuged at 12,000 rpm for 5 min. The pellet was subjected to freeze-thaw thrice. The nuclear proteins were extracted with 50 μL of extraction buffer [500 mmol/L Tris-HCl (pH, 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.02% sodium azide, protease inhibitor, and phosphatase inhibitors]. The proteins were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membrane. The immunoreactive bands were visualized using the enhanced chemiluminescence method. Each membrane was stripped and reprobed with anti-actin antibody to correct for differences in protein loading. Changes in protein levels were quantified by densitometric scanning of the immunoreactive bands.

Immunoprecipitation

To determine the effect of honokiol treatment on the interaction between cyclin D1 and Cdk4, desired cells were treated with DMSO or 40 μmol/L honokiol for 8 or 24 h, washed twice with ice-cold PBS, and lysed with lysis buffer containing 50 mmol/L Tris (pH, 8.0), 150 mmol/L NaCl, and 1% NP40. Aliquot containing 250–500 μg of lysate protein was incubated overnight at 4°C with 10 μg of anti–cyclin D1 antibody. Protein A/G plus agarose (50 μL, Santa Cruz Biotechnology) was subsequently added to each sample, and the incubation was continued for an additional 3 h at 4°C with gentle shaking. The immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting using anti-Cdk4 or anti–cyclin D1 antibody.

Luciferase Reporter Assay

The PC-3 and LNCaP cells were transiently co-transfected with 1 μg of pGL2-E2F1-Luc plasmid and 0.1 μg of pRL-CMV

plasmid using FuGENE 6 transfection reagent. The E2F1-luciferase reporter construct containing −728/+77 region of E2F1 gene promoter was a generous gift from Dr. Stephen Safe (Texas A&M University, College Station, TX; ref. 31). Twenty-four hours after transfection, the cells were treated with honokiol for the desired time period, washed with ice-cold PBS, and harvested in reporter lysis buffer. The samples were centrifuged, and a 20-μL supernatant fraction was used for measurement of dual luciferase activity (Promega) using a luminometer. The luciferase activity normalized against protein concentration was expressed as a ratio of firefly luciferase to *Renilla* luciferase units.

RNA Interference

The LNCaP cells were seeded in six-well plates and transfected at 50% confluency with 100 nmol/L p21 siRNA or p53 siRNA using OligofectAMINE according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with DMSO (control) or specified concentration of honokiol for 24 h. The cells were then collected and processed for immunoblotting and analysis of cell cycle distribution.

Measurement of ROS Generation

ROS generation in control and honokiol-treated cells was measured by flow cytometry following staining with DCFDA. Briefly, desired cell line was seeded in six-well plates (1×10^5 cells per well), allowed to attach overnight and exposed to DMSO (control) or desired concentrations of honokiol for specified time periods. The cells were stained with 5 μmol/L DCFDA for 30 min at 37°C, and the fluorescence was measured by flow cytometry as described previously (32). In some experiments, cells were pretreated for 2 h with 4 mmol/L NAC before honokiol exposure and analysis of ROS generation, cell cycle distribution, or trypan blue dye exclusion assay.

Statistical Analysis

Statistical significance of differences in measured variables between control and treated groups were determined by paired *t* test or one-way ANOVA followed by Dunnett's or Bonferroni's multiple comparison tests. Difference was considered significant at $P < 0.05$.

Results

Honokiol Treatment Decreased Viability of Human Prostate Cancer Cells

Initially, we determined the effect of honokiol treatment (Fig. 1A for the structure of honokiol) on cell viability using LNCaP and PC-3 cell lines, which, respectively, are well-characterized representatives of androgen-responsive and androgen-independent human prostate cancers. The effect of honokiol on cell viability was determined by trypan blue dye exclusion assay, and the results are summarized in Fig. 1B and C (LNCaP and PC-3 cells, respectively). The drug concentrations used in the present study were within the range used previously to document cellular effects of honokiol (e.g., growth suppression and apoptosis induction) in other cell lines (5–16). As can be seen in Fig. 1B and C, honokiol treatment decreased viability of both cell lines

