Honokiol, a Natural Plant Product, Inhibits the Bone Metastatic Growth of Human Prostate Cancer Cells

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BACKGROUND. Honokiol, a soluble nontoxic natural product derived from *Magnolia* spp., has been shown to induce apoptosis in malignant cells. The effect of honokiol and the combined therapy with docetaxel on prostate cancer (PCa) growth and bone metastasis was investigated in experimental models.

METHODS. The in vitro proapoptotic effects of honokiol on human androgen-dependent and -independent PCa, bone marrow, bone marrow-derived endothelial, and prostate stroma cells were investigated. Honokiol-induced activation of caspases was evaluated by Western blot and FACS analysis. To confirm the cytotoxicity of honokiol, mice bone was inoculated in vivo with androgen-independent PCa, C4-2 cells and the effects of honokiol and/or docetaxel on PCa growth in bone were evaluated. Daily honokiol (100 mg/kg) and/or weekly docetaxel (5 mg/kg) were injected intraperitoneally for 6 weeks. PCa growth in mouse bone was evaluated by radiography, serum prostate-specific antigen (PSA) and tissue immunohistochemistry.

RESULTS. Honokiol induced apoptosis in all cell lines tested. In PCa cells honokiol induced apoptosis via the activation of caspases 3, 8, and 9 and the cleavage of poly-adenosine diphosphate ribose polymerase in a dose- and time-dependent manner. Honokiol was shown to inhibit the growth and depress serum PSA in mice harboring C4-2 xenografts in the skeleton and the combination with docetaxel showed additive effects that inhibited further growth without evidence of systemic toxicity. Immunohistochemical staining confirmed honokiol exhibited growth-inhibitory, apoptotic, and antiangiogenic effects on PCa xenografts.

CONCLUSIONS. The combination of honokiol and low-dose docetaxel may be used to improve patient outcome in androgen-independent prostate cancer with bone metastasis. ***Cancer* 2007;109:1279–89.** © *2007 American Cancer Society.*

KEYWORDS: Honokiol, prostate cancer, bone metastasis, apoptosis.

Honokiol is a plant lignan isolated from the bark and seed cones of the magnolia tree.[1] Previous studies demonstrated that honokiol markedly inhibited endothelial cell growth in culture,[2] angiosarcoma formation in mice,[2] cultured B cell chronic lymphocytic leukemia cells isolated from patients,[3] and a human colorectal carcinoma cell line in vitro.[4] The antineoplastic effects of honokiol have been extended to many other forms of malignancies including lung cancer and multiple myeloma.[2,4–6] Park et al.[7] showed that honokiol induces apoptosis via cytochrome c release and caspase activation in rat hepatic satellite cells. Honokiol was shown to inhibit Akt/phosphoinositide-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling in a cultured angiosarcoma cell line.[2] In addition to its antitumor properties, honokiol also blocks phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2) in human endo-

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thelial cells and honokiol treatment results in blockade of VEGF-induced Rac activation.[2,8]

Bone is a common site of prostate cancer metastasis and therapeutic options are limited. The attractive features of honokiol as an antiangiogenic drug are that it not only induces apoptosis in a variety of cancer cells[2–6] but also inhibits nuclear factor kappa B (NF-κB) activation,[9] which promotes cancer cell survival, and reverts p-glycoprotein activation, associated with decreased cancer cell sensitivity to chemotherapeutic drugs.[10,11] Honokiol also inhibits osteoclastogenesis, a process often associated with increased bone turnover and cancer cell bone colonization through enhanced lytic and blastic reactions when cancer cells are residing in bone.[9] Despite much study of honokiol's effect on the growth of cancer cells and cancer-associated vascular endothelial cells, there is no report on whether honokiol has cytotoxic effects on the cells in the tumor microenvironment. Cells such as marrow stromal and prostate stromal cells are known to be closely associated with tumor cells in the primary and their subsequent metastasis to bone in patients.

Current therapy for metastatic prostate cancer includes docetaxel, a semisynthetic member of the taxane family. It has shown clinical activity in a wide spectrum of solid tumors including breast, lung, ovarian, and prostate cancers.[12–14] Docetaxel is a second-generation taxane with improved pharmacokinetic, pharmacodynamic, and cytotoxicity profiles, and due to differences in drug efflux is retained intracellularly for a longer period.[15,16] Early in vitro studies revealed that docetaxel has a number of unique preclinical characteristics compared with other chemotherapeutic agents, including the taxane paclitaxel. We evaluated the combined effects of honokiol plus low-dose docetaxel to avoid host systemic toxicity and determined their combined effects on the growth of hormone-refractory human prostate cancer cells in the mouse skeleton. We demonstrated that honokiol inhibits growth and induces apoptosis of prostate cancer and associated stromal cells, human bone marrow cells, and human bone marrow-derived endothelial cells via caspase-dependent pathways, and showed additive effects with low-dose docetaxel in vivo without inducing systemic toxicity in mice. Our study provides a rationale for the use of honokiol in aggressive hormone-refractory prostate cancer.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Chemical Reagents

The androgen-dependent (AD) AR-positive parental LNCaP human prostate cancer cell line, its lineage-derived androgen-independent (AI), AR-positive, and metastatic C4-2 subline, and AI and AR-negative human prostate cancer PC-3 were used for this study as previously described.[17] Immortalized paired human prostate-associated stromal cells from normal/benign lesions, NPF (normal/benign prostate fibroblasts) cells, and cancer-associated lesions, CPF (cancer-associated prostate fibroblast) cells, were established from the same patient using Emory University-approved Institutional Review Board (IRB) protocols. Attaching stromal cells were cultured in T-medium (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO). Cell cultures were routinely used at less than 6 passages. Immortalized normal human bone marrow stromal cells (HS27A) were obtained from the American Type Culture Collection (ATCC; CRL-2496; Manassas, VA), whereas transformed human bone marrow endothelial cells (TrHBMEC) were obtained as a gift from Dr. Weksler.[18] Unless otherwise stated, these cell lines were cultured in T-medium supplemented with 5% FBS, 100 IU/L penicillin G, and 100 μg/L streptomycin at 37°C under 5% CO_2. Honokiol (Wako Chemical, Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL stock solution and its purity was determined to be a minimum of 99% by high-performance liquid chromatography.[3,19] This reagent was stored at −20°C and diluted by media just before use. Docetaxel (Sanofi-Aventis, Bridgewater, NJ) was dissolved in ethanol at 100 μM and stored at 4°C.

