Honokiol, a Small Molecular Weight Natural Product, Inhibits Angiogenesis *in Vitro* and Tumor Growth *in Vivo**

**Xianhe Bai‡, Francesca Cerimele‡, Masuko Ushio-Fukai§,
Muhammad Waqas§, Paul M. Campbell¶, Baskaran Govindarajan‡, Channing J. Der¶,
Traci Battle‖, David A. Frank‖, Keqiang Ye**, Emma Murad‡, Wolfgang Dubiel‡‡,
Gerald Soff§§, and Jack L. Arbiser‡¶¶**

*From the Departments of ‡Dermatology, §Cardiology, and **Pathology, Emory University School of Medicine, Atlanta,
Georgia 30322, ¶Lineberger Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599,
‖Department of Adult Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115,
‡‡Department of Surgery and Division of Molecular Biology, Medical Faculty Charité, Humboldt University,
Berlin, Germany, and §§Northwestern University School of Medicine, Chicago, Illinois 60208*

Natural products comprise a major source of small molecular weight angiogenesis inhibitors. We have used the transformed endothelial cell line SVR as an effective screen of natural product extracts to isolate anti-angiogenesis and anti-tumor compounds. Aqueous extracts of Magnolia grandiflora exhibit potent activity in our SVR proliferation assays. We found that the small molecular weight compound honokiol is the active principle of magnolia extract. Honokiol exhibited potent anti-proliferative activity against SVR cells *in vitro*. In addition, honokiol demonstrated preferential inhibition of primary human endothelial cells compared with fibroblasts and this inhibition was antagonized by antibodies against TNFα-related apoptosis-inducing ligand. *In vivo*, honokiol was highly effective against angiosarcoma in nude mice. Our preclinical data suggests that honokiol is a systemically available and non-toxic inhibitor of angiogenesis and should be further evaluated as a potential chemotherapeutic agent.

Angiogenesis inhibitors have been derived from a number of sources, including cleaved proteins, monoclonal antibodies, and natural products. Natural products contain a variety of chemopreventive compounds that have been shown to prevent the development of malignancies (1, 2). We and others have discovered that some of these chemopreventive agents have anti-angiogenic activities, which may account in part for their chemopreventive effects. These compounds include curcumin from Curcuma longa, epicatechin gallate from tea, genistein from soybeans, and resveratrol from grapes and red wine (3–6).

These compounds exert anti-angiogenic and chemopreventive properties through a variety of mechanisms. Curcumin inhibits angiogenesis by both direct effects on endothelium as well as by inhibiting the COP9 signalosome-associated kinase activity, which regulates the degradation of the c-Jun oncogene with consequent downstream effects on the synthesis of the potent angiogenic factor, vascular endothelial growth factor (VEGF)[1] (7, 8). Epicatechin gallate works in part through inhibiting the activity of the 26 S proteasome, which may also regulate the synthesis of VEGF (4–9). Genistein and resveratrol are broad spectrum protein kinase inhibitors that inhibit tumor promotion (1, 2, 10, 11). However, few of these compounds actually exhibit activity against established tumors *in vivo*.

We have developed a simple bioassay amenable to large-scale screening and fractionation of natural products, namely inhibition of proliferation of the transformed endothelial cell line SVR (12). Using this bioassay on extracts of the seed cone of Magnolia grandiflora, we have shown that one of the active components of this extract is the small molecule honokiol. We demonstrate that honokiol inhibits angiogenesis by interfering with phosphorylation of VEGFR2 in human endothelial cells. In addition, honokiol inhibits the growth of transformed epithelial cells *in vitro*, thus demonstrating that it has both anti-angiogenic and anti-tumor activity. Honokiol is well tolerated and effective against sarcomas in mice, making it an attractive candidate for clinical trials.

EXPERIMENTAL PROCEDURES

Extraction of Magnolia Grandiflora Seed Cones—Magnolia grandiflora seed cones were collected and ground. The powdered magnolia cones (100 g) were extracted with 500 ml of boiling water for 30 min and then allowed to cool to room temperature. The crude aqueous extract was clarified using a 0.45-microfilter followed by ultrafiltration with 3000 nominal molecular weight limits. The ultrafiltrate was lyophilized and then reconstituted in distilled water to give a final concentration of 500 mg/ml. The material was then fractionated by high pressure liquid chromatography, and fractions were lyophilized and reconstituted as 10 mg/ml solutions. These fractions were tested on proliferation assays on SVR cells as described below. Honokiol and magnolol were obtained from Wako Chemical Company (Tokyo, Japan), and unsubstituted biphenyls were obtained from Aldrich.