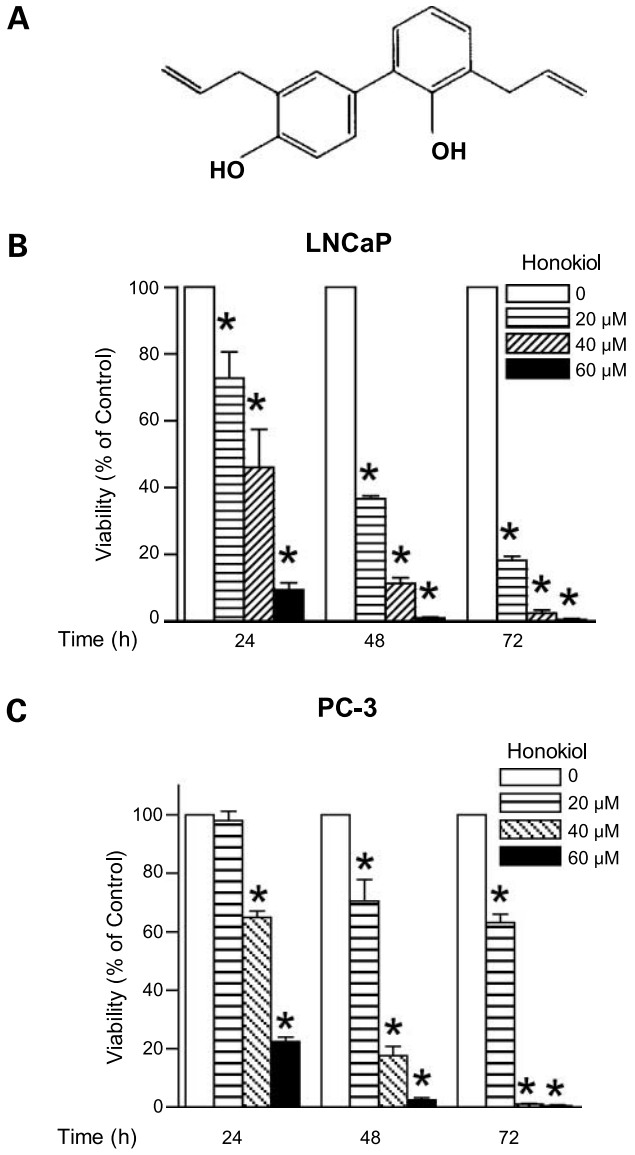


Figure 1. **A,** chemical structure of honokiol. Effect of honokiol treatment on viability of (**B**) LNCaP and (**C**) PC-3 cells as determined by trypan blue dye exclusion assay. The desired cell line was treated with DMSO (control) or honokiol (20, 40, and 60 μmol/L) for the indicated time periods. In **B** and **C**, *columns,* mean ($n = 3$); *bars,* SE. *, $P < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. Similar results were observed in replicate experiments.

in a concentration- and time-dependent manner. The viability of PC-3 or LNCaP cells was not affected at 5–10 μmol/L honokiol concentration (results not shown). These results indicated that honokiol treatment decreased viability of prostate cancer cells irrespective of their androgen responsiveness or p53 status.

Honokiol Treatment Caused G_0-G_1 Phase Cell Cycle Arrest

Suppression of cancer cell growth by many cancer chemopreventive agents including dietary phytochemicals

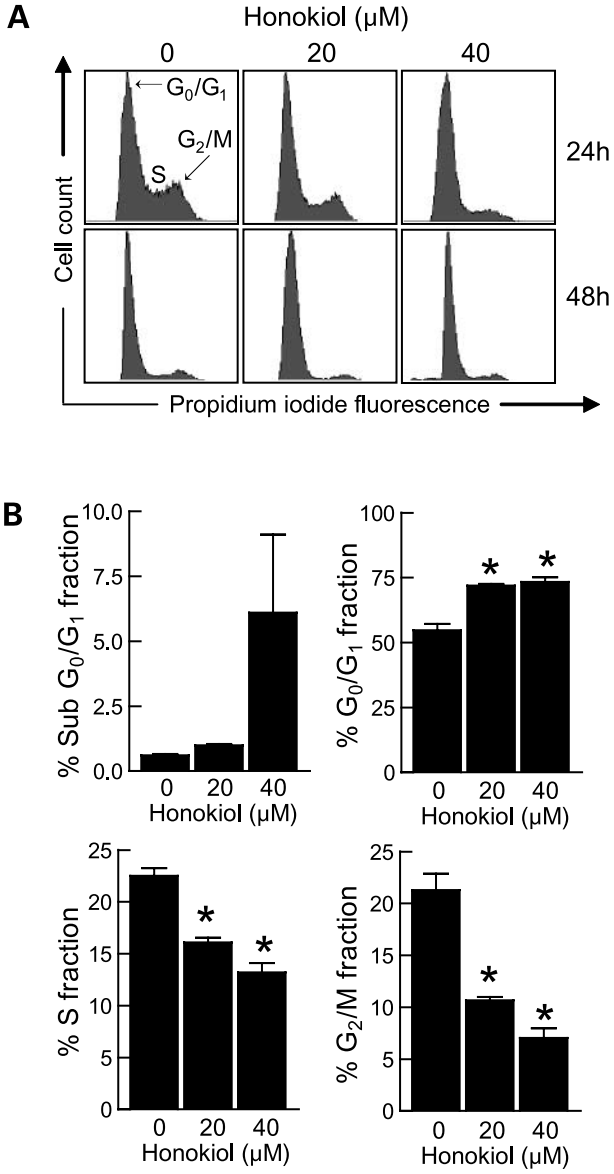
derived from garlic and cruciferous vegetables correlates with perturbations in the cell cycle progression (29, 33–35). To gain insights into the mechanism of cell growth inhibition by honokiol, we determined its effect on cell cycle distribution. Representative flow histograms depicting cell cycle distribution in LNCaP cultures following a 24- or 48-h exposure to DMSO (control) or honokiol (20 or 40 μmol/L) are shown in Fig. 2A. Exposure of LNCaP cells to honokiol resulted in the enrichment of G_0-G_1 fraction with 2N DNA content, which was accompanied by a decrease in both S phase and G_2-M phase cells (Fig. 2B). The honokiol-treated PC-3 cells were also arrested in the G_0-G_1 phase of the cell cycle in a concentration-dependent manner (Fig. 2C). Thus, honokiol-mediated growth inhibition of both LNCaP and PC-3 cells correlated with G_0-G_1 phase cell cycle arrest.