Cell Proliferation

Cell proliferation was measured using the CellTiter 96 AQ nonradioactive cell proliferation assay (MTS assay; Promega, San Luis Obispo, CA). Briefly, cells suspended in T media plus 5% FBS were added to the 96-well plate at 5000 cells/well in triplicate. After 24 hours of culture, honokiol and/or docetaxel were added in various concentrations. Cells were cultured for 24 or 48 hours, then added to 20 μL/well of combined MTS/PMS solution (Promega). After incubation for 1 hour at 37°C in a humidified incubator with 5% CO_2 atmosphere, the OD 490 nm was recorded using an enzyme-linked immunosorbent assay (ELISA) plate reader. Data represent mean ± standard deviation (SD) of triplicate cultures from 2 independent experiments. The survival fractions of cells were expressed as relative survival with controls recorded as 1.0.

Cell Apoptosis

Cells plated at 3×10^5 cells/well in 6-well plates with T media plus 5% FBS were cultured for 24 hours and added with various concentrations of honokiol. Cells were cultured for an additional 24 hours. Apo-

ptosis was assessed by flow cytometry using annexin V, Alexa Fluor 350 conjugate (Molecular Probes, Eugene, OR) following the manufacturer's protocol. These assays were repeated twice. Data were expressed as the average of these determinations.

Western Blot

Cultured cells that reached 70% to 80% confluence were harvested in a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% NaN_3, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing 1.5 mM phenylmethanesulfonyl fluoride (PMSF) and Protease Inhibitor Cocktail Complete (Roche Diagnostics, Mannheim, Germany). Protein concentrations in the supernatants were measured with the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Fifty μg of total protein was loaded to each lane and resolved on a 12% NUPAGE Bis-Tris-buffered (pH 7.0) polyacrylamide gel (Invitrogen) and transferred onto a nitrocellulose membrane. The membrane was incubated with anticaspase 3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anticaspase 8 antibody (BD Biosciences, San Jose, CA), anticaspase 9 antibody (Santa Cruz), and Poly-ADP Ribose Polymerase (PARP) antibody (Cell Signaling Technology, Danvers, MA) mixed with anti-EF1-α antibody serving as the loading control. The reactive bands were visualized by ECL (Amersham Biosciences, Buckinghamshire, UK). In some studies, Western blot membranes were stripped by submerging the membranes in restored Western blotting stripping buffer (Pierce), incubated at 37°C for 30 minutes with occasional agitation, and washed with washing buffer (1× PBS, 0.1% Tween 20) before the secondary blotting.

Assessment of In Vivo Therapeutic Effects of Honokiol, Docetaxel, and the Combination of Honokiol and Docetaxel on Human Prostate Tumor Xenografts in Mouse Bone

A total of 1.0×10^6 C4-2 cells were inoculated in mouse bilateral tibia using a previously established procedure.[17,20] Blood specimens (70 μL) were obtained from the retroorbital sinus vein every 2 weeks for serum prostate-specific antigen (PSA) determination. Serum PSA was determined by microparticle ELISA using an Abbott IMx instrument (Abbott Laboratories, Abbott Park, IL). A total of 16 athymic male nude mice (BALB/c nu/nu; National Cancer Institute, Bethesda, MD) were divided into 4 groups: vehicle control group ($n = 4$); honokiol-alone treatment group ($n = 4$); docetaxel-alone treatment group; and the combination of honokiol and docetaxel treatment group ($n = 4$). The treatments were initiated at 3 weeks after tumor cell inoculation with confirmed tumors in bone by x-ray and positive serum PSA.[17] Honokiol was dissolved in 100% ethanol containing 20% Intralipid (Baxter Healthcare, Deerfield, IL) as previously described.[2] Mice were administered daily with honokiol at 2.5 mg per mouse (approximately 100 mg/kg) via the intraperitoneal route for a 6-week period. Docetaxel was administered similarly to mice at a dose of 5 mg/kg once a week. Control mice received vehicle injections for the same duration. Mice were weighed every week and tumor growths in bilateral tibia were followed by serum PSA and x-ray every 2 weeks. Mice were sacrificed 6 weeks after the initiation of treatment. The bilateral tibia were removed, kept in 10% formalin for 48 hours, and then decalcified in EDTA (pH 7.2) for 15 days. Tibia specimens were dehydrated and paraffin-embedded for histopathologic (hematoxylin and eosin [H&E] staining) and immunohistochemical analyses.

