Honokiol, a Constituent of Oriental Medicinal Herb *Magnolia officinalis*, Inhibits Growth of PC-3 Xenografts *In vivo* in Association with Apoptosis Induction

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Abstract

Purpose: This study was undertaken to determine the efficacy of honokiol, a constituent of oriental medicinal herb *Magnolia officinalis*, against human prostate cancer cells in culture and *in vivo*.

Experimental Design: Honokiol-mediated apoptosis was assessed by analysis of cytoplasmic histone-associated DNA fragmentation. Knockdown of Bax and Bak proteins was achieved by transient transfection using siRNA. Honokiol was administered by oral gavage to male nude mice s.c. implanted with PC-3 cells. Tumor sections from control and honokiol-treated mice were examined for apoptotic bodies (terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay), proliferation index (proliferating cell nuclear antigen staining), and neovascularization (CD31 staining). Levels of Bcl-2 family proteins in cell lysates and tumor supernatants were determined by immunoblotting.

Results: Exposure of human prostate cancer cells (PC-3, LNCaP, and C4-2) to honokiol resulted in apoptotic DNA fragmentation in a concentration- and time-dependent manner irrespective of their androgen responsiveness or p53 status. Honokiol-induced apoptosis correlated with induction of Bax, Bak, and Bad and a decrease in Bcl-xL and Mcl-1 protein levels. Transient transfection of PC-3 cells with Bak- and Bax-targeted siRNAs and Bcl-xL plasmid conferred partial yet significant protection against honokiol-induced apoptosis. Oral gavage of 2 mg honokiol/mouse (thrice a week) significantly retarded growth of PC-3 xenografts without causing weight loss. Tumors from honokiol-treated mice exhibited markedly higher count of apoptotic bodies and reduced proliferation index and neovascularization compared with control tumors.

Conclusion: Our data suggest that honokiol, which is used in traditional oriental medicine for the treatment of various ailments, may be an attractive agent for treatment and/or prevention of human prostate cancers.

The root and stem bark of the oriental herb *Magnolia officinalis* (also known as Houpo) have been used in traditional Chinese and Japanese medicine for the treatment of various ailments due to its muscle relaxant, anti–gastric ulcer, antiallergic, antibacterial, and antithrombotic properties (1). Honokiol, one of the bioactive constituents of *M. officinalis*, has attracted a great deal of research interest due to its diverse biological effects (2–10). The known pharmacologic effects of honokiol include inhibition of platelet aggregation, protection of the myocardium against ischemic damage, inhibition of ventricular arrhythmia, and antibacterial, anti-inflammatory, antifungal, antioxidative, and antiallergic effects (2–10). For example, honokiol has been shown to offer protection against lipid peroxidation in rat heart mitochondria (11). Honokiol also protects the rat brain from focal cerebral ischemia-reperfusion injury by suppressing neutrophil infiltration and production of reactive oxygen species (12). Honokiol administration at 2.5 mg/kg dose has been shown to alleviate experimental mesangial proliferative glomerulonephritis in rats (13).

Recent studies, including those from our laboratory, have revealed that honokiol can suppress proliferation of cancer cells in culture (14–25). For example, honokiol inhibited migration of HT-1080 human fibrosarcoma cells (14). Honokiol treatment caused apoptotic cell death in the CH27 human squamous lung cancer cell line in association with down-regulation of Bcl-xL protein expression, release of cytochrome *c* from mitochondria to the cytosol, and activation of caspases (15). Honokiol exhibited potent antiproliferative and anti-angiogenic activities against transformed angiosarcoma cell line SVR (16). Mobilization of free calcium to the cytosol through a phospholipase C–mediated pathway in rat cortical neurons and human neuroblastoma SH-SY5Y cells on treatment with honokiol has also been described (17). Honokiol displayed

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anticancer activity against the human colorectal carcinoma cell line RKO *in vitro* and *in vivo* and prolonged life span of tumor-bearing nude mice (18). Honokiol overcame resistance to conventional anticancer drugs through induction of caspase-dependent and caspase-independent apoptosis in human multiple myeloma cells (20). Honokiol treatment was found to increase 1,25-dihydroxyvitamin D3– and retinoic acid–induced differentiation in leukemia cells (22).

More recently, honokiol was shown to inhibit tumor necrosis factor-α–stimulated nuclear factor-κB (NF-κB) activation in cancer cells (23). Honokiol treatment potentiated apoptosis, suppressed osteoclastogenesis, and inhibited invasion through inhibition of NF-κB activation (24). NF-κB is a transcription factor involved in the regulation of various genes including inflammatory cytokines, chemokines, cell adhesion molecules, growth factors, and IFNs (26). NF-κB regulates gene expression of a number of antiapoptotic proteins including cIAP1, cIAP2, Bfl-1/A1, Bcl-2, Bcl-xL, etc. (27–29). Interestingly, NF-κB is constitutively activated in a variety of hematologic and solid tumor cells, including prostate cancer cells (30–32).

Because honokiol suppresses NF-κB activation (23, 24), we hypothesized that this phytochemical might promote apoptotic cell death in human prostate cancer cells. In the present study, we tested this hypothesis using PC-3 (an androgen-independent cell line lacking functional p53), LNCaP (an androgen-responsive cell line with wild-type p53), and C4-2 (an androgen-independent variant of the LNCaP cell line) human prostate cancer cell lines as a model. We show that honokiol causes apoptosis in human prostate cancer cells irrespective of their androgen responsiveness or p53 status. Honokiol-induced apoptosis correlates with induction of Bax, Bak, and Bad protein expression and a decrease in Bcl-xL and Mcl-1 protein levels. Knockdown of Bak and Bax using siRNA or ectopic expression of Bcl-xL confers partial yet significant protection against cell death caused by honokiol. Moreover, oral gavage of honokiol significantly retards growth of PC-3 xenografts, which correlates with increased apoptosis and decreased cell proliferation in the tumor tissue.

Materials and Methods

Reagents. Honokiol was supplied by LKT Laboratories as a white powder of ~99.6% purity as indicated in the supplier's certificate of analysis and verified in our laboratory by reverse-phase HPLC analysis. 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; F-12 K media, trypsin-EDTA solution, antibiotic mixture, sodium pyruvate, HEPES buffer, and fetal bovine serum were from Life Technologies, Inc.; OligoFECTAMINE was from Invitrogen; FuGENE6 and Cell Death Detection ELISA^{PLUS} kit were from Roche; protease inhibitor cocktail and anti–E-cadherin antibody were from BD Biosciences PharMingen; and phosphatase inhibitor cocktail was from Sigma. The antibodies against Bad, Bak, Bax, Bcl-xL, CD31, and Mcl-1 were from Santa Cruz Biotechnology. Antibodies against Bcl-2 and proliferating cell nuclear antigen (PCNA) were from DakoCytomation. Bak- and Bax-targeted siRNAs were from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. The sequences of the Bak- and Bax-targeted siRNA are not revealed by the manufacturer. However, these siRNAs have been validated previously in our laboratory (33). A control nonspecific siRNA (UUCUCCGAACGUGUCACGUdTdT) was from Qiagen. H&E stain was from Anatech. ApopTag Plus Peroxidase *In situ* Apoptosis kit was from Chemicon.