In Vitro Proliferation Assays—10,000 SVR cells were plated in 24-well dishes. The next day, the medium was replaced with fresh medium containing the inhibitors or vehicle controls. Cells were incubated at 37 °C for 72 h (12, 13), and cell number was determined in triplicate using a Coulter Counter (Hialeah, FL). Immortalized and K-Ras trans-

¶¶ To whom correspondence should be addressed: Dept. of Dermatology, Emory University School of Medicine, WMB 5309, 1639 Pierce Dr., Atlanta, GA 30322. Tel.: 404-727-5063; Fax: 404-727-0923; E-mail: jarbise@emory.edu.

[1] The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; ANOVA, analysis of variance; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; KDR, VEGFR2.

formed rat epithelial cells (RIEpZip and RIEpZipK-Ras12V) and fibroblasts (NIH3T3 pZip and NIH3T3 pZipK-Ras12V) were maintained at 37 °C, 10% CO_2, in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (RIE) or 10% calf serum (NIH3T3) (14, 15). Cells were plated at 10^5/well in six-well plates. Vector and Ras-transformed NIH3T3 and RIE cells were treated with either vehicle (20 μl of Me_2SO) or increasing concentrations (5, 10, 20, and 40 μg/ml) of honokiol (from a 2 mg/ml Me_2SO stock) and observed for morphology changes after 24 h.

Apoptosis Assays—SVR cells were plated at 125,000 cells/100-mm plate in 5% fetal bovine serum/Dulbecco's modified Eagle's medium. After 24 h, cells were treated with 10 μg/ml magnolol or honokiol or left untreated as control. At 18 h and 48 h of treatment, two plates per condition were analyzed. Adherent cells were washed with PBS, and the cells were suspended with trypsin/EDTA treatment. Floating cells were also collected by centrifugation of the conditioned medium, and the total cell population was analyzed. Cell surface annexin V was measured by flow cytometry using the ApoAlert annexin V kit (Clontech, Palo Alto, CA) as described by the manufacturer. The cells were washed in 1× Binding Buffer by centrifugation and then resuspended in 200 μl of 1× Binding Buffer containing annexin V (0.1 μg/ml) and propidium iodide (0.5 μg/ml). After incubation at room temperature for 15 min., the cells were analyzed by flow cytometry for the presence of annexin V and propidium iodide.

Analysis of PI 3-Kinase and MAPK Signaling—SVR angiosarcoma cells were cultured in low glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For experimental cultures, honokiol was added from a 10 mg/ml stock solution made in Me_2SO and used at final concentrations of 20–45 μg/ml as indicated. Cells were incubated with honokiol for 1 h at 37 °C prior to harvesting cells for Western blot analysis. The PI 3-kinase inhibitor, LY294002, and the MAPK kinase (MKK1) inhibitor, U0126 (Cell Signaling Laboratories, Beverly, MA) were used at final concentrations of 50 μM. Whole cell extracts were prepared by lysing cells in buffer containing 50 mM Tris-Cl, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml pepstatin. Protein concentrations were determined by the Bradford assay (Bio-Rad). Equal amounts of protein (80 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Blots were incubated with an antibody specific for the phosphorylated form of Akt (Ser-473), p44/42 MAPK (Thr-202/Tyr-204), or Src (Tyr-416) (Cell Signaling Laboratories, Beverly, MA) using a 1:5,000 dilution of the antibodies. Blots were stripped and reprobed with antibodies that recognizes unphosphorylated and phosphorylated Akt, p44/42 MAPK, or v-Src (Oncogene Research Products, San Diego, CA) using a 1:5,000 dilution (Akt and p44/42 MAPK antibodies) or 1:40 dilution (v-Src) of the antibodies. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Calbiochem). Detection was performed using the Renaissance chemiluminescent ECL kit (PerkinElmer Life Sciences) followed by autoradiography.

Lipid Kinase Assays—5 μg each of hemagglutinin-p85 and Myc-p110 were cotransfected into human embryonic kidney 293 cells according to previously published methods (16, 17). After 24 h, cells were treated with 10 μM honokiol or same volume of Me_2SO as control for another 24 h. After removal of the culture medium, cells were washed with 5 ml of ice-cold PBS twice, lysed in 0.5 ml of lysis Buffer A (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A) and was centrifuged for 10 min at 14,000 × g at 4 °C. P110 was immunoprecipitated with anti-Myc antibody from 500 μl of the supernatant. The immunoprecipitate was washed with the following buffers: three times with Buffer B (PBS, 1% Nonidet P-40, and 1 mM dithiothreitol); twice with Buffer C (PBS, 0.5 M LiCl, and 1 mM dithiothreitol); and twice with Buffer D (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, and 1 mM dithiothreitol). After washing, samples were aspirated completely and resuspended in 100 μl of kinase buffer (40 mM Tris-HCl, 150 mM NaCl, 20 mM $MgCl_2$, and 1 mM dithiothreitol). 10 μl of propidium iodide substrate (2 mg/ml in HEPES, pH 7.6, 1 mM EDTA, and 0.1% cholate) was added, and samples were incubated at room temperature for 10 min. Reactions were initiated by adding 30 ml of reaction buffer (70 μM ATP in kinase buffer with 10 μCi of [γ-^{32}P]ATP/reaction) to each sample. After 10-min incubation at room temperature, the mixture solubilized in 8 μl of 37% HCl was added and vortexed for a few seconds. 150 μl of 1:1 $CHCl_3$:CH_3OH was introduced and mixed and then centrifuged for 5 min. The bottom organic layer was removed into a fresh tube and air-dried overnight. The next morning 10 μl of methanol was added to dissolve the lipid, and then it was spotted onto a TLC plate and the lipids were separated by 65:35 (v/v) 2-propanol, 2 M acetic acid. After the TLC plate was dried, it was exposed to a film.