Immunohistochemical Analysis

Histopathology of the tumor specimens in mouse tibia was assessed in H&E-stained slides. Immunohistochemical (IHC) staining was performed as follows: cell proliferation, vascular endothelial, and cell death profiles in tumor-bearing bone specimens were assessed by IHC using a Dako Autostainer Plus system (Dako, Carpinteria, CA). The respective antibodies used in IHC analyses were mouse monoclonal antibody against Ki67 (used at 1:1000, Santa Cruz), rabbit polyclonal antibody against PECAM-1 (CD-31) (H-300; 1:200, Santa Cruz), and mouse monoclonal antibody against M30 CytoDeath (1:600, DiaPharma Group, West Chester, OH). Tissues were deparaffinized, rehydrated, and subjected to 5-minute pressure-cooking antigen retrieval, 10-minute double endogenous enzyme block, and 30-minute primary antibody reaction followed by 30-minute EnVision plus Dual Link System (peroxidase) incubation at room temperature. Antigenic signals were detected by adding diaminobenzidine as a chromogen of horseradish peroxidase and slides were counterstained by hematoxylin. All reagents were obtained from Dako unless otherwise indicated. For quantification, 200 cells at 3 randomly selected areas at ×100 magnification were assessed and the positively Ki67- and M30-stained cells were recorded; positive CD31 stains were recorded by counting the number of positively stained microvessels in the definite area. Data are presented as microvessel density with a control value of 1.0.[21]

Statistical Analysis

Statistical analysis was conducted using Student *t*-test and Welch *t*-test with JSTAT (Java Virtual Machine

Statistics Monitoring Tool; Sun Microsystems, Santa Clara, CA). Statistical significance was established at $P < .05$.

RESULTS

Honokiol Inhibits the Growth of Human Prostate Cancer, Prostate Stromal, Marrow Stromal, and Bone Marrow-Derived Endothelial Cells In Vitro

Previous studies have established honokiol as a nontoxic antitumor drug for several human blood-borne and solid tumors.[6–10] To explore the potential use of honokiol as a treatment option for hormone-refractory human prostate cancer bone metastasis, we evaluated the cytotoxic effects of honokiol on androgen-dependent (LNCaP), androgen-independent (C4-2, PC-3) human prostate cancer cells and those cells known to closely interact with human prostate cancer either at the primary or bone metastatic sites, specifically human prostate stromal fibroblasts (NPF and CPF), marrow stromal cells (HS27A), and transformed human bone marrow endothelial cells (TrHBMEC). These cells were exposed to the indicated concentrations of honokiol for 24 hours and cell growth was determined by MTS assay. Honokiol was found to inhibit the growth of prostate cancer cells, LNCaP, C4-2, and PC-3, with 50% inhibition (IC_{50}) observed at 5 to 10 μg/mL, which is equivalent to 18.75 to 37.5 μM. Honokiol also inhibited the growth of both normal/benign and cancer-associated prostate stromal cells established from patients (NPF and CPF). Typically, we observed that honokiol was more effective in inhibiting the growth of cancer-associated than normal/benign-associated stromal fibroblasts with 10 or 20 μg/mL honokiol treatment. At 10 or 20 μg/mL honokiol the growth inhibitions observed in CPF were 91.0 ± 0.11% and 89.4 ± 1.10% as opposed to NPF, which were 85.2 ± 0.16% and 83.3 ± 0.400% ($P < .002$). Honokiol also inhibited the growth of normal immortalized human marrow stromal cells (HS27A) and transformed human bone marrow endothelial cells (TrHBMEC), with IC_{50}s of 5 and 10 μg/mL, respectively (Fig. 1). These results, taken together, suggest that honokiol is cytotoxic to prostate cancer cells and to supporting cells in the cancer microenvironment at a concentration achievable in vivo (see below).

Honokiol Induced Caspase-Dependent Apoptosis in Human Prostate Cancer Cells

To elucidate how honokiol inhibits cell viability, particularly in a condition that mimics human prostate cancer bone metastasis, we analyzed annexin V expression, an indicator of apoptosis, in LNCaP, C4-2, HS27A, and TrHBMEC cells cultured in the presence of 0, 5, 10, and 20 μg/mL honokiol for 24 hours. Honokiol treatment significantly induced apoptosis in all cell types studied in a dose-dependent manner. Greater than 50% cell death can be achieved with 10 μg/mL honokiol (Fig. 2). We next focused our investigation on the apoptotic cell death mechanism induced by honokiol treatment. C4-2 cells were treated with 5, 10, and 15 μg/mL honokiol for 24 and 48 hours. Activation of caspases 3, 8, and 9 and cleavage