Cell culture. The PC-3 and LNCaP cell lines were purchased from the American Type Culture Collection. The C4-2 cell line, an androgen-independent variant of LNCaP cells, was obtained from UroCor. The C4-2 cell line, which was generated through coculture of parental LNCaP cells with human bone fibroblasts *in vivo* in castrated male athymic mice, displays elevated prostate-specific antigen (PSA) expression and increased anchorage-independent growth in soft agar (34, 35). Monolayer cultures of PC-3, LNCaP, and C4-2 cells were maintained as previously described by us (33, 36). Each cell line was maintained at 37°C in an atmosphere of 5% CO_2 and 95% air.

Apoptotic DNA fragmentation assay. Honokiol-mediated apoptotic cell death, characterized by cytoplasmic histone-associated DNA fragmentation, in PC-3, LNCaP, and C4-2 cells was determined with a Cell Death Detection ELISA^{PLUS} kit according to the manufacturer's instructions. Briefly, desired cell line was plated in six-well plates and allowed to attach by overnight incubation. The cells were then exposed to DMSO (control) or desired concentrations of honokiol for 24, 48, or 72 h. Both floating and attached cells were collected and processed for analysis of cytoplasmic histone-associated DNA fragmentation as described by the manufacturer. Results are expressed as enrichment of cytoplasmic histone-associated DNA fragments relative to DMSO-treated control.

Immunoblotting. Cells were treated with DMSO or honokiol and lysed using a solution containing 50 mmol/L Tris (pH 8.0), 1% Triton X-100, 0.1% SDS, and 150 mmol/L NaCl as previously described by us (33). The cell lysates were cleared by centrifugation at 14,000 rpm for 30 min. The tumor tissues harvested from the control and the honokiol-treated mice were suspended in PBS containing protease and phosphatase inhibitors, minced into small pieces, and homogenized using a polytron. The homogenates were centrifuged at 14,000 rpm for 40 min and the resultant supernatant fractions were used for immunoblotting. The proteins were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membrane. Immunoblotting for different proteins was done essentially as previously described (33, 36). Immunoreactive bands were visualized by enhanced chemiluminescence. Each membrane was stripped and reprobed with anti-actin antibody to ensure equivalent protein loading. Immunoblotting for each protein was done at least twice using independently prepared lysates. Changes in protein levels were quantified by densitometric scanning of the immunoreactive band and corrected for actin loading control.

RNA interference of Bax and Bak. PC-3 cells were seeded in six-well plates at a density of 1.5×10^5 per well and transfected at 50% confluency with 200 nmol/L control nonspecific siRNA or Bax- or Bak-specific siRNA using OligoFECTAMINE according to the manufacturer's instructions. Twenty-four hours after transfection, cells were treated with DMSO (control) or 40 μmol/L honokiol for 24 h. The cells were then collected and processed for immunoblotting or analysis of cytoplasmic histone-associated DNA fragmentation.

Ectopic expression of Bcl-xL. PC-3 cells were plated in 12-well plates at a density of 1.25×10^5 per well and transiently transfected at 50% to 60% confluency with 0.5 μg pSFFV-Bcl-xL or pSFFV-neo plasmids generously provided by the late Dr. Stanley J. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA) using FuGENE6 transfection reagent. Twenty-four hours after transfection, the cells were treated with DMSO or honokiol for 24 h, collected, and processed for immunoblotting to confirm overexpression of Bcl-xL and analysis of cytoplasmic histone-associated DNA fragmentation.

Xenograft assay. Male nude mice (6-8 weeks old) were purchased from Harlan Sprague-Dawley and housed in accordance with the Institutional Animal Care and Use Committee guidelines. The use of mice for studies described herein was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The mice were divided into groups of six mice. The mice were gavaged orally with 1, 2, or 3 mg honokiol (in 0.1 mL PBS) thrice per week (Monday,

Wednesday, and Friday) for 2 weeks before the tumor cell implantation. The control mice were gavaged orally with 0.1-mL vehicle with a similar dosing schedule. After 2 weeks of pretreatment, exponentially growing PC-3 cells were mixed in a 1:1 ratio with Matrigel (Becton Dickinson) and a 0.1-mL suspension containing 2×10^6 cells was injected s.c. on both left and right flanks above the hind limb of each mouse. Treatment continued for the duration of the experiment. Tumor size was measured thrice a week using a caliper and tumor volume was calculated as previously described by us (37, 38). Body weights of the control and honokiol-treated mice were recorded throughout the experiment. Mice of each group were also monitored for other symptoms of side effects including food and water withdrawal and impaired posture or movement. At the termination of the experiment, the tumor tissues were harvested and used for immunohistochemistry or immunoblotting.

Histologic analysis of apoptotic bodies. Tumor tissues from control and honokiol-treated mice were fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, and sectioned at 4- to 5-μm thickness. Apoptosis in tumor sections of control and honokiol-treated mice was visualized by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay using ApopTag Plus Peroxidase *In situ* Apoptosis kit according to the manufacturer's recommendations.

Immunohistochemical analysis for PCNA, CD31, and E-cadherin. Representative tumor sections from control and honokiol groups were fixed in acetone for 10 min at 4°C. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 15 min, the sections were treated with normal rabbit serum for 20 min. The sections were then incubated with the primary anti-CD31 (1:750 dilution), anti–E-cadherin (1:2,000 dilution), or anti-PCNA antibody (1:800 dilution) for 1 h at room temperature and washed with TBS. Biotinylated antigoat IgG was applied for 30 min at room temperature. Color was developed by 10-min incubation with 3,3′-diaminobenzidine (DakoCytomation). The sections were counterstained with H&E. The slides were examined under a Leica microscope at ×40 magnification.