Effect of Honokiol Treatment on Levels of G_1-S Phase–Specific Cyclins and Cdks

Eukaryotic cell cycle progression involves sequential activation of Cdks whose association with corresponding regulatory cyclins is necessary for their activation (36, 37). For instance, the G_1-S transition is regulated by complexes formed by cyclin D and Cdk4 or Cdk6 and cyclin E and Cdk2 (36, 37). We determined the effect of honokiol treatment on protein levels of G_1-S–specific cyclins and Cdks by immunoblotting to gain insights into the mechanism of honokiol-induced cell cycle arrest in our model. As can be seen in Fig. 3A, honokiol treatment caused a rapid and marked decrease in protein level of cyclin D1 in LNCaP cells, which was evident as early as 4 h after treatment. The honokiol-treated LNCaP cells exhibited a decrease in protein levels of Cdk4 and cyclin E, whereas constitutive expression of Cdk6 was very low in this cell line (Fig. 3A). The honokiol-mediated down-regulation of cyclin D1 and Cdk4 protein expression was also observed in PC-3 cells (Fig. 3B). In addition, a marked decrease in protein levels of Cdk6 and Cdk2 was evident in honokiol-treated PC-3 cells especially at the 24- and 48-h time points (Fig. 3B). These results indicated that honokiol-mediated cell cycle arrest in LNCaP and PC-3 cells was associated with a decrease in protein levels of cyclins and Cdks.

Because the effect of honokiol was most pronounced on cyclin D1 and Cdk4 protein expression in both cell lines (Fig. 3A and B), we raised the question of whether honokiol treatment affected interaction between these proteins. We addressed this question by immunoprecipitation of cyclin D1 from equal amounts of lysate proteins from control and honokiol-treated (40 μmol/L for 8 or 24 h) PC-3 and LNCaP cells, followed by immunoblotting using anti-Cdk4 antibody. As can be seen in Fig. 3C, the binding of cyclin D1 with Cdk4 was suppressed by honokiol treatment in both cell lines. However, the honokiol-mediated suppression of cyclin D1 and Cdk4 interaction was relatively more pronounced in the LNCaP cell line than in PC-3 cells (Fig. 3C). These results indicated that the honokiol-mediated cell cycle arrest in prostate cancer cells was associated with the suppression of complex formation between cyclin D1 and Cdk4.

Several possibilities exist to explain honokiol-mediated decline in the levels of cell cycle regulatory proteins,



C

Honokiol (μM)	Percent cells in phase			
	Sub G_0/G_1	*G_0/G_1*	*S*	*G_2/M*
0	1.5 ± 0.1	50 ± 0.4	21 ± 0.2	27 ± 0.4
20	2.7 ± 0.2*	59 ± 0.1*	19 ± 0.2*	19 ± 0.4*
40	1.9 ± 0.1	71 ± 0.7*	13 ± 0.4*	14 ± 0.4*

Figure 2. **A,** representative flow histograms depicting cell cycle distribution in LNCaP cultures treated for 24 or 48 h with DMSO (control) or honokiol (20 or 40 μmol/L). **B,** cell cycle distribution in LNCaP cultures treated for 24 h with DMSO (control) or 20 and 40 μmol/L honokiol. *Columns,* mean (n = 3); *bars,* SE. *, $P < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. **C,** percentage of cells in different phases of the cell cycle in PC-3 cultures treated for 24 h with DMSO (control) or honokiol (20 or 40 μmol/L). Results are mean ± SE (n = 3). *, $P < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test.