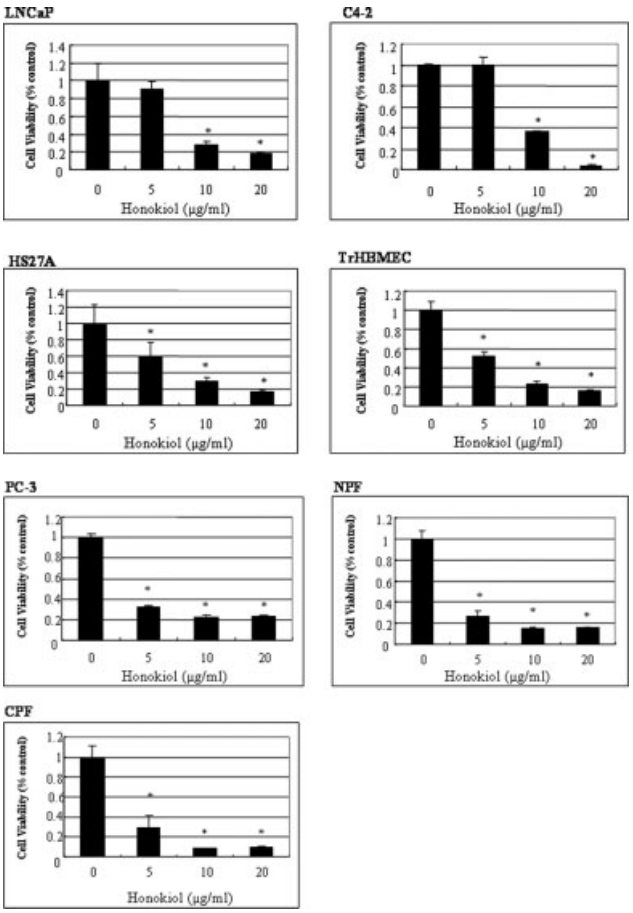

FIGURE 1. Honokiol induces cytotoxicity in androgen-dependent, LNCaP, and androgen-independent, C4-2 and PC-3 prostate cancer cell lines, human bone marrow stromal (HS27A), human bone marrow-associated endothelial cells (TrHBMEC), and paired human prostate associated stromal fibroblasts from normal/benign lesions, NPF (normal/benign prostate fibroblasts) and cells established from cancer-associated lesions, CPF (cancer associated prostate fibroblasts). Growth inhibition by honokiol in these cell lines was assessed by cell proliferation assay after 24-hour culture. Data represent mean ± the standard deviation (SD) of triplicate cultures from 2 independent experiments. Horizontal axis shows honokiol concentration up to 20 μg/mL (75 μM). The vertical axis shows the comparative cell proliferation ratio indicated as cell viability (percent control) with vehicle control. *Significant inhibition of cell proliferation observed after honokiol treatment compared with vehicle controls ($P < .05$).

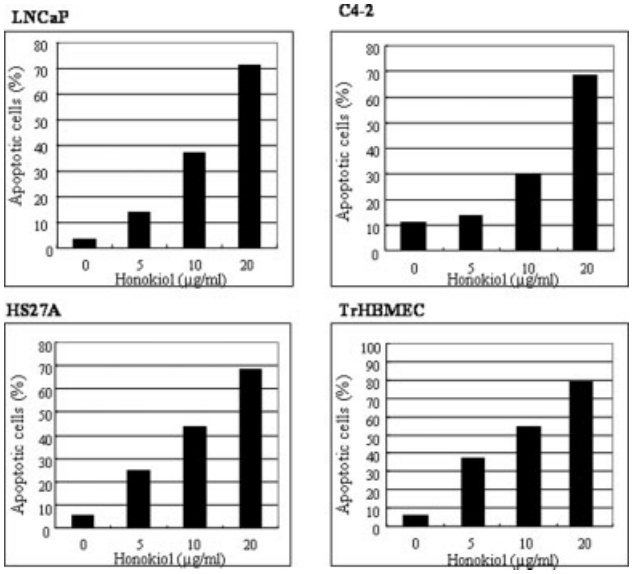

FIGURE 2. Honokiol induces apoptosis in prostate cancer cells, human bone marrow cells, and human bone marrow-associated endothelial cells. Apoptosis was assessed in the tested cells after honokiol treatment: Annexin V FACS analysis after honokiol treatment. Horizontal axis shows honokiol concentration. Vertical axis shows percent of apoptotic cells. Cells were subjected to honokiol treatment for 24 hours and percent of apoptotic cells induced by honokiol correlated with honokiol concentrations in the culture media (5–20 μg/mL).

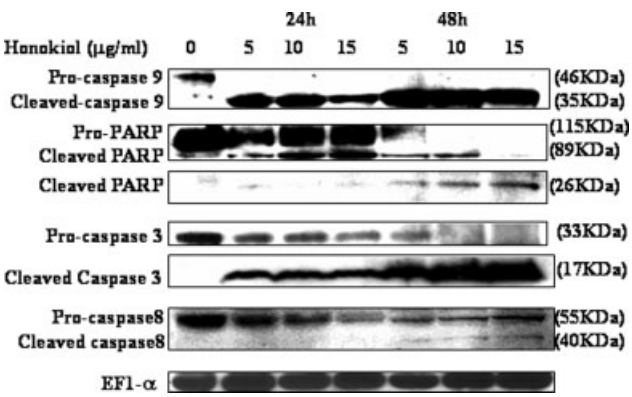

FIGURE 3. Honokiol induced apoptosis through the activation of caspase 3, 8, 9, and PARP pathways. C4-2 cells were exposed to 5 to 15 μg/mL of honokiol for 24 and 48 hours. Activated caspases 3, 8, and 9 as detected by the increased bandings of proteins at 17, 40, and 35 kDa, respectively, and increased cleavage of PARP are dose- and time-dependent.

of PARP were analyzed by Western blot. After honokiol treatment, we observed activated caspases 3, 8, and 9, as exhibited by increased intensity of protein bands at 17, 40, and 35 kDa, respectively, and increased cleavage of PARP (Fig. 3) in a dose- and time-dependent manner. These results are consistent with the published reports showing that honokiol