Results

Honokiol treatment caused apoptotic DNA fragmentation in prostate cancer cells. We have previously shown that honokiol treatment decreases viability of LNCaP (androgen receptor positive and wild-type p53) and PC-3 (androgen receptor null and p53 null) human prostate cancer cells at 20 to 60 μmol/L concentrations as judged by trypan blue dye exclusion assay (25). In the present study, we used the same cell lines to test whether honokiol promotes apoptosis. In addition, we used an androgen-independent variant of LNCaP cells (C4-2 cell line) to test whether honokiol-induced apoptosis in human prostate cancer cells was influenced by androgen responsiveness. We validated these cells for their androgen responsiveness and other known genetic differences (e.g., p53 and androgen receptor expression). As expected, proliferation of androgen-responsive LNCaP cell line was suppressed when cultured in charcoal-stripped fetal bovine serum (results not shown). On the other hand, the culture of androgen-independent variant C4-2 in charcoal-stripped fetal bovine serum did not have any appreciable effect on its growth (results not shown). Finally, as expected, expression of androgen receptor and p53 was observed in the LNCaP cell line but not in PC-3 as judged by immunoblotting (results not shown). Honokiol-mediated apoptosis was examined by analysis of cytoplasmic histone-associated DNA fragmentation, a well-accepted technique for detection of apoptotic cell death. Honokiol treatment resulted in a concentration- and/or time-dependent increase in cytoplasmic histone-associated DNA fragmentation in both LNCaP (Fig. 1A) and PC-3 (Fig. 1B) cells. These results suggested that the honokiol-induced apoptosis was most likely not influenced by androgen responsiveness, which we confirmed by comparing sensitivities of LNCaP cells and its androgen-independent variant C4-2 toward honokiol-induced apoptotic DNA fragmentation. As can be seen in Fig. 1C, the apoptotic DNA fragmentation caused by honokiol was more or less comparable in LNCaP and C4-2 cells. Consistent with the results of the DNA fragmentation assay, exposure of LNCaP and PC-3 cells to honokiol (40 μmol/L) resulted in cleavage of poly-(ADP-ribose)-polymerase (results not shown), a hallmark of apoptotic cell death. These results indicated that honokiol caused apoptotic DNA fragmentation in prostate cancer cells irrespective of their androgen responsiveness or p53 status.

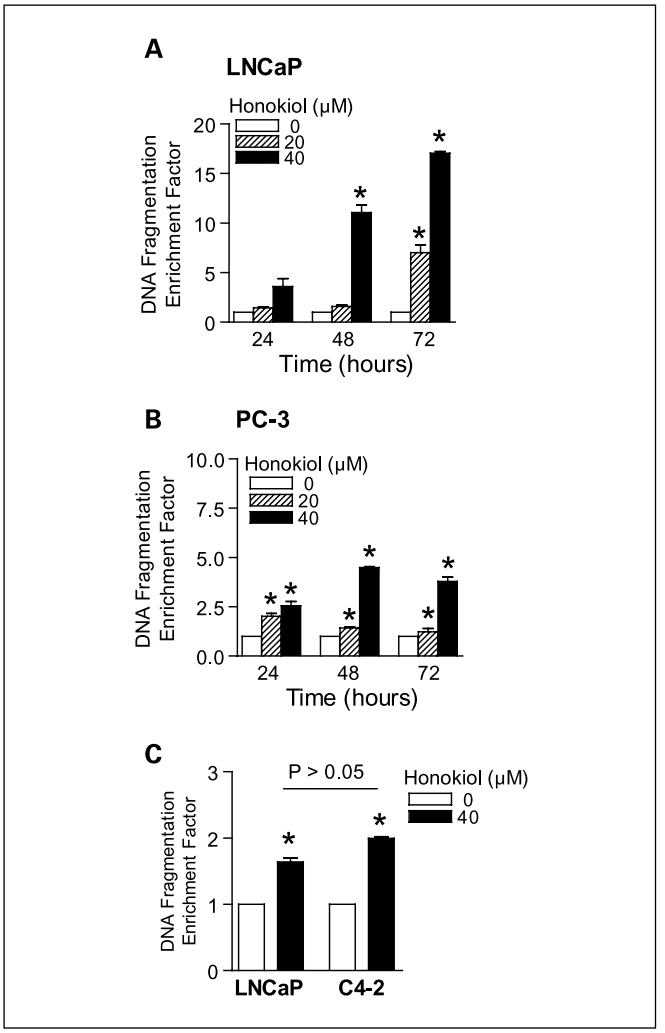

Fig. 1. Honokiol treatment caused apoptotic DNA fragmentation in human prostate cancer cells irrespective of their androgen responsiveness or p53 status. Cytoplasmic histone-associated DNA fragmentation in LNCaP (androgen receptor positive, androgen responsive, and wild-type p53; *A*), PC-3 (androgen receptor and p53 null; *B*), and LNCaP and C4-2 (androgen independent variant of LNCaP cells; *C*) human prostate cancer cell lines exposed to DMSO (control) or honokiol (20 or 40 μmol/L) for 24, 48, or 72 h (*A* and *B*) or 24 h (*C*). Both floating and adherent cells were collected and processed for apoptotic DNA fragmentation assay. Columns, mean (n = 2-3); bars, SE. *, $P < 0.05$, significantly different compared with control by one-way ANOVA. The honokiol-mediated cytoplasmic histone-associated DNA fragmentation was confirmed in at least two independent experiments in each cell line.

Honokiol-induced apoptosis was associated with alterations in levels of Bcl-2 family proteins. Bcl-2 family proteins have emerged as critical regulators of apoptosis by functioning either as promoters (e.g., Bax and Bak) or inhibitors (e.g., Bcl-2 and Bcl-xL) of the cell death process (39). We proceeded to determine the effect of honokiol treatment on levels of Bcl-2 family proteins to gain insights into the mechanism of apoptosis in our model. As can be seen in Fig. 2A (LNCaP) and Fig. 2B (PC-3), honokiol treatment (40 μmol/L) resulted in a modest increase (1.5- to 3.0-fold increase over control) in protein levels of multidomain proapoptotic Bcl-2 family members Bax and Bak especially at the 24- to 48-h time points. The level of proapoptotic protein Bad and antiapoptotic protein Bcl-2 was also modestly increased on treatment of LNCaP and PC-3 cells with honokiol. Honokiol-treated LNCaP and PC-3 cells exhibited a 50% to 70% decrease in levels of antiapoptotic protein Mcl-1 relative to control (Fig. 2A and B). Whereas constitutive expression of Bcl-xL was very low in LNCaP cells (results not shown), honokiol treatment caused a decrease in its level in PC-3 cells (Fig. 2B). Collectively, these results indicated that the honokiol-induced apoptotic DNA fragmentation in LNCaP/PC-3 cells correlated with alterations in protein levels of Bcl-2 family proteins.