VEGFR2 Phosphorylation Analysis—Human recombinant $VEGF_{165}$ was purchased from R&D Systems (Minneapolis, MN). Anti-vascular endothelial growth factor R2 (KDR) antibody, anti-phosphotyrosine (PY99) antibody, and protein A-G-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). HUVECs were obtained from Emory Skin Diseases Research Center. Cells were grown on plates coated with 0.1% gelatin in EGM-MV BulletKit (Clonetics, San Diego, CA), 10% fetal bovine serum in endothelial basic medium with 12 μg/ml bovine brain extract, 1 μg/ml hydrocortisone, 1 μl/ml GA-1000, and human endothelial growth factor. Experiments were performed using cells between passages 2 and 5. Growth-arrested HUVECs were stimulated with agonists at 37 °C, and cells were lysed with 500 μl of ice-cold lysis buffer, pH 7.4 (in mM: 50 HEPES, 5 EDTA, and 50 NaCl), 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin), and phosphatase inhibitors (in mM: 50 sodium fluoride, 1 sodium orthovanadate, and 10 sodium pyrophosphate). For immunoprecipitation, cell lysates (600 μg) were precipitated with antibody overnight at 4 °C and then incubated with 25 μl of protein A-G-agarose beads for 1.5 h at 4 °C. Cell immunoprecipitates (500 μg) were separated using SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, blocked overnight in PBS containing 6% nonfat dry milk and 0.1% Tween 20, and incubated for 1 h with primary antibodies as described previously (20). After incubation with secondary antibodies, proteins were detected by ECL chemiluminescence. The amount of KDR in each cell extract was assessed by immunoblotting with anti-KDR antibody. Results were expressed as mean ± S.E. Statistical significance was assessed by Student's paired two-tailed t test or analysis of variance on untransformed data followed by comparison of group averages by contrast analysis using the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA). A p value of <0.05 was considered to be statistically significant.

Rac Activation Assay—HUVECs were grown to confluence and made quiescent in 0.5% fetal bovine serum for 12 h before stimulation with VEGF (20 ng/ml). Cells were lysed with ice-cold lysis buffer, pH 7.5, containing 25 mmol/liter HEPES, 150 mmol/liter NaCl, 1% IGEPAL CA-630, 0.25% sodium deoxycholate, 10 mmol/liter $MgCl_2$, 10% glycerol, 25 mmol/liter NaF, 1 mmol/liter EDTA, 1 mmol/liter sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mmol/liter phenylmethylsulfonyl fluoride. Activated (GTP-bound) Rac was affinity-precipitated with p21-activated kinase-1 protein binding domain peptide, which binds only to Rac-GTP and not Rac-GDP. p21-Activated kinase-1 protein binding domain-agarose (7.5 μg/mg cell lysate) was added, and the reaction mixture was gently rocked at 4 °C for 60 min. The agarose beads were collected by pulsing for 5 s in a microcentrifuge at 14,000 × g, and the beads were washed three times with 0.5 ml of lysis buffer. The agarose beads were resuspended in 40 μl of 1× SDS sample buffer and boiled for 5 min. The supernatant was separated by SDS-PAGE on a 12% gel, and the proteins were transferred to nitrocellulose membrane. After blocking for 1 h in PBS containing 5% nonfat dry milk and 0.1% Tween 20, the membrane was incubated with anti-Rac antibody (1:1000 dilution) overnight. After incubation with the secondary antibody, Rac was detected by enhanced chemiluminescence.

Statistical Analysis—Results are expressed as mean ± S.E. Statistical significance was assessed by Student's paired two-tailed t test or analysis of variance on untransformed data, followed by comparison of group averages by contrast analysis, using the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA). A p value of <0.05 was considered to be statistically significant.