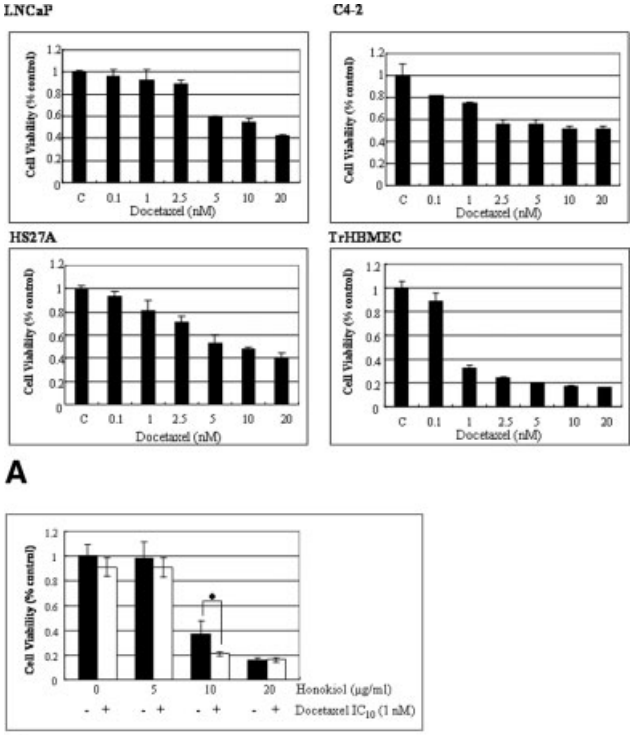

FIGURE 4. (A) Docetaxel inhibits the proliferation of prostate cancer cells, human bone marrow cells, and human bone marrow-associated endothelial cells in a dose-dependent manner with an IC_{10} of 1–10 nM and IC_{50} of 1–10 nM and shows additive inhibiting effects on androgen-independent prostate cancer cell line (C4-2) with honokiol in vitro. (B) The combined therapy of IC_{10} docetaxel (1 nM) plus 10 μg/mL honokiol indicated significantly more inhibiting effects on the growth of C4-2 cells than 10 μg/mL honokiol alone ($P < .05$). Cells were treated with (A) 0.1–20 nM docetaxel and (B) 0–20 μg/mL honokiol in the presence or absence of IC_{10} docetaxel (1 nM) for 48 hours. Cell proliferation was determined by MTS assay. The vertical axis shows comparative cell proliferation ratio indicated as cell viability (percent control) with vehicle control. *Significant inhibition of cell proliferation observed with the combined treatment of IC_{10} docetaxel with honokiol compared with honokiol alone in (B) ($P < .05$).

caused apoptotic death in human tumor and endothelial cells.[1–6,22]

Combined Honokiol and Docetaxel Treatments Augment Growth Inhibition of C4-2 Cells In Vitro and Tumors in Mouse Skeleton

We evaluated the effects of docetaxel and the combined effects of honokiol and docetaxel on the growth of LNCaP, C4-2, HS27A, and TrHBMEC cells in vitro. As shown in Figure 1, honokiol alone inhibited the growth of prostate cancer cells and those cells in the cancer microenvironment like HS27A and TrHBMEC, with IC_{50}s that ranged from 5 to 10 μg/mL. Docetaxel exposure alone for 48 hours was also shown to inhibit the growth of these cells, with IC_{50}s from 1 to 10 nM approximately (Fig. 4). A combina-

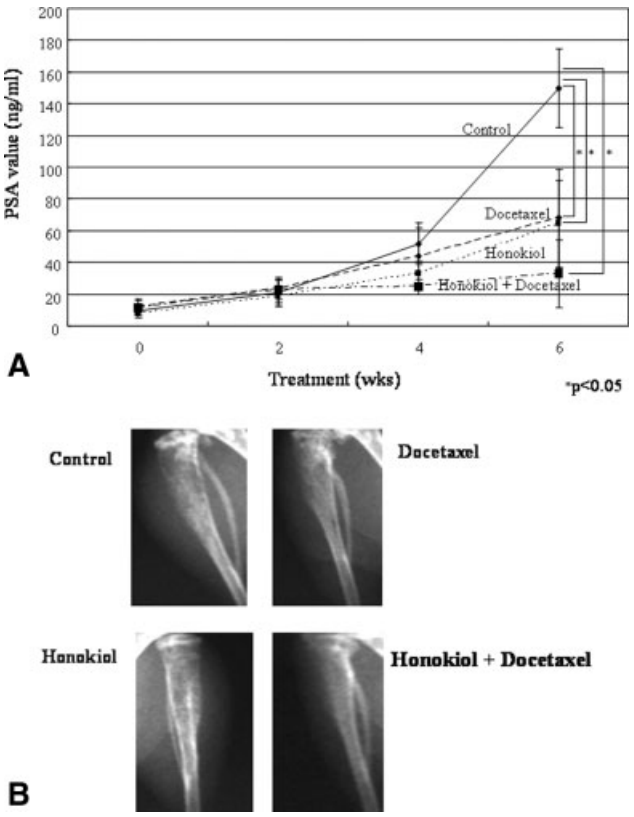

FIGURE 5. Honokiol and/or docetaxel significantly decreased serum prostate-specific antigen (PSA) levels in mice bearing C4-2 prostate tumor bone xenografts. (A) Honokiol, when injected intraperitoneally, reduced serum PSA in mice bearing C4-2 tumors in mouse skeleton: honokiol alone, docetaxel alone, and honokiol and docetaxel combined therapy showed a significant decrease of serum PSA in comparison with control group after 6 weeks treatments ($P < .05$). (B) Representative chromatograms of the bone-bearing tumors in each group as detected by x-ray, showing that honokiol plus docetaxel further improves the tumor x-ray appearances in comparison to honokiol or docetaxel treatment alone. These x-ray results correspond with the finding that the combination treatment was more effective than honokiol or docetaxel treatment alone in lowering serum PSA in mice bearing C4-2 tumors in tibia.

tion of low doses of honokiol (0.3125-1.25 μg/mL) plus docetaxel at defined concentrations (IC_{10} and IC_{50}) resulted in an additive interaction between these 2 drugs as calculated by the combined index (Calcusyn software, Biosoft, Ferguson, MO) (data not shown). Figure 4B shows that the combination therapy of IC_{10} docetaxel (1 nM) with honokiol had significantly more inhibiting effects on C4-2 cell growth than honokiol alone at 10 μg/mL.

The antitumor effects of honokiol and/or docetaxel were evaluated in vivo in mice harboring C4-2 tumors. Tumor growth and responsiveness to therapy were determined by serum PSA and x-ray of the skeleton. These results were confirmed by histopathologic, IHC, and histomorphometric analyses of the harvested tumor specimens at the termination of the experiments.