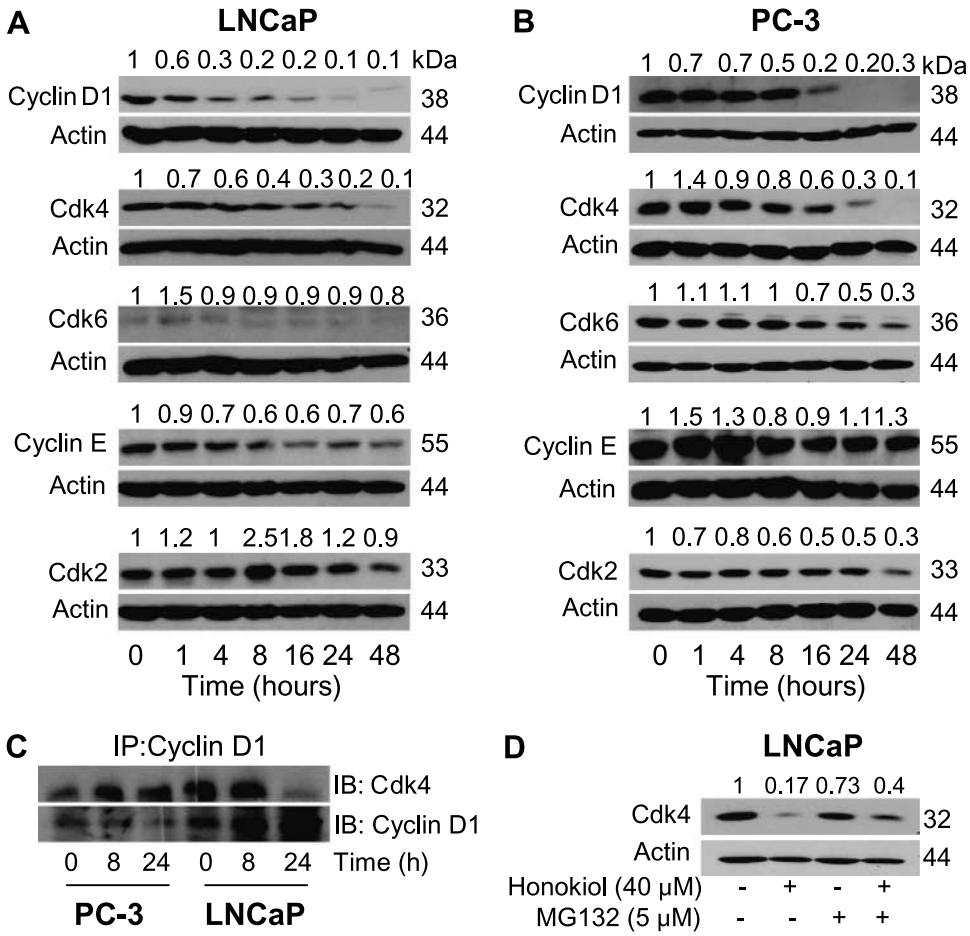


Figure 3. Immunoblotting for cyclin D1, Cdk4, Cdk6, cyclin E, and Cdk2 using lysates from (**A**) LNCaP and (**B**) PC-3 cells treated with 40 μmol/L honokiol for the indicated time periods. Immunoblotting for each protein was done twice or more using independently prepared lysates with similar results. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. *Numbers on top of the bands,* changes in protein levels compared with control as determined by densitometric scanning of the immunoreactive bands and corrected for actin loading control. **C,** immunoblotting for Cdk4 or cyclin D1 using immunoprecipitated complexes with anti–cyclin D1 antibody from equal amounts of lysate proteins from PC-3 and LNCaP cells treated with 40 μmol/L honokiol for the indicated time periods. Similar results were observed in replicate experiments. **D,** immunoblotting for Cdk4 using lysates from LNCaP cells treated for 24 h with 40 μmol/L honokiol in the absence or presence of a noncytotoxic dose (5 μmol/L) of proteasomal inhibitor MG132 (2 h pretreatment).

including general inhibition of protein synthesis, decreased transcription, and/or increased proteasomal degradation. However, the results shown in Fig. 3 argue against general protein synthesis inhibition as a likely mechanism for cell cycle arrest in our model because honokiol treatment did not cause a decrease in the level of every cell cycle regulatory protein examined. For instance, the level of cyclin E was not decreased in honokiol-treated PC-3 cells (Fig. 3B). We therefore tested the possibility whether honokiol-mediated decrease in protein levels of Cdk4 and cyclin D1 was due to their increased degradation. The honokiol-mediated decrease in protein levels of Cdk4 (Fig. 3D) and cyclin D1 (results not shown) was partially attenuated in the presence of a noncytotoxic concentration of a well-known proteasomal inhibitor MG132. These results indicated that the honokiol-mediated decrease in levels of Cdk4 and cyclin D1 was in part caused by increased proteasomal degradation of these proteins.

Honokiol Treatment Caused Induction of p21 Protein Expression and Suppressed Protein Level and Phosphorylation of Rb

The Cdk inhibitor p21 plays an important role in the regulation of G_1-S transition by binding to and inhibiting kinase activity of Cdk/cyclin complexes (36–38). The cyclin D1/Cdk4 and cyclin D1/Cdk6 kinase complexes hyperphosphorylate Rb protein, leading to its dissociation from transcription factor E2F1, which regulates expression of genes necessary for cell cycle progression (36–38). To gain further insights into the mechanism of honokiol-induced G_0-G_1 phase cell cycle arrest, we determined its effect on protein levels and/or phosphorylation of p21 and Rb by immunoblotting. As shown in Fig. 4A (*left*), honokiol treatment resulted in the induction of p21 protein expression in LNCaP cells, which was clearly evident at 16–24-h time points. In addition, honokiol treatment caused a decrease in protein levels of Rb and E2F1 (nuclear extract) and suppression of Rb phosphorylation at $Ser^{807/811}$ in LNCaP cells (Fig. 4A, *left*).

The p53 tumor suppressor is inert in the absence of stress, but can be stabilized by different stimuli and transcriptionally regulates expression of certain genes whose protein products are involved in the regulation of cell cycle progression (e.g., p21; refs. 38, 39). As can be seen in Fig. 4A (*left*), the honokiol-treated LNCaP cells exhibited an increase in protein level and Ser^{15} phosphorylation of p53. Thus, honokiol-mediated G_0-G_1 phase cell cycle arrest in LNCaP cells was associated with the induction of p21

protein level, suppression of protein level and phosphorylation of Rb, and an increase in level and Ser^{15} phosphorylation of p53 (Fig. 4A, *left*). It is interesting to note that the honokiol-mediated increase in protein levels of both p21 and p53, but not Ser^{15} phosphorylation of p53, was markedly suppressed at the 48-h time point. Although the precise mechanism for this effect remains obscure, it is possible that the longer incubation of honokiol-treated cells leads to increased degradation of p21 and p53 proteins. Further studies are needed to verify this possibility.

To address the question of whether honokiol-mediated induction of p21 protein expression was a p53-regulated response, we determined the effect of honokiol treatment on the p21 protein level using PC-3 cell line which lacks functional p53. As can be seen in Fig. 4A (*right*), honokiol-mediated induction of p21 was also observed in PC-3 cells. Similar to LNCaP cells, honokiol treatment resulted in the suppression of protein level and phosphorylation of Rb in PC-3 cells that was clearly evident at 8–48-h time points.

Next, we designed experiments to test whether the honokiol-mediated decrease in Rb protein level was due to an increase in its degradation mediated by the proteasome. The honokiol-mediated decrease in protein level of Rb in LNCaP (Fig. 4B) and PC-3 cells (results not shown) was partially yet markedly attenuated in the presence of proteasomal inhibitor MG132. These results indicated that similar to Cdk4 and cyclin D1, the honokiol-mediated decrease in the level of Rb protein was, at least in part, caused by its increased proteasomal degradation.