TRAIL Inhibition Studies (Endothelial Proliferation, VEGFR2 Phosphorylation, and Rac Activation)—Human dermal microvascular endothelial cells (Emory Skin Disease Research Center) were cultured in 24-well plates with 10,000 cells/well for 24 h. Plates were washed by PBS, and 0.5 ml of fresh microvascular endothelial cell medium (18) with 0, 1, 6, or 9 μg/ml honokiol was added. Cells were incubated for 30 min, and 30 μg of TRAIL antibody/well (Alexis 804–296-C100) or 30 μg of isotype IgG control antibody (sc-2050, Santa Cruz Biotechnology) was added to the control plate according to the method of Clarke *et al.* (19). 30 μl of PBS was used as a vehicle control. The cells were incubated for 48 h, and cells were counted using a Coulter Counter. To determine whether TRAIL blockade could inhibit the effect of honokiol on VEGFR2 phosphorylation and Rac activation, HUVECs were treated with TRAIL antibody or mouse IgG (30 μg/ml) for 15 h before the addition of honokiol (10 μg/ml) for 1 h in 0.5% fetal bovine serum containing cultured medium. Cells were then stimulated with VEGF (20 ng/ml) for 3 min. Cell lysates were assessed for measurement of tyrosine phosphorylation of VEGFR2 or Rac activity as described above.

COP9 Signalosome-associated Kinase Assays—Kinase reaction was carried out in a final volume of 20 μl in the presence of 1 μg of recombinant c-Jun and [γ-^{32}P]ATP and isolated COP9 signalosome from human erythrocytes. The reaction mixture was incubated for 60 min at 37 °C. The complete reaction mixture was then separated by SDS-PAGE. The gel was dried and autoradiographed. Percent activity was determined by densitometry. As negative controls, assays were performed in the absence of compounds, which represent 100% activity (7, 8). Compounds were tested in two concentrations (10 and 50 μM).

Kinase Inhibition Assays—Honokiol and magnolol were tested *in vitro* for inhibitory activity against the following enzymes according to the method of Cohen *et al.* (20): MKK1, MAPK2/ERK2, c-Jun N-terminal kinase/SAPK1c, SAPK2a/p38, SAPK2b/p38b2, SAPK3/p38g, SAPK4/p38d, MAPKAP-K1a, MAPKAP-K2, MSK1, PRAK, protein kinase A, protein kinase Ca, PDK1, protein kinase Ba, SGK, S6K1, GSK3b, ROCK-II, AMPK, CHK1, CK2, Phosphorylase kinase, Lck, CSK, CDK2/cyclin A, CK1, DYRK1a, and PP2a. We acknowledge the assistance of Dr. Philip Cohen of the University of Dundee with kinase assays.

In Vivo Tumorigenesis—SVR (1×10^6) cells were injected into the flank of 6-week-old nude male mice obtained from Charles River Breeding Laboratories. When tumors became visible at approximately 1 week after inoculation, mice received 3 mg/day honokiol or vehicle control suspended in 20% Intralipid (Baxter Healthcare, Deerfield, IL) in a total volume of 0.3 ml intraperitoneally. Tumor volume was measured using the formula (width^2 × length) × 0.52 where width represents the shortest dimension (11). No weight loss or other toxicities were observed in honokiol or control mice.

RESULTS

Fractionation of Magnolia Extracts—Aqueous magnolia extract displayed potent inhibitory effects on SVR cells (data not shown). High pressure liquid chromatography fractions of magnolia extracts corresponded to fractions known to contain magnolol and honokiol (21–23).

Effect of Purified Magnolia Compounds on SVR Proliferation—Given the potential importance of natural products as anti-tumor and anti-angiogenesis agents, honokiol and magnolol were tested for their effects on the survival and proliferation of SVR cells and a steep decline in cell number was seen between 4 and 8 μg/ml honokiol (Fig. 1*A*). A dose-dependent decrease in cell number was seen at higher concentrations of magnolol, but given the higher potency of honokiol in our proliferation assay, we chose to focus on honokiol. Both honokiol and magnolol are substituted hydroxybiphenyls, thus we tested the effect of non-substituted hydroxybiphenyls (Fig. 1*B*). The unsubstituted biphenyls are essentially inactive in the SVR bioassay, suggesting that the substitution is essential for bioactivity.

Effects of Magnolia Compounds on Apoptosis—SVR cells were treated with magnolol and honokiol at 10 μg/ml. As noted above, the cellular growth rates were reduced by both agents. At 18 h of honokiol treatment (10 μg/ml), there was a 2-fold increase in the early apoptotic cells as measured by annexin V-positive, propidium iodide-negative. This further increased to a 7.7-fold increase in early apoptotic cells by 48 h of treatment to 10.8% of total cells (Fig. 2). In contrast, magnolol at comparable concentrations did not induce apoptosis as assessed by annexin V positivity. These data indicate that honokiol exerts much of its suppressive effect on SVR cells by the induction of apoptosis.