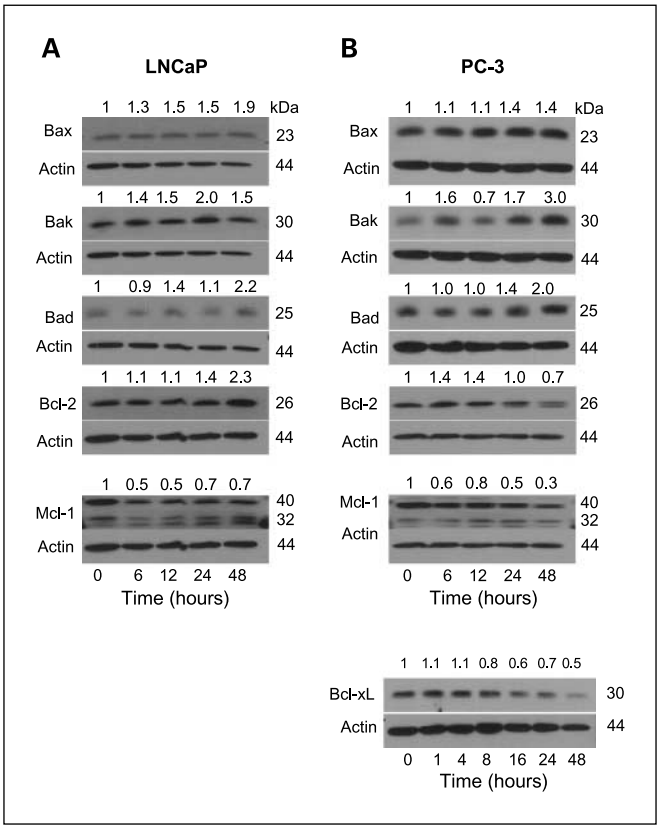

Fig. 2. Honokiol treatment alters levels of Bcl-2 family proteins in PC-3 and LNCaP cells. Immunoblotting for Bax, Bak, Bad, Bcl-2, Mcl-1, and/or Bcl-xL using lysates from LNCaP cells (*A*) and PC-3 cells (*B*) treated with 40 μmol/L honokiol for the indicated time periods. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein level. Densitometric scanning data after correction for actin loading control are shown on top of each band. Immunoblotting for each protein was done at least twice using independently prepared lysates and the results were consistent.

Bcl-xL overexpression and Bax or Bak knockdown in PC-3 cells conferred partial protection against honokiol-induced DNA fragmentation. Although honokiol-mediated induction of Bax expression and Bcl-xL down-regulation have been described in other cellular systems (15, 20, 21, 24), direct experimental evidence for their involvement in the cell death caused by honokiol is lacking. To probe into this question, we determined the effect of ectopic expression of Bcl-xL, a Bcl-2 homologue that functions to inhibit cell death by different stimuli (39), on honokiol-induced apoptosis using PC-3 cells. The level of Bcl-xL protein was higher by ~1.32-fold in PC-3 cells transiently transfected with pSFFV-Bcl-xL plasmid (PC-3/Bcl-xL cells) than in the empty vector–transfected control PC-3/Neo cells (Fig. 3A). The cytoplasmic histone-associated DNA fragmentation resulting from a 24-h exposure to 40 μmol/L honokiol was evident in both the empty vector–transfected control cells and PC-3/Bcl-xL cells (Fig. 3B). However, the Bcl-xL–overexpressing PC-3 cells were significantly more resistant to apoptosis induction by honokiol compared with PC-3/Neo cells (Fig. 3B).

Because honokiol treatment resulted in induction of Bax and Bak protein expression, albeit modestly (Fig. 2), we designed experiments using Bax- and Bak-targeted siRNAs to test the role of these proteins in regulation of honokiol-induced apoptosis. The levels of Bax and Bak proteins were decreased in PC-3 cells transiently transfected with Bax- and Bak-targeted siRNAs (Fig. 3C). The cytoplasmic histone-associated DNA fragmentation resulting from a 24-h treatment with 40 μmol/L honokiol was statistically significantly lower in PC-3 cells with knockdown of Bax and Bak proteins compared with cells transiently transfected with a control nonspecific siRNA (Fig. 3D). In addition, Bak knockdown conferred a relatively greater protection against honokiol-induced apoptosis compared with Bax depletion. Nonetheless, these results indicated that Bax, Bak, and Bcl-xL were targets of honokiol-induced apoptosis.

Oral administration of honokiol inhibited PC-3 xenograft growth in male nude mice. Next, we designed animal experiments to test (*a*) whether honokiol administration inhibits growth of PC-3 xenografts *in vivo* and (*b*) if honokiol administration causes apoptosis in tumors *in vivo*. To address these questions, we determined the effect of honokiol administration by oral gavage (thrice a week) on PC-3 xenograft growth. The honokiol doses were selected based on a previous study, which showed that intragastric administration of 2 mg honokiol significantly inhibited the growth of RKO colorectal cancer cells in nude mice (18). A 2-week pretreatment schedule was adopted to mimic the prevention protocol. The body weights of the control and experimental groups of mice were recorded periodically to determine the toxicity, if any, of honokiol. As can be seen in Fig. 4A, the average body weights of the control and honokiol-treated mice did not differ significantly throughout the experimental protocol. Moreover, the honokiol-treated mice otherwise seemed to be healthy and did not exhibit any other signs of distress such as impaired movement and posture, indigestion, and areas of redness or swelling.

The average tumor volume in 1 mg honokiol–treated mice was generally lower compared with control mice on each day of tumor measurement, although the difference did not reach statistical significance at $P = 0.05$ (Fig. 4B). On the other hand, the average tumor volume in mice treated with 2 mg honokiol was statistically significantly lower compared with vehicle-treated control mice starting on day 28 (Fig. 4B). For example,