Honokiol Treatment Inhibited the Transcriptional Activity of E2F1

Because phosphorylation of Rb affects the transcriptional activity of E2F1 (36, 37) and honokiol treatment resulted in the suppression of Rb phosphorylation (Fig. 4A), we determined its effect on the transcriptional activity of E2F1 by luciferase reporter gene assay. As can be seen in Fig. 4C, honokiol treatment caused a significant decrease in E2F1-associated luciferase activity in LNCaP cells that was evident as early as 4 h after treatment with 40 μmol/L concentration. The honokiol-mediated inhibition of transcriptional activity of E2F1 in PC-3 cells was evident at

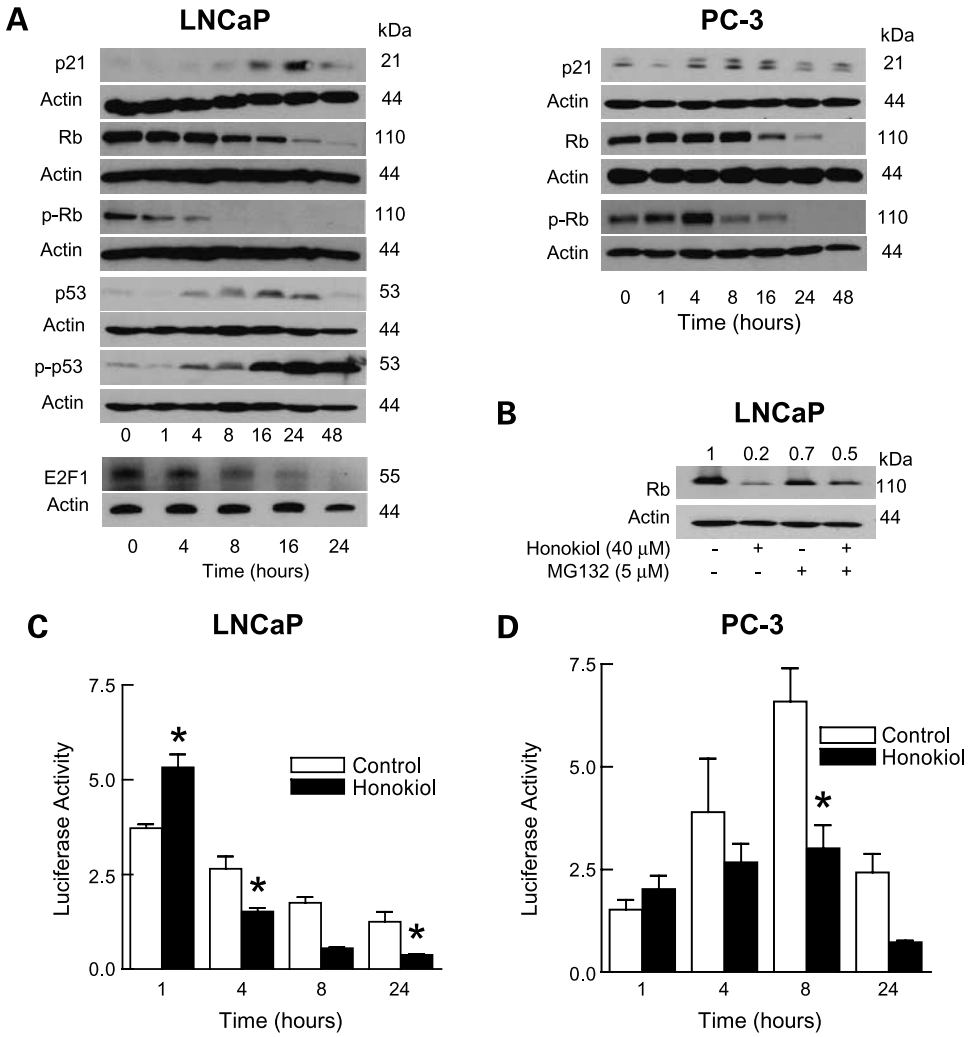


Figure 4. **A,** immunoblotting for p21, total Rb, phospho-Rb, p53, phospho-p53, and/or E2F1 using whole cell lysates or nuclear extracts (E2F1) from LNCaP (*left*) and PC-3 cells (*right*) treated with 40 μmol/L honokiol for the indicated time periods. Immunoblotting for each protein was done twice or more using independently prepared lysates, and the results were similar. The blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading. **B,** immunoblotting for total Rb using lysates from LNCaP cells treated for 24 h with 40 μmol/L honokiol in the absence or presence of 5 μmol/L proteasomal inhibitor MG132 (2 h pretreatment). Transcriptional activity of E2F1 as determined by luciferase reporter gene assay in (**C**) LNCaP and (**D**) PC-3 cells treated with DMSO (*white columns*) or 40 μmol/L honokiol (*black columns*) for the indicated time periods. *Columns,* mean (n = 3); *bars,* SE. *, $P < 0.05$, significantly different compared with DMSO-treated control by paired t test.