Mechanistic Studies of Honokiol—Honokiol was found to exhibit inhibitory activity against the COP9 signalosome-associated kinases of 13 μg/ml comparable to curcumin, an anti-angiogenic compound known to inhibit COP9 signalosome kinase activity (3, 8).

The phosphoinositol 3-kinase and p44/42 MAPK signal transduction pathways are known to be important in cell growth and survival and may play a particularly important role in angiogenesis (24, 25). Both Akt and p44/42 MAPK were
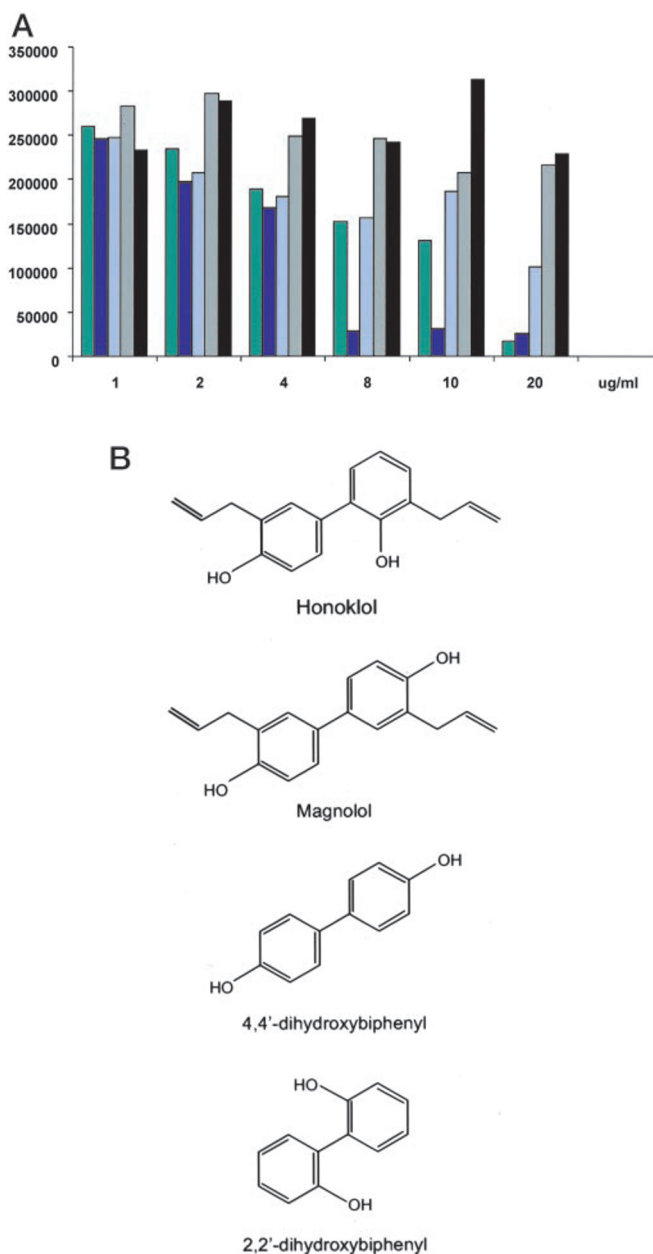

FIG. 1. *A*, inhibition of SVR proliferation by pure honokiol and magnolol and unsubstituted derivatives. SVR cells were treated and counted using a Coulter Counter assay. The *y* axis represents cell number, whereas the *x* axis represents concentrations of test compounds. The *green column* represents magnolol, the *dark blue column* represents honokiol, the *pale blue column* represents 4,4′-dihydroxybiphenyl, the *gray column* represents 2,2′-dihydroxybiphenyl, and the *black column* represents vehicle control (Me$_2$SO). *B*, structure-function relationship of honokiol, magnolol, and parental dihydroxybiphenyls.

constitutively activated in SVR cells. Treatment of these cells with the PI 3-kinase inhibitor LY294002 or the MKK inhibitor U0126 inhibited phosphorylation of Akt and p44/42 MAPK, respectively (Fig. 3*A*).

To determine whether honokiol could modulate these constitutively active signaling pathways involved in cell growth and survival, SVR cells were incubated with increasing amounts of honokiol *in vitro* and analyzed for changes in activated Akt and p44/42 MAPK. These dose response experiments demonstrated that 30 μg/ml (112.5 μM) honokiol inhibited Akt phosphorylation. Although incubation of SVR cells with lower concentrations of honokiol (2.7–13.3 μg/ml; *i.e.* 10–50 μM) for extended times (2–24 h) did not affect p44/42 MAPK phosphorylation,
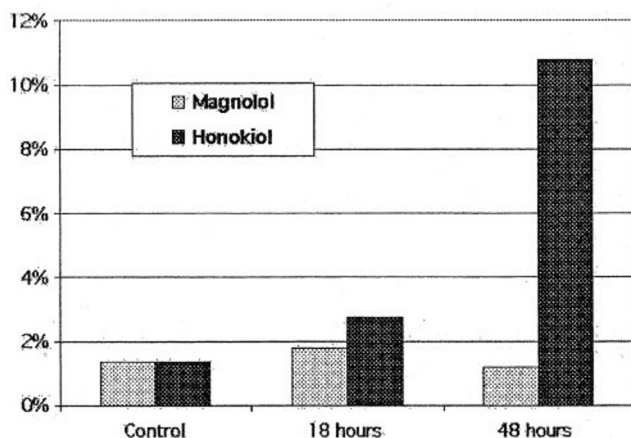
Apoptotic Cells