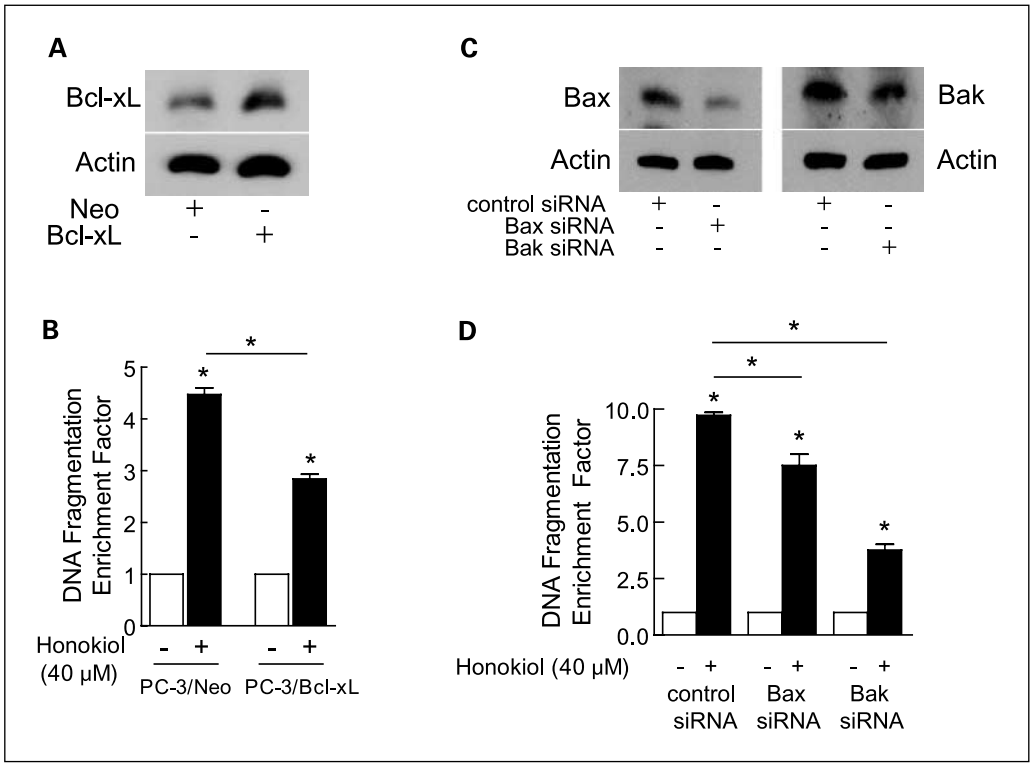

Fig. 3. Honokiol-induced apoptotic DNA fragmentation was partially but significantly attenuated by ectopic expression of Bcl-xL and Bax and Bak knockdown in PC-3 cells. *A,* immunoblotting for Bcl-xL protein using lysates from PC-3 cells transiently transfected with empty vector (PC-3/Neo cells) or pSFFV-Bcl-xL plasmid (PC-3/Bcl-xL cells). The blot was stripped and reprobed with anti-actin antibody to ensure equal protein loading. The level of Bcl-xL protein was ~1.32-fold higher in PC-3/Bcl-xL cells compared with PC-3/Neo as revealed by densitometric scanning of the immunoreactive band after correction for actin loading control. *B,* cytoplasmic histone-associated DNA fragmentation in PC-3/Neo and PC-3/Bcl-xL cells following 24-h treatment with DMSO (control) or 40 μmol/L honokiol. *C,* immunoblotting for Bax and Bak using lysates from PC-3 cells transiently transfected with a nonspecific control siRNA and Bax- or Bak-targeted siRNA. The blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading. Transient transfection of PC-3 cells with Bax and Bak siRNAs resulted in a marked knockdown of these proteins as revealed by densitometric scanning of the immunoreactive bands after correction for actin loading control. *D,* cytoplasmic histone-associated DNA fragmentation in PC-3 cells transiently transfected with a control siRNA and Bax or Bak siRNA and treated for 24 h with either DMSO (control) or 40 μmol/L honokiol. *B* and *D,* columns, mean (n = 2-3); bars, SE. *, $P < 0.05$, significantly different compared with corresponding control or between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. Similar results were observed in two independent experiments.

on the 77th day of tumor measurement, the average tumor volume in control mice (379 ± 235 mm^3) was ~6-fold higher compared with honokiol-treated mice. Consistent with tumor volume data, the average wet weight of the tumors was significantly lower in 2 mg honokiol–treated mice compared with control mice as judged by one-way ANOVA followed by Bonferroni's multiple comparison test ($P < 0.05$, control versus 2 mg honokiol–treated mice; Fig. 4C). These results indicated that honokiol administration at 2 mg dose significantly inhibited PC-3 xenograft growth without causing any side effects to the mice.

Honokiol administration increased apoptosis in tumors. To test whether honokiol-mediated inhibition of PC-3 xenograft growth *in vivo* at the 2 mg dose was due to increased apoptosis, tumor tissues from control and honokiol-treated mice were examined for histologic evidence of apoptosis. The apoptotic bodies in tumor sections of control and honokiol-treated mice were visualized by TUNEL staining and representative micrographs are shown in Fig. 5. The tumors from honokiol-treated mice exhibited a markedly higher count of apoptotic bodies compared with control tumors. The tumor sections from control and honokiol-treated mice were also stained with H&E. Eosin is an acidic stain that interacts with cellular proteins rich in basic amino acids and commonly used for cytoplasmic staining. The eosin-protein complex is characterized by a vivid pink cytoplasmic staining. Hematoxylin is a nuclear stain that interacts with negatively charged phosphate groups of nuclear DNA forming a blue-purple color. The staining for H&E was relatively brighter and more intense in tumors of control mice compared with tumors from honokiol-treated mice (Fig. 5). These results suggested a relatively higher proliferation index in control tumors than in the tumors from honokiol-treated mice. We confirmed this speculation by analysis of PCNA expression, a well-known proliferation marker. The brown-color PCNA staining was relatively more intense in control tumors compared with the tumors from honokiol-treated mice. These results indicated that honokiol administration inhibited PC-3 cell proliferation *in vivo*.

Previous studies have shown that honokiol inhibits angiogenesis *in vitro* (16). To test whether 2 mg honokiol treatment caused suppression of neovascularization, the tumor sections from control and honokiol-treated mice were stained for angiogenic marker CD31 (also known as platelet endothelial adhesion molecule 1). Tumors from honokiol-treated mice exhibited a decrease in number of CD31-positive blood vessels compared with control tumors. These results indicated that honokiol administration inhibited neovascularization in tumor tissue.

E-cadherin is considered to be a suppressor of invasion and growth of many epithelial cancers (40). Some anticancer agents function by causing up-regulation of E-cadherin expression (41). We therefore compared expression of E-cadherin in tumors of control and 2 mg honokiol–treated mice. Expression of E-cadherin was lower in tumors of honokiol-treated mice compared with control tumors. Collectively, these results indicated that honokiol-mediated suppression of PC-3 xenograft growth *in vivo* was associated with increased apoptosis and decreased proliferation index as well as neovascularization.