8- and 24-h time points (Fig. 4D). Collectively, these results indicated that the honokiol-mediated G_0-G_1 phase cell cycle arrest in LNCaP and PC-3 cells was associated with the inhibition of transcriptional activity of E2F1.

p21 and p53 Proteins Were Dispensable for Honokiol-Induced G_0-G_1 Phase Cell Cycle Arrest

We proceeded to determine the functional significance of p21 and p53 protein induction in honokiol-mediated cell cycle arrest by using siRNA technology. Similar to untransfected LNCaP cells (Fig. 4A, *left*), honokiol treatment caused the induction of p21 and p53 protein expression in control nonspecific siRNA-transfected LNCaP cells (Fig. 5A). Transient transfection of LNCaP cells with p21- and p53-targeted siRNA resulted in the near-complete abrogation of honokiol-mediated induction of p21 and p53 protein expression, respectively (Fig. 5A). Next, we determined the effect of p21 knockdown on honokiol-mediated cell cycle arrest. As can be seen in Fig. 5B, the honokiol-mediated G_0-G_1 phase cell cycle arrest was observed both in control nonspecific siRNA-transfected LNCaP cells and LNCaP cells transfected with a p21-targeted siRNA. Likewise, p53 protein knockdown did not have any appreciable effect on honokiol-induced cell cycle arrest in LNCaP cells (Fig. 5C). Collectively, these results indicated that p21 and p53 proteins were dispensable for honokiol-induced cell cycle arrest.

Honokiol Treatment Caused ROS Generation

Cellular responses to many naturally occurring anticancer agents correlate with ROS generation (32, 33, 40). We raised the question of whether honokiol-mediated cell cycle arrest in prostate cancer cells was linked to ROS generation. To address this question, initially, we determined the effect of honokiol treatment on ROS generation by flow cytometry after staining the cells with DCFDA. The DCFDA is cell permeable, cleaved by nonspecific cellular esterases, and oxidized in the presence of H_2O_2 and other peroxides to yield fluorescent 2′,7′-dichlorofluorescein (DCF). The honokiol-treated LNCaP and PC-3 cells exhibited a statistically significant increase in DCF fluorescence, which was relatively more pronounced in LNCaP cells than in PC-3 (Fig. 6A). The honokiol-mediated increase in DCF fluorescence in both cell lines was significantly attenuated in the presence of antioxidant NAC (Fig. 6B). Moreover, NAC conferred partial yet statistically significant protection against honokiol-induced cell cycle arrest in both LNCaP and PC-3 cells (Fig. 6C).

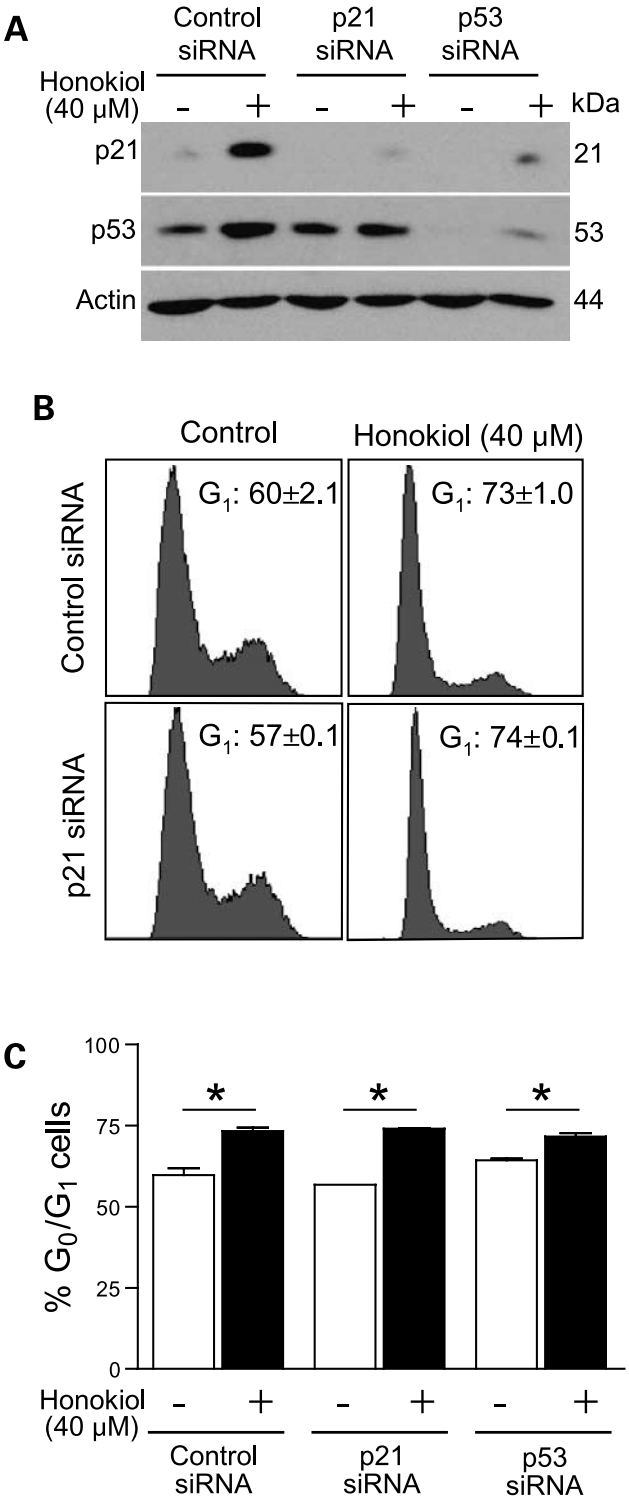


Figure 5. **A,** immunoblotting for p21 and p53 using lysates from control nonspecific siRNA-transfected LNCaP cells and LNCaP cells transfected with p21- and p53-specific siRNA and treated for 24 h with DMSO (control) or 40 μmol/L honokiol. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. **B,** representative flow histograms depicting cell cycle distribution in control nonspecific siRNA-transfected LNCaP cells and p21-specific siRNA-transfected LNCaP cells following a 24-h treatment with DMSO (control) or 40 μmol/L honokiol. **C,** percentage of G_0-G_1 fraction in control nonspecific siRNA-transfected LNCaP cells and p21- and p53-specific siRNA-transfected LNCaP cells following a 24-h treatment with DMSO (control) or 40 μmol/L honokiol. *Columns,* mean (n = 3); *bars,* SE. *, $P < 0.05$, significantly different compared with DMSO-treated control by paired t test. Experiment was repeated with similar results.