FIG. 2. **Effect of honokiol and magnolol on apoptosis.** The *light columns* represent SVR cells treated with magnolol, and the *dark columns* represent SVR cells treated with honokiol. The *control lanes* represent cells immediately after treatment compared with 18 and 48 h of treatment.

Akt phosphorylation was inhibited by 2.7 μg/ml within 24 h (Fig. 3*B*).

The oncoprotein Src can activate the phosphoinositol 3-kinase and MAPK pathways. To determine whether inhibition of Akt and MAPK phosphorylation by honokiol was due to upstream inhibition of Src, SVR cells were incubated with honokiol and then examined for changes in Src phosphorylation. Honokiol at high concentrations inhibited phosphorylation of c-Src in SVR cells (Fig. 3*A*). Treatment with lower concentrations of honokiol for extended times did not cause inhibition of Src phosphorylation (Fig. 3, *A* and *B*).

The effect of honokiol treatment on SVR cells suggested a preferential effect on PI 3-kinase signaling compared with MAPK signaling, as phosphorylation of Akt was inhibited by lower doses than that of MAPK. To determine whether honokiol had a direct effect on production of inositol phosphates, we examined the effect of honokiol on synthesis of these phosphates. Honokiol treatment led to ~50% inhibition of levels of phosphorylated inositol (data not shown). To determine whether honokiol directly antagonized Ras, the ability of honokiol to inhibit the growth of immortalized and Ras-transformed epithelial and mesenchymal cells was tested. Although honokiol exhibited dose-dependent inhibition of cell growth, there was no significant difference in inhibition between immortalized and Ras-transformed cells (data not shown). In addition, morphologic reversion that occurs in these cells when Ras is specifically inhibited was not observed as a result of honokiol treatment. These findings suggest that honokiol has activity against both preneoplastic (immortalized) and neoplastic (Ras-transformed) tumor cells but does not specifically inhibit Ras signaling. These findings, along with the preferential activity against PI 3-kinase over MAPK signaling, made us consider TRAIL as a potential intermediary of honokiol activity.

Honokiol Preferentially Inhibits Growth of Primary Human Endothelial Cells over Fibroblasts—For a molecule to be considered an angiogenesis inhibitor, it must have preferential inhibitory activity against endothelial cells *versus* non-endothelial primary cells. To test whether this is the case for honokiol, we tested the ability of honokiol to inhibit the growth of primary fibroblasts and dermal endothelial cells. Honokiol exhibited preferential inhibition of endothelial cells over fibroblasts in a dose-dependent fashion (Fig. 4).

Honokiol-mediated Inhibition of Endothelial Cell Growth Is Mediated by TRAIL—The lack of specific effects of honokiol on
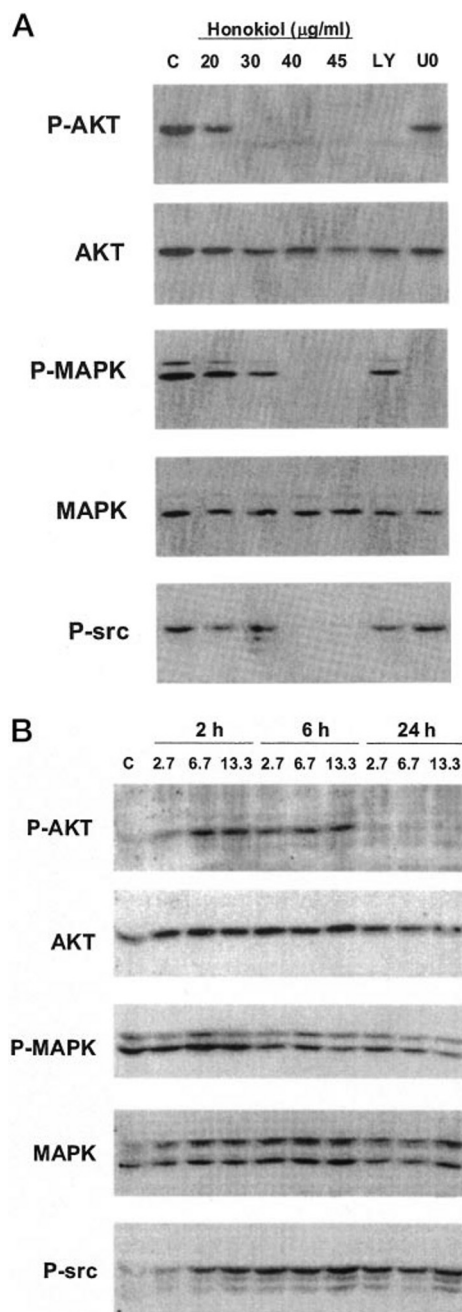