Serum PSA significantly decreased in all treated groups compared with control for 6 weeks treatment ($P < .05$). The combined treatment group had lower serum PSA values than the docetaxel treatment alone but the difference was not statistically significant. In these studies we did not observe any obvious toxicities induced by honokiol treatment as reflected by the lack of body weight loss or infections in mice. We evaluated bone lesions induced by prostate cancer cells in the skeleton and the effects of various treatments. We correlated these results with histomorphometric analyses of the skeleton. The results revealed that 6 weeks after combined treatment with honokiol plus docetaxel the ratios of tumor areas were significantly decreased, and the ratios of cortical bone and bone marrow were significantly increased to the values found in normal bone. In addition, the tumor specimens were composed predominantly of granulation tissue or marrow with less significantly demarcated tumor areas. The vehicle controls, in contrast, had greatly increased tumor mass, areas of extensive osteolytic bone destruction, osteoblastic areas, and much less granulation tissue or marrow space. We observed that the honokiol- or docetaxel-alone treatment groups had intermediate ratios of tumor and cortical bone and marrow space, with intermediate areas of granulation tissue within each histopathologic specimen (Figs. 5A,B, 6A).

IHC Analysis of Human Prostate Cancer Xenografts Subjected to Honokiol and/or Docetaxel Treatment

IHC staining of mouse tibia at the end of the treatment revealed: 1) compared with vehicle controls, markedly decreased cell proliferation (Ki67) was observed in the combined treatment group; 2) similar observations were noted in the combined treatment group with respect to decreased vascular density (CD31) and increased apoptosis (M30) compared with vehicle controls; and 3) docetaxel treatment alone generally yields an intermediate level of inhibition of cell proliferation ($P = .0060$), decreased vascular density ($P = .0027$), and increased apoptosis ($P = .0074$) when compared with the combined treatment group. These differences are statistically significant (Fig. 6B).

DISCUSSION

Honokiol, a component of traditional tea extracts, is known for its low toxicity, favorable oral absorption