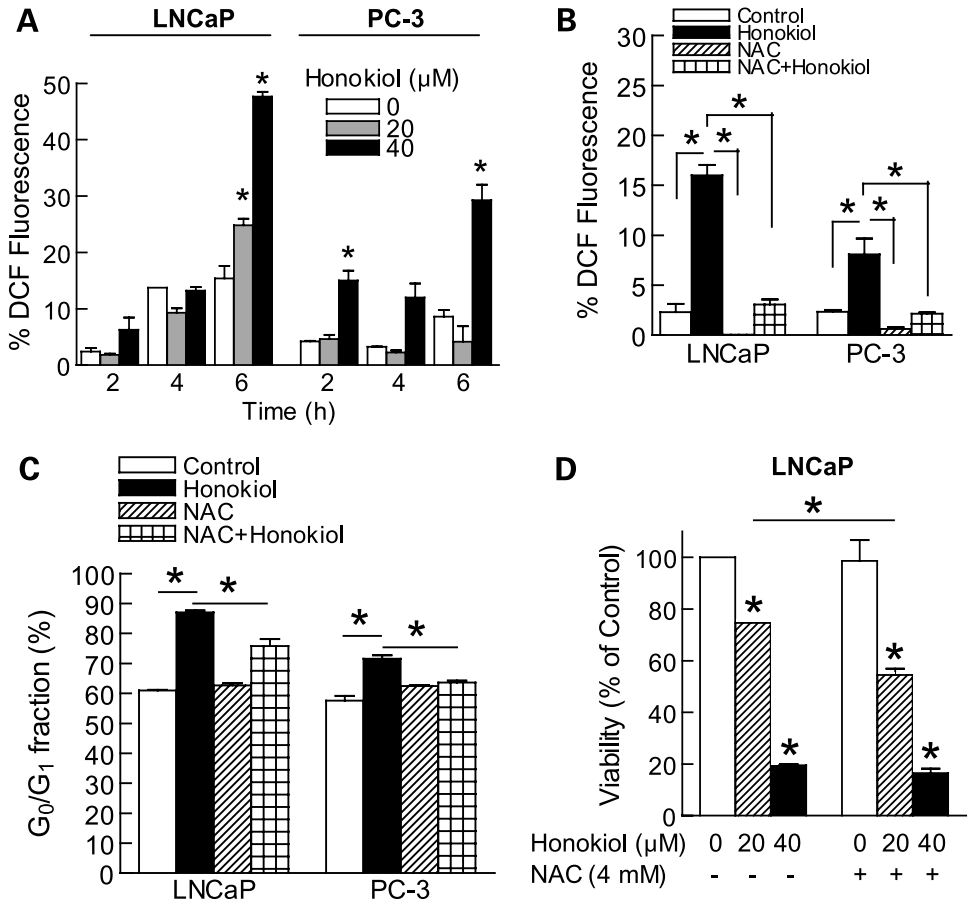


Figure 6. **A,** percentage of DCF-positive cells (an indicator of ROS generation) in LNCaP and PC-3 cultures treated with DMSO (control) or the indicated concentrations of honokiol for different time periods. *Columns,* mean (n = 3); *bars,* SE. *, $P < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. **B,** percentage of DCF-positive cells in LNCaP and PC-3 cultures treated for 6 h with DMSO (control) or 40 μmol/L honokiol in the absence or presence of 4 mmol/L NAC (2 h pretreatment). *Columns,* mean (n = 3); *bars,* SE. **C,** percentage of G_0-G_1 cells in LNCaP and PC-3 cultures treated for 24 h with DMSO (control) or 40 μmol/L honokiol in the absence or presence of 4 mmol/L NAC (2 h pretreatment). *Columns,* mean (n = 3); *bars,* SE. **D,** effects of 24-h treatments with honokiol (20 or 40 μmol/L) and/or 4 mmol/L NAC (2 h pretreatment) on LNCaP cell viability as determined by trypan blue dye exclusion assay. *Columns,* mean (n = 3); *bars,* SE. In **B** to **D**, *, $P < 0.05$, significantly different between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test.

We also determined the effects of honokiol and/or NAC treatments on LNCaP cell viability by trypan blue dye exclusion assay, and the results are shown in Fig. 6D. The growth-inhibitory effect of honokiol at 20 μmol/L concentration was modestly but significantly increased in the presence of NAC, although the difference in cell viability between honokiol and honokiol-plus-NAC groups did not reach statistical significance at the 40-μmol/L concentration. These results suggest that the cell cycle arrest, at least at the 20-μmol/L concentration, may serve to protect or delay the growth-suppressive effect of honokiol. Further studies are needed to systematically explore this possibility.