FIG. 3. *A*, honokiol inhibits phosphorylation of AKT, p44/42 MAPK, and Src. SVR cells were incubated with 20 (75 μM), 30 (112.5 μM), 40 (150 μM), or 45 μg/ml (169 μM) honokiol for 1 h. SVR cells were also incubated with 50 μM LY294002 (*LY*) or 50 μM U0126 (*U0*) for 2 h. Cells were lysed and analyzed by Western blotting using antibodies specific for the phosphorylated (P-AKT, P-MAPK, and P-Src) or unphosphorylated forms of AKT and MAPK. *B*, honokiol inhibits phosphorylation of Akt at low concentrations but not p44/42 MAPK or Src. SVR cells were incubated with 2.7 (10 μM), 6.7 (25 μM), or 13.3 μg/ml (50 μM) honokiol for 2, 6, or 24 h. Cells were lysed and analyzed by Western blotting using antibodies specific for the phosphorylated (P-Akt, P-MAPK, and P-Src) or unphosphorylated forms of Akt and MAPK.

Ras antagonism led us to explore alternative mechanisms of honokiol. In addition, the known antagonism that PI 3-kinase shows against TRAIL activity along with the known induction of MAPK activation by TRAIL made TRAIL a candidate for the effect of honokiol on cell growth. We examined the effect of antibodies against TRAIL on the effect of honokiol on primary human endothelial cells (Fig. 5). Treatment with antibodies against TRAIL inhibited the activity of honokiol against endothelium, whereas isotype control antibodies had no effect.
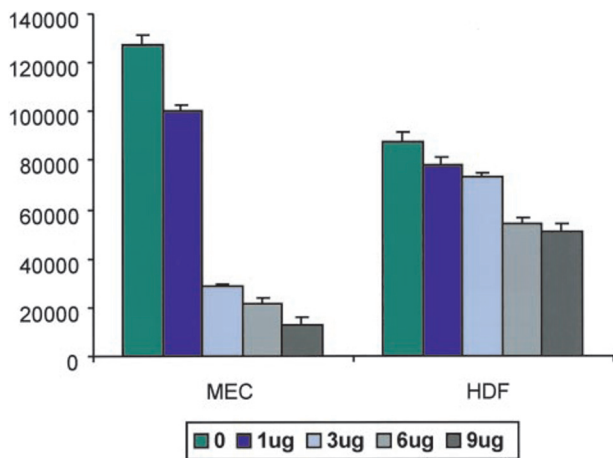
FIG. 4. **Honokiol preferentially inhibits endothelial proliferation compared with fibroblast proliferation.** The *x* axis represents dosage of honokiol, whereas the *y* axis represents cell number. The *color scale* at *bottom* shows the doses of honokiol used in micrograms/milliliter.
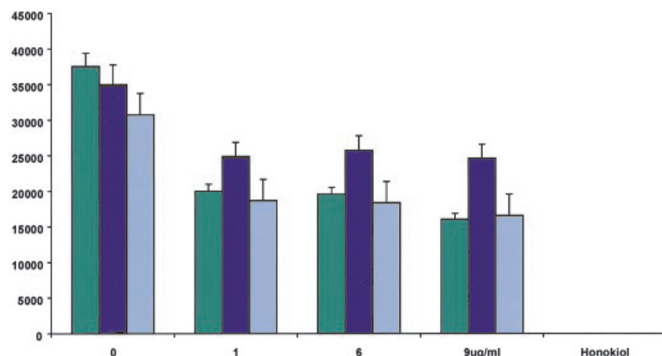

FIG. 5. **Honokiol inhibition of endothelial proliferation is TRAIL-dependent.** 10^4/well microvascular endothelial cells were cultured in 24-well plates for 24 h. The next day, cells were washed by PBS and pretreated with 0.5 ml/well fresh MEC medium with 0, 1, 6, or 9 μg/ml honokiol for 30 min before addition of TRAIL or isotype control antibody (30 μg/well). Cells were incubated for 48 h after the addition of reagents and were counted with a Coulter Counter. The *green bars* represent endothelial cells treated with honokiol alone, the *dark blue bars* represent cells treated with honokiol and TRAIL antibody, and the *light blue bars* represent cells treated with honokiol and isotype control antibody. The differences in honokiol-treated endothelium in the presence or absence of TRAIL antibody are significant ($p < 0.05$).