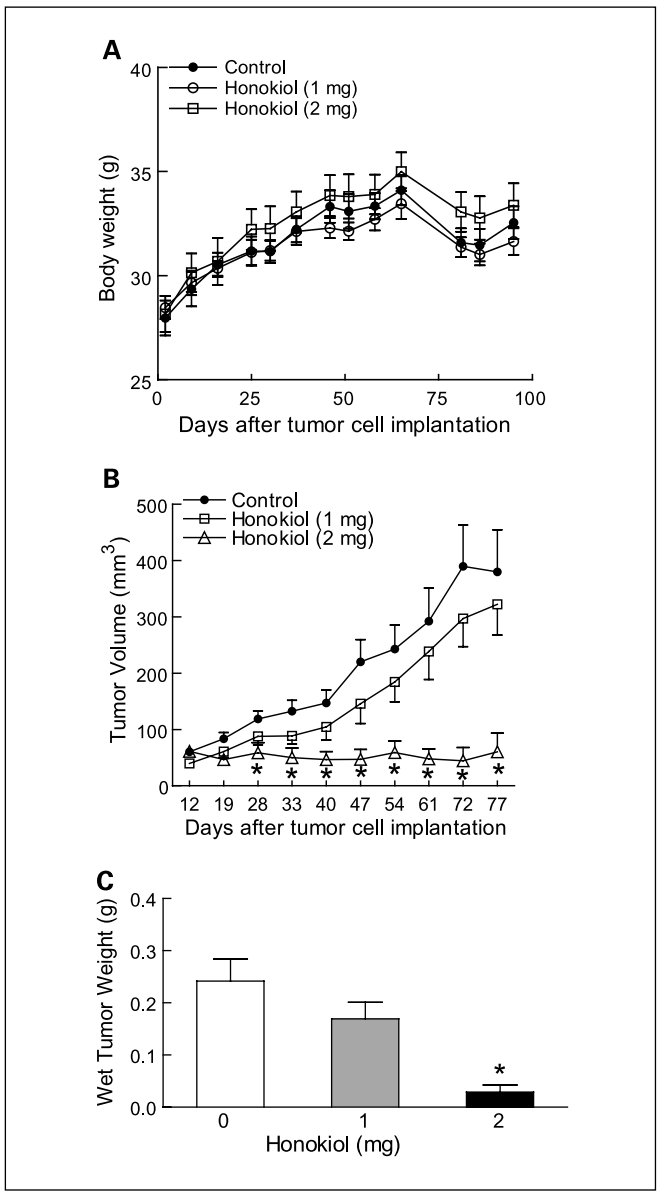

Fig. 4. Oral administration of honokiol suppressed growth of PC-3 cells s.c. implanted in male nude mice. Effect of oral administration of 1 or 2 mg honokiol (Monday, Wednesday, and Friday) on body weight of mice (*A*), tumor volume (*B*), and wet tumor weight (*C*). Mice were pretreated with honokiol or vehicle for 2 wk before tumor cell injection to mimic the prevention protocol. Points, mean [n = 5-6 (*A*) and n = 10-12 (*B* and *C*); 6 mice per group with tumor cells injected on both left and right flanks of each mouse]. Please note that one mouse from the control group died of unknown cause on the 44th day after tumor cell implantation. *, $P < 0.05$, significantly different compared with control by one-way ANOVA followed by Bonferroni's or Tukey's multiple comparison test.

Expression of Bcl-2 family proteins in tumor specimens. To gain insights into the mechanism of honokiol-mediated suppression of PC-3 xenograft growth, we compared the levels of Bcl-2 family proteins in tumors from control and 2 mg honokiol–treated mice. Representative blots for Bax, Bak, Bad, Bcl-xL, and Mcl-1 expression using tumor supernatants from control and honokiol-treated mice are shown in Fig. 6A. Change in protein level was quantified by densitometric scanning of the immunoreactive bands and corrected for actin loading control. As can be seen in Fig. 6B, the level of each protein examined including Bax and Bak was relatively higher in tumors from honokiol-treated mice compared with control tumors, although the difference did not reach statistical significance due to large data scatter ($P > 0.05$, paired t test). These results indicated that the honokiol-mediated alterations in expression of Bcl-2 family proteins observed in cultured cells were not necessarily correlated *in vivo*.

Discussion

The present study reveals that honokiol causes apoptotic DNA fragmentation in human prostate cancer cells irrespective of their androgen responsiveness or p53 status. The honokiol-mediated growth inhibition and apoptosis induction are not restricted to the cultured prostate cancer cells because suppression of PC-3 xenograft growth by honokiol administration, at least at the 2 mg/mouse dose, correlated with increased apoptosis (TUNEL positive apoptotic bodies) and decreased proliferation index (PCNA staining). Moreover, the tumors from honokiol-treated mice exhibited decreased number of CD31-positive blood vessels, supporting previous speculations that honokiol may function as an antiangiogenic agent (16).

Growth inhibition, suppression of NF-κB activation, cell cycle arrest, and apoptosis induction by honokiol in cultured cancer cells have been observed at the 10 to 150 μmol/L concentrations (15–24). It is hard to predict whether such concentrations of honokiol are achievable *in vivo* because pharmacokinetic parameters for this agent have not yet been determined in humans. Chen et al. (18) conducted a pharmacokinetic study with i.p. administration of 250 mg/kg honokiol in female BALB/c mice. The peak plasma concentration of honokiol was observed at ~27 ± 6 min after administration with an absorption half-life of ~10 ± 3 min and elimination half-life of ~5 ± 0.5 h (18). The maximal plasma concentration of honokiol was found to be ~1,100 μg/mL (18). Even 12 h after honokiol administration, the plasma concentration of honokiol exceeded the concentrations shown to be effective against cultured cancer cells *in vitro* (15–24). It is therefore possible that the plasma concentrations of honokiol required for cancer cell growth inhibition and apoptosis induction may be achievable in humans. However, a carefully designed pharmacokinetic study in humans is needed to verify this possibility.

The present study reveals that the honokiol-mediated apoptosis in PC-3 and LNCaP cells correlates with a modest increase in protein levels of proapoptotic proteins Bax, Bak, and Bad and a decrease in levels of Bcl-xL and Mcl-1 proteins. In addition, the honokiol-mediated suppression of PC-3 xenograft growth *in vivo* correlates with a modest increase in protein levels of Bax and Bak in the tumor specimen, although the difference did not reach statistical significance due to large data

scatter. Honokiol-mediated alteration in levels of Bcl-2 family proteins has been described in other cells types. For instance, apoptosis induction by honokiol in B-cell chronic lymphocytic leukemia cells correlated with up-regulation of Bax and down-regulation of Mcl-1 expression (21). In multiple myeloma cells, honokiol-induced apoptosis correlated with cleavage of Mcl-1 and up-regulation of Bad protein expression (20). However, the levels of Bid, Bak, Bax, Bcl-2, and Bcl-xL proteins were not affected by honokiol treatment in multiple myeloma cells (20). Similar to the results in PC-3 cells (present study), honokiol-induced apoptosis in CH27 human squamous lung cancer cell line was accompanied by down-regulation of Bcl-xL protein expression (15). These results suggest that the molecular targets of honokiol-mediated apoptosis probably vary in different cancer cells. Nonetheless, the present study points toward an important role of Bax, Bak, and Bcl-xL in honokiol-induced