Discussion

Previous studies have shown that honokiol suppresses growth of cancer cells *in vitro* in association with apoptosis induction and G_0-G_1 phase cell cycle arrest (5–14). An understanding of the mechanisms by which honokiol causes apoptotic cell death and inhibits cell cycle progression may facilitate its further clinical development because this knowledge could lead to the identification of mechanism-based biomarkers potentially useful in future clinical trials. Although considerable progress has been made toward the delineation of the signaling pathways responsible for honokiol-mediated apoptosis, the mechanism by which this agent inhibits cell cycle progression is not fully understood. The present study was undertaken to gain insights into the mechanism of cell cycle arrest by honokiol using LNCaP and PC-3 human prostate cancer cells as a model. We show that honokiol treatment causes G_0-G_1 phase cell cycle arrest in prostate cancer cells irrespective of their androgen responsiveness and p53 status. We also found that the honokiol-mediated accumulation of G_0-G_1 fraction is most likely caused by the inactivation of Cdk4/cyclin D1 kinase complex. This conclusion is supported by the following observations: (*a*) honokiol treatment causes the most dramatic effect on cyclin D1 and Cdk4 protein expression in both cell lines; (*b*) honokiol treatment reduces complex formation between cyclin D1 and Cdk4 in these cells; and (*c*) honokiol treatment suppresses the phosphorylation of Rb, which is a downstream target of Cdk4/cyclin D and Cdk6/cyclin D complexes (36, 37). The honokiol-mediated decrease in protein levels of Cdk4 and cyclin D1 is, at least in part, due to increased proteasome-dependent degradation of these proteins.

The Rb family proteins are critical downstream targets of G_1-specific Cdk/cyclin complexes (41). In hypophosphorylated state, the Rb family proteins associate with and inhibit the activity of E2F family transcription factors, which are involved in the transcription of key cell cycle regulatory

proteins (37, 42, 43). Upon growth stimulus, the G_1-specific Cdks/cyclins phosphorylate Rb proteins on multiple residues, causing the release of E2F family transcription factors (41). We found that the honokiol-mediated cell cycle arrest in both LNCaP and PC-3 cells correlates with not only the hypophosphorylation of Rb but also the inhibition of transcriptional activity of E2F1 as revealed by the luciferase reporter gene assay.

The p21 protein, a transcriptional target of p53 tumor suppressor, regulates G_1-S transition by inhibiting Cdks (38, 39, 44, 45). The present study reveals that honokiol treatment causes the induction of p21 protein level in a p53-independent manner because this effect is observed in both LNCaP and PC-3 cells. However, the induction of p21 protein does not contribute to the cell cycle arrest in our model because the honokiol-mediated accumulation of G_0-G_1 fraction is maintained in cells even after knockdown of p21 protein level. Thus, we conclude that both p21 and p53 are dispensable to the honokiol-mediated cell cycle arrest at least in human prostate cancer cells.

Recent studies have indicated that ROS generation is a critical event in cellular responses to many naturally occurring anticancer agents (32, 33, 40). For instance, the cell cycle arrest caused by a garlic-derived cancer chemopreventive agent (diallyl trisulfide) in human prostate cancer cells correlates with ROS generation (33, 40). The diallyl trisulfide–induced cell cycle arrest is significantly attenuated in the presence of antioxidants such as NAC (33, 40). The present study reveals that honokiol treatment causes ROS generation in LNCaP and PC-3 cells. The honokiol-mediated cell cycle arrest in both cell lines is significantly attenuated in the presence of NAC. These results suggest that ROS are probably important signaling intermediates in honokiol-mediated cell cycle arrest. The growth-inhibitory effect of honokiol is modestly but significantly increased in the presence of NAC at least at lower concentrations of honokiol. It is possible that the cell cycle arrest serves to protect or delay the growth-suppressive effect of honokiol at lower concentrations. Further studies are needed not only to systematically explore this possibility but also to gain insights into the mechanism of honokiol-induced ROS production.

In the present study, the inhibition of LNCaP/PC-3 cell growth and cell cycle progression are observed at 20–60 μmol/L honokiol concentrations. It is hard to predict whether such concentrations of honokiol are achievable *in vivo* in the absence of pharmacokinetic data in humans. Nonetheless, the honokiol concentrations used in the present study are within the range employed in previous studies to document cellular effects of this agent. Honokiol has also been shown to suppress the growth of cancer cells *in vivo*. For instance, the growth of transplanted SVR angiosarcoma cells in male nude mice was inhibited by ~50% by i.p. injection of 3 mg honokiol/day starting after 1 week of tumor cell inoculation (8). No weight loss or other toxicities were reported in honokiol-treated mice (8). Similarly, i.p. injection of 100 mg honokiol/kg body weight sensitized androgen-independent C4-2 human prostate cancer cells inoculated in mouse bilateral tibia to docetaxel without causing any systemic toxicity (46). Unpublished studies from our laboratory also indicate that oral gavage of 2 mg honokiol per mouse (thrice a week) statistically significantly inhibits the growth of PC-3 xenografts in male nude mice without causing weight loss or any other side effects.[1] The honokiol-mediated suppression of PC-3 xenograft growth correlates with increased apoptosis and the suppression of proliferating cell nuclear antigen and Ki-67 staining in the tumor tissue.[1] Collectively, these results suggest that honokiol should be seriously considered for further clinical investigation to determine its possible chemopreventive and/or therapeutic efficacy against prostate cancer in humans.

In summary, the present study indicates that honokiol suppresses the growth of androgen-responsive (LNCaP) as well as androgen-independent (PC-3) human prostate cancer cells in association with G_0-G_1 phase cell cycle arrest. The honokiol-mediated cell cycle arrest is independent of p21, but correlates with the suppression of Rb protein level/phosphorylation and inhibition of transcriptional activity of E2F1.

[1] Hahm E, Arlotti JA, Marynowski SW, Singh SV, Honokiol inhibits growth of PC-3 human prostate cancer xenograft *in vivo* (submitted for publication).

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