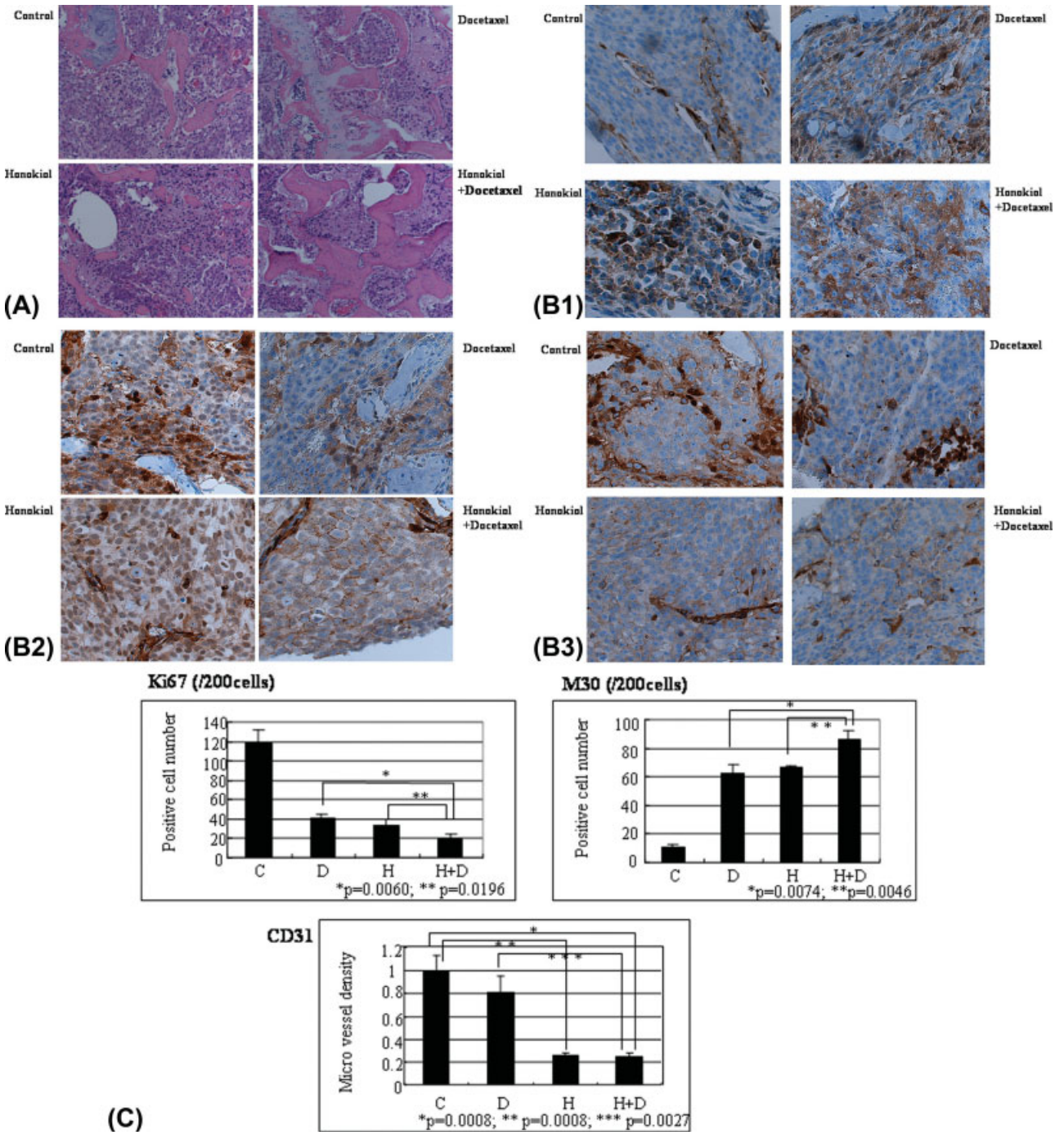

FIGURE 6. Honokiol and/or docetaxel exhibited growth inhibitory, proapoptotic, and antiangiogenic activities against C4-2 tumor xenografts in mice. (A) Honokiol and/or docetaxel inhibited tumor growth and provoked reparative granulation in tumor tissues when subjected to histopathologic analysis. The combined therapy induced more reparative granulation tissues in the bone marrow areas harbored with C4-2 tumors than that of the control or the honokiol- or docetaxel-alone groups (×100). (B) Honokiol and/or docetaxel induced anticell-proliferation, proapoptosis, and antiangiogenesis in vivo as analyzed by immunohistochemical analysis. The combined therapy induced greater anticell-proliferation (Ki-67), proapoptosis (M-30), and antiangiogenesis (CD-31) effects than control, honokiol, or docetaxel treatment alone groups (×200). (C) Detailed comparative quantification of the combined therapy as opposed to controls or individual therapies on the expression of markers indicative of cell proliferation, apoptosis, and angiogenesis are shown ($P < .05$). C indicates control; D, docetaxel treatment; H, honokiol treatment; H + D, combination of honokiol plus docetaxel.

and tissue distribution, and multiple pharmacologic actions including antioxidative, antiangiogenic, and antitumor effects and other less well-defined effects on behavior and the central nervous system.[1–3,23] Because of its reported antiangiogenic and antitumor effects through the induction of apoptosis, we sought to define if honokiol can be used alone or in combination with docetaxel to treat hormone-refractory human prostate cancer. Our results showed that: 1) honokiol has broad growth inhibitory effects on both androgen-dependent and -independent human prostate cancer cells; 2) honokiol also inhibits the growth of cells in the tumor microenvironment commonly associated with tumor metastasis and modulates the growth of localized and disseminated prostate cancers, such as prostate stromal cells, marrow stromal cells, and bone marrow-associated vascular endothelial cells; 3) honokiol induces apoptotic death in androgen-independent and bone metastatic human prostate cancer C4-2 cells; 4) honokiol exerts additive growth inhibitory effects with docetaxel on prostate cancer cells and cells in the cancer microenvironment, both in culture and in immune-compromised mice; and 5) the additive antitumor effects of honokiol and docetaxel were demonstrated in androgen-independent human prostate cancer xenografts in mouse tibia. In this study, we noted the growth inhibitory concentrations (5–10 μg/mL) of honokiol in cell culture, which can be achieved in vivo based on the dose of 100 mg/kg. Our results collectively suggest that honokiol is an effective agent that can be considered for use in combination with docetaxel as a new treatment protocol for hormone-refractory human prostate cancer bone metastasis.

Honokiol, a hydroxylated biphenyl compound, is also known to have antioxidant activity and is capable of scavenging hydroxyl free radicals.[24] Therefore, honokiol can antagonize H_2O_2-induced membrane damage and mitochondrial dysfunction.[22] By effectively antagonizing TNF-α-induced NF-κB activation in various cell types, honokiol inhibited cell survival mediated by Akt activation.[25] By interrupting NF-κB activation, honokiol also blocked NF-κB downstream activation and assembly of reactive oxygen species (ROS). Because NF-κB and ROS activation correlate with the initiation of carcinogenesis, the antioxidative effects of honokiol could justify its use to prevent cancer development.[26]

Exposure of B-cell chronic lymphatic leukemia (B-CLL) cells to honokiol caused apoptosis by up-regulating Bcl-2-associated death protein (Bax) and down-regulating the expression of the key survival protein, myeloid-cell leukemia sequence 1 (Mcl-1). Honokiol overcame interleukin-4-mediated B-CLL cell survival.[3] However, honokiol induced B-CLL apoptotic cell death through up-regulation of Bad and Bcl-X_L, whereas it had no effect on the levels of Bcl-2, Bcl-Xs, Bag-1, Bax, and Bak proteins.[3] In human CH27 squamous lung cancer cells, honokiol activated apoptosis by induction of caspase-3 and cleavage of PARP.[6] Although activation of caspases 3, 7, 8, and 9 was triggered by honokiol, the pan-caspase inhibitor z-VAD-fmk failed to abrogate honokiol-induced apoptosis in human multiple myeloma cells. Honokiol also induced the release of apoptosis-inducing factor (AIF) from the mitochondria of myeloma cells. This suggests that honokiol promotes both caspase-dependent and -independent apoptosis.[21] A number of possible mechanisms have been suggested in the enhancement of TNF-α-induced cell death by honokiol: 1) suppression of NF-κB-regulated antiapoptotic signaling[27]; 2) activation of caspase-8, the triggering caspase in the TNF-α apoptotic pathway[28]; 3) promotion of c-Jun N-terminal kinase signaling (JNK)[29]; 4) accumulation of ROS[30]; 5) inhibition of p38 MAPK activation[31]; and 6) inhibition of protein synthesis,[32] specifically the expression of NF-κB-regulated antiapoptotic proteins.[33] In this study, we showed honokiol-induced apoptosis in androgen-independent prostate cancer cells through activation of caspase 3, 8, and 9, and PARP and implicated the involvement of mitochondrial and death receptor signaling pathways.