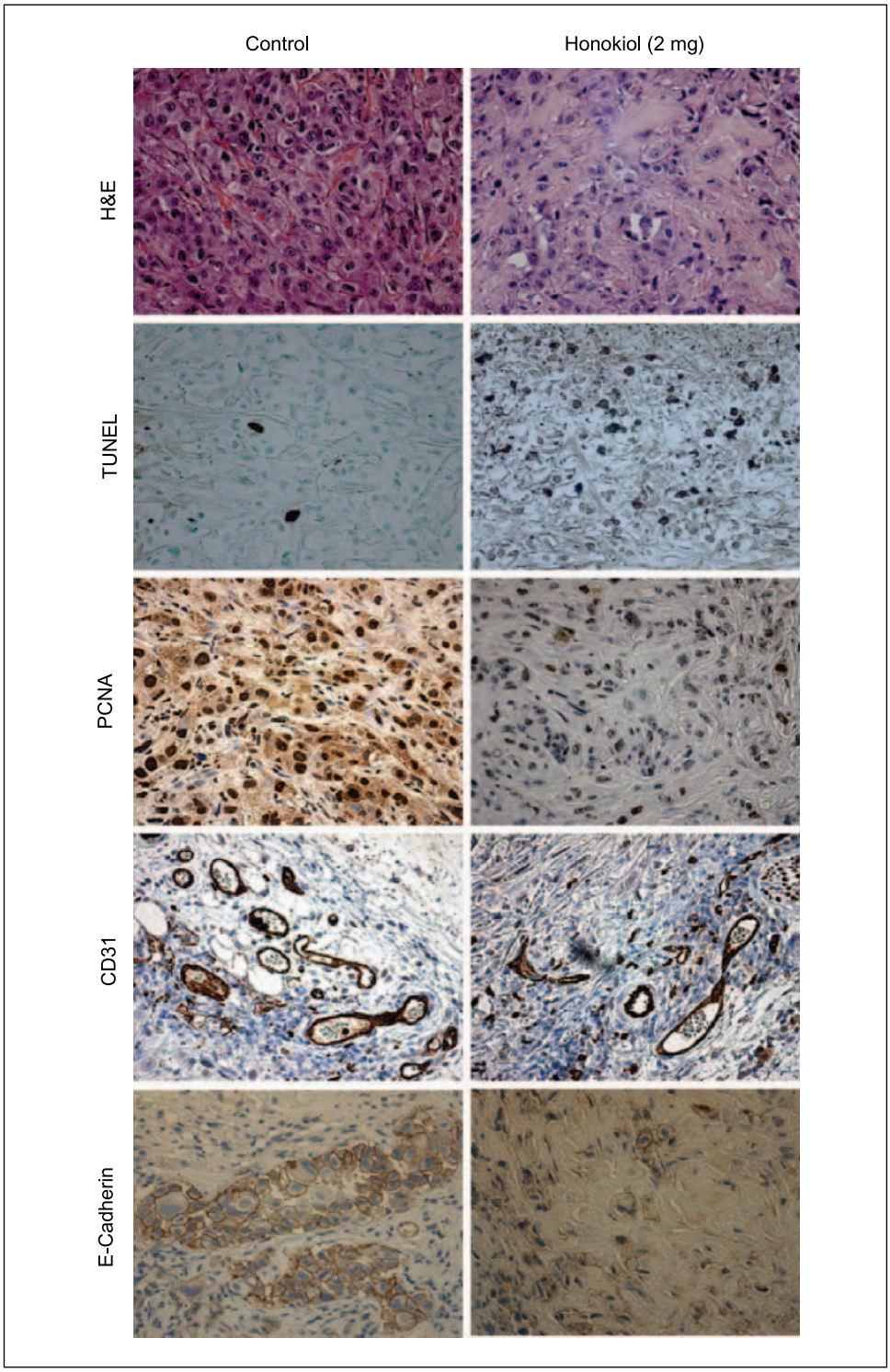

Fig. 5. Tumors harvested from honokiol-treated mice exhibited increased apoptosis and reduced proliferation compared with control tumors. Analysis of tumors from control and honokiol-treated mice for histology (H&E staining), apoptotic bodies (TUNEL staining), proliferation index (PCNA staining), neovascularization (CD31 staining), and E-cadherin expression. Tumor sections from four separate mice of control and 2 mg honokiol – treated groups were examined for H&E, TUNEL, PCNA, CD31, and E-cadherin staining; representative staining patterns in tumor sections from a control mouse and a 2 mg honokiol – treated mouse are shown.

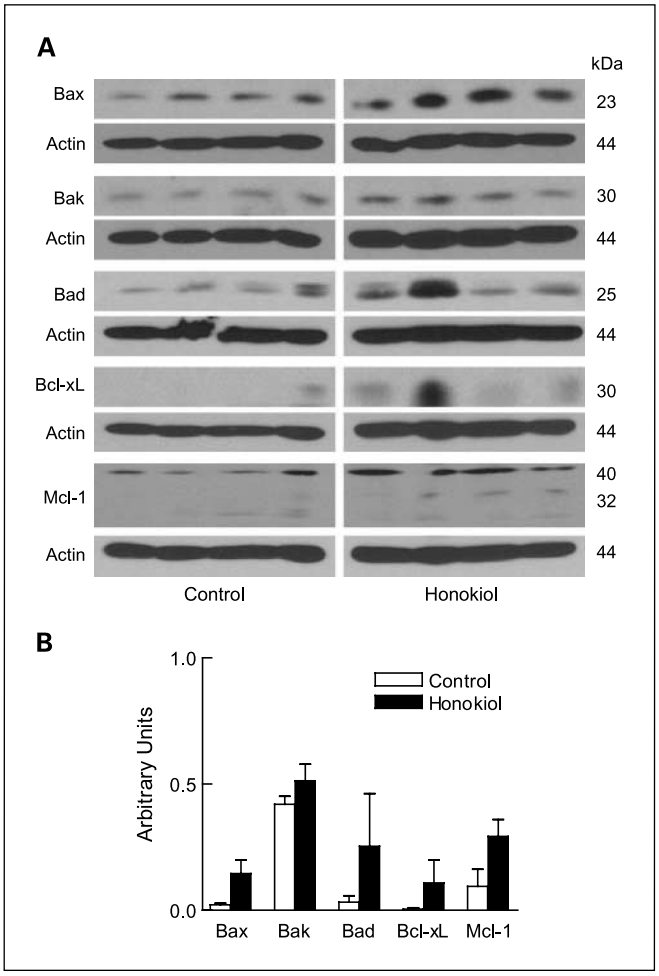

Fig. 6. Analysis of Bcl-2 family protein levels in tumors harvested from control and honokiol-treated mice. *A,* immunoblotting for Bcl-2 family proteins using tumor supernatants from control and honokiol-treated mice. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. *B,* densitometric scanning data for Bcl-2 family protein levels in tumors from control and honokiol-treated mice. Tumor tissues from four mice of each group were used for immunoblotting. Columns, mean ($n = 4$); bars, SE. The difference in expression of Bcl-2 family proteins between control and 2 mg honokiol – treated group did not reach statistical significance due to large data scatter.