The application of honokiol together with chemotherapy has added advantages. Honokiol has been shown to down-regulate the expression of P-glycoprotein, a multiple drug resistance (MDR) gene, at mRNA and protein levels in a human breast cancer MCF-7 cell line. The antiangiogenic properties of honokiol also deserve emphasis when considering the treatment of prostate cancer bone metastasis because, histomorphologically, neovascularization has been shown to support cancer skeletal metastases.[34,35]

In addition, the antiangiogenic activity of honokiol could have general importance in eradicating cancer growth locally. For example, during prostate cancer progression, markers of angiogenesis such as VEGF, platelet-derived growth factor (PDGF), or transforming growth factor (TGF) are more highly expressed in prostate carcinoma than in nonmalignant prostate. One measure of angiogenic activity, microvessel density, is higher in primary prostate cancer tissue than in adjacent benign hyperplastic tissue. When measured in patients with concomitant metastases, microvessel density in primary tumors is higher in patients with metastatic disease compared with those with localized disease without concomi-

tant metastases. Expression of angiogenic markers has also been correlated with high-grade primary tumors.[36] Considering these facts collectively, the next therapeutic approach for hormone-refractory prostate cancer should be antiangiogenesis plus chemotherapy. A clinical trial with docetaxel and bevacizumab, a humanized monoclonal antibody that targets VEGF, has been initiated.[37] As demonstrated in the present study, honokiol, an effective antiangiogenic and proapoptotic drug with minimal systemic toxicity, could be considered as an effective adjuvant therapy for the treatment of both localized and disseminated prostate cancers.

Chen et al.[38] used a pharmacokinetics study with 250 mg/kg honokiol administered via the intraperitoneal route in nude mice and demonstrated 80 mg/kg honokiol as the therapeutic dose for the mice bearing colorectal cells RKO-incubated tumor. They showed that 250 mg/kg honokiol administered via the intraperitoneal route in nude mice yielded a maximal plasma honokiol concentration of approximately 1100 μg/mL at 27.179 ± 6.252 minutes after honokiol administration. The plasma pharmacokinetic curve indicated an absorption half-life of 10.121 ± 2.761 minutes and an elimination half-life of 5.218 ± 0.461 hours, with duration of more than 12 hours where the serum concentrations were found above the therapeutic concentrations of 5 to 10 μg/mL. In addition, Bai et al.[2] demonstrated that 100 mg/kg honokiol administered via the intraperitoneal route in nude mice revealed significant inhibition of tumor growth in vivo without any systemic toxicities. Based on these publications, we selected 100 mg/kg as the appropriate dose for the current study.

Whereas honokiol also inhibited the growth of immortalized normal human marrow stromal cells (HS27A), immortalized human prostate-associated stromal cells from normal/benign lesions (NPF), and transformed human bone marrow endothelial cells (TrHBMEC), with an IC_{50} of 5 and 10 μg/mL, respectively (Fig. 1), when administered to mice honokiol did not cause any systemic toxicity. These results, taken together, suggest that honokiol can be administered safely in mice but with cytotoxic effects on prostate cancer cells and their supporting cells in the cancer microenvironment at a concentration achievable in vivo (see above). This interpretation is supported also by our previous work[2] and our present study, where we observed that mice treated with honokiol increased their body weight like their paired controls, and the histopathology of animal tissues revealed no systemic toxicity. Instead, we observed that honokiol inhibited the growth of prostate tumors in bone and restored in part the normal histomorphology of the bone in mice harboring prostate tumor xenografts in bone, as evidenced by histomorphometric analysis of the bone in mice treated with honokiol either with or without docetaxel (Figs. 5, 6). These findings are supported by previous work on B-CLL where honokiol induced caspase-dependent cell death in all kinds of the B-CLL cells examined and was more toxic to B-CLL cells than normal mononuclear cells, suggesting greater susceptibility of the malignant cells.[3] Because of the exhibited growth inhibitory effects not only on tumor cells but also on supporting cells in the tumor microenvironment, we propose that honokiol is promising for the treatment of localized and metastatic prostate cancer.

Docetaxel binds to tubulin and deranges the equilibrium between microtubule assembly and disassembly during mitosis.[39] In metastatic breast, lung, and ovarian cancers, randomized trials have shown that docetaxel-containing therapies are superior to or as effective as established standard chemotherapeutic regimens and are often associated with an improved safety profile.[13] Clinical trials have also found that weekly docetaxel treatment in patients with metastatic hormone-refractory prostate cancer is associated with improvements in clinical outcome.[40,41] Docetaxel is comparatively active in heavily pretreated patients with paclitaxel-resistant hormone-refractory prostate cancer despite significant toxicity found in a small subject population.[42] Docetaxel is currently considered to be among the most important and effective anticancer drugs. Because of its toxicity, a lower dose of docetaxel, in combination with other effective agents such as honokiol, could be a viable option. In addition to interfering with microtubular functions, docetaxel also induces apoptosis with down-regulation of bcl_{XL} and bcl-2 and up-regulation of $p21^{WAF1}$ and p53.[43,44] For these reasons, we chose to test low-dose docetaxel, 5 mg/kg, in combination with honokiol. We report here that honokiol in combination with docetaxel had significant inhibitory effects on human prostate tumor xenografts grown in mouse skeleton as evaluated by mouse serum PSA, x-ray of mouse bone, and the histomorphometric analysis of the mouse skeleton (Figs. 5, 6). The cytotoxic effects of the combined action of honokiol and docetaxel were confirmed by histopathology and IHC analyses of the specimens harvested at the end of the experiments (Fig. 6).[45]

In conclusion, we have shown that prostate cancer cells that failed to respond to hormone withdrawal responded to honokiol-induced apoptosis. Honokiol also significantly induced death in cells surrounding primary and metastatic prostate cancers, the prostate stromal fibroblasts, marrow stromal

cells, and bone marrow-associated endothelial cells. Honokiol is a promising nontoxic agent that could be used as an adjuvant with low-dose docetaxel for the treatment of hormone-refractory prostate cancer and its distant bone metastases.

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