apoptosis because the cytoplasmic histone-associated DNA fragmentation caused by this agent is partially yet significantly attenuated by ectopic expression of Bcl-xL as well as knockdown of Bax and Bak protein levels. The partial protection against honokiol-mediated apoptotic DNA fragmentation by Bax and Bak depletion is probably attributable to partial knockdown of these proteins. At the same time, the possibility that Bax and Bak induction only partially accounts for honokiol-induced DNA fragmentation cannot be fully discarded.

Because preclinical *in vivo* efficacy testing of potential cancer therapeutic/preventive agents is a key step in their clinical development, we determined the effect of honokiol on growth of PC-3 cell s.c. implanted in male nude mice. We found that oral administration of 2 mg honokiol/mouse significantly retards growth of PC-3 xenografts *in vivo*. During the preparation of this article, Shigemura et al. (42) showed that honokiol suppresses growth and causes apoptosis not only in prostate cancer cells (LNCaP, C4-2, and PC-3) but also in cells known to closely interact with prostate cancer cells either at the primary site or bone metastasis site, including human prostate stromal fibroblasts, marrow stromal cells (HS27A), and transformed human bone marrow endothelial cells. These investigators also showed that daily i.p. injection of 100 mg honokiol/kg body weight over a 6-week period inhibited metastatic growth of C4-2 cells inoculated in mouse bilateral tibia and decreased serum PSA levels (42). We also found that treatment of LNCaP cells with honokiol causes a decrease in intracellular levels of both androgen receptor and PSA proteins (results not shown). Honokiol administration also inhibited bone metastasis by C4-2 cells, characterized by a decrease in area of tumor as judged by X-ray, an increase in the ratio of cortical bone and bone marrow to the values found in control animals, and increased granulation (42). In addition, honokiol treatment potentiated anticancer activity of docetaxel *in vitro* as well as *in vivo* (42). Inhibition of RKO colorectal carcinoma cell growth in female nude mice by i.p. injection of 2 mg honokiol/mouse has also been observed (18). Moreover, honokiol administration at 80 mg/kg dose significantly increased mean survival time (50.9 days) by ~71% compared with control mice (18). However, the present study is the first published report to document (*a*) *in vivo* anticancer activity of orally administered honokiol against PC-3 cells and (*b*) increased apoptosis and decreased cell proliferation and neovascularization in tumors derived from honokiol-treated mice.

It is interesting to note that the honokiol-mediated changes in levels of Bcl-2 family proteins in cultured cells do not correlate *in vivo*. For instance, honokiol treatment causes a decrease in levels of Bcl-xL and Mcl-1 in cultured PC-3 cells, whereas the levels of these proteins are not reduced in tumors from honokiol-treated mice compared with control tumors. Several possibilities exist to explain inconsistencies in cellular and *in vivo* results. One possibility is the difference in metabolism of honokiol between cultured cancer cells and cancer cells *in vivo*. It is also possible that the timing of tumor tissue collection is not optimal to detect honokiol-mediated *in vivo* changes in levels of Bcl-2 family proteins. Carefully designed experiments, including a time course analysis of the effect of honokiol administration, on levels of Bcl-2 family of apoptosis regulatory proteins in tumors *in vivo* are needed to systematically explore these possibilities.

Honokiol qualifies as a "promiscuous" rather than a "selective" agent based on its known pharmacologic effects (2–25). However, promiscuity is not unique to honokiol because many known successful drugs as well as a number of promising natural anticancer agents are promiscuous. The best known example of a widely used drug that can be classified as promiscuous is aspirin. In addition to its pain-relieving and antiarthritic effects, aspirin exhibits many other pharmacologic effects including blood thinning, reduction of platelet aggregation, prevention of preeclampsia (a hypertensive disorder during pregnancy), and anticancer effects (43). Likewise, recent studies including those from our own laboratory have revealed that many promising dietary cancer chemopreventive agents (e.g., garlic constituent diallyl trisulfide and cruciferous vegetable constituent sulforaphane) target multiple signal transduction pathways in various cell types to inhibit cancer cell growth *in vitro* and *in vivo* (reviewed in refs. 44, 45). Because the etiology and pathogenesis of cancer is highly complex involving abnormalities in multiple cellular checkpoints and signal

transduction pathways, we are in agreement with the arguments of Arbiser (46) that promiscuity may be an advantageous feature of potential anticancer agents because recently developed anticancer agents having a high degree of selectivity have failed to live up to the expectations (e.g., specific epidermal growth factor receptor inhibitor Iressa).

A unified mechanism to explain multiple pharmacologic effects of honokiol may not be plausible. However, it seems reasonable to conclude that some of the cellular responses to honokiol leading to cell cycle arrest (25) and apoptosis induction (present study) in human prostate cancer cells may be linked to suppression of NF-κB activation. For instance, NF-κB is known to regulate expression of multiple genes whose protein products are involved in the regulation of cell cycle progression and apoptosis, including cyclin D1, Bcl-2, Bcl-xL, XIAP, and survivin (26). We have previously shown that honokiol-mediated G_0-G_1 phase cell cycle arrest in PC-3 and LNCaP cells correlates with a decrease in protein level of cyclin D1 (25). The honokiol-mediated apoptosis in PC-3 and/or LNCaP cells is associated with down-regulation of Bcl-xL (Fig. 2) and survivin protein expression (results not shown). However, suppression of NF-κB activation may be one of the mechanisms in cell cycle arrest and apoptosis by honokiol and may not fully explain other molecular changes such as a decrease in protein level of retinoblastoma Rb tumor suppressor (25).

In conclusion, the results of the present study show that honokiol treatment causes apoptotic DNA fragmentation in cultured human prostate cancer cells, irrespective of their androgen responsiveness or p53 status, and inhibits growth of PC-3 xenografts in mice without causing weight loss or any other side effects. Honokiol-mediated suppression of PC-3 xenograft growth *in vivo* correlates with increased apoptosis as well as decreased proliferation index and neovascularization.

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