FIG. 6. *A*, effect of honokiol on VEGF-induced KDR autophosphorylation in HUVECs. HUVECs were preincubated with vehicle or honokiol (5 and 10 μg/ml) for 60 min and then stimulated with 20 ng/ml VEGF for 5 min. Lysates were immunoprecipitated (*IP*) with anti-phosphotyrosine (pTyr) antibody followed by immunoblotting (*IB*) with anti-KDR antibody (*top panel*). *Bottom panel* represents averaged data expressed as fold change over basal (the ratio in untreated cells was set to 1). Values are the means ± S.E. for three independent experiments. *, $p < 0.05$ for increase in phosphorylation by VEGF in the presence of inhibitor *versus* VEGF alone. *B*, effect of honokiol on VEGF-induced Rac activation. HUVECs were preincubated with vehicle or honokiol (10 μg/ml) for 60 min and then stimulated with 20 ng/ml VEGF for 3 min. Rac activity was measured by p21-activated kinase-1-protein binding domain affinity precipitation as described under "Experimental Procedures." *Top*, representative immunoblot of GTP-bound Rac. *Bottom*, densitometric analysis (mean ± S.E.) of immunoblots from three experiments expressed as fold increase over control. *, $p < 0.01$ compared with VEGF alone.

Thus, the activity of honokiol activity is mediated in part by TRAIL.

Honokiol Inhibits VEGF-induced KDR Autophosphorylation in Human Endothelial Cells—The mitogenic and chemotactic effects of VEGF on endothelial cells are mainly mediated through the VEGFR2 tyrosine kinase, KDR. Because we have previously demonstrated that reactive oxygen species are involved in VEGF-induced KDR autophosphorylation in endothelial cells (26), we next examined the effect of honokiol on this response in cultured HUVEC. As shown in Fig. 4, VEGF induces a 3.8-fold increase ($p < 0.05$) in phosphorylation of KDR in HUVECs at the peak of 5 min (Fig. 6*A*) and honokiol significantly inhibited VEGF-induced response in a dose-dependent manner. Treatment of endothelial cells with honokiol in the presence of TRAIL antibodies did not antagonize the effect of honokiol on phosphorylation of VEGFR2 (data not shown).

Honokiol Inhibits VEGF-induced Rac1 Activation in Human Endothelial Cells—We have previously demonstrated that Rac1 activation is required for VEGF-induced production of reactive oxygen species derived from NAD(P)H oxidase and subsequent KDR autophosphorylation in HUVEC (26). Be-
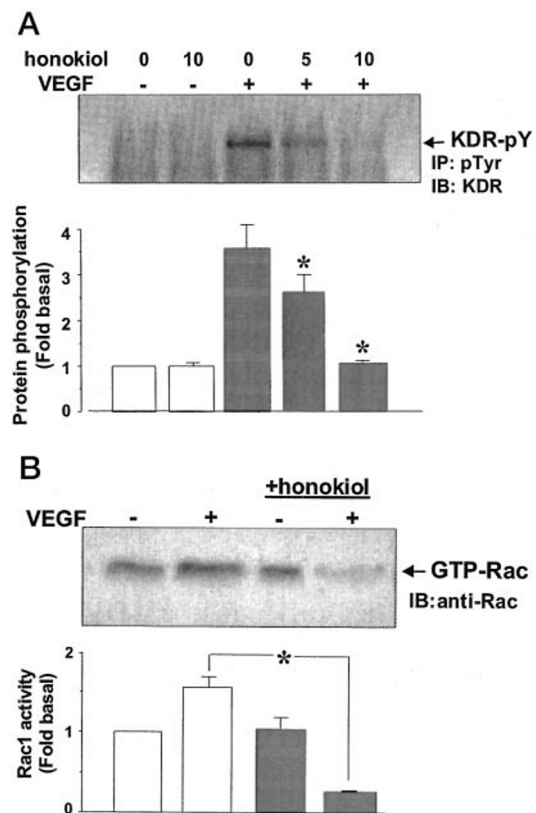
cause honokiol inhibited VEGF-induced KDR autophosphorylation, we next examined whether this effect is mediated through the inhibition of Rac1. As shown in Fig. 6*B*, honokiol (10 μg/ml) that almost completely blocked KDR autophosphorylation dramatically reduced VEGF-stimulated Rac1 activity without affecting its basal levels. These results suggest that honokiol may act as an anti-oxidant mainly through inhibition of Rac1, a critical component of NAD(P)H oxidase, in endothelial cells. Treatment of endothelial cells with honokiol in the presence of TRAIL antibodies did not antagonize the effect of honokiol on activation of Rac1 (data not shown). Prior studies have suggested the induction of reactive oxygen both prior to and following phosphorylation of KDR (26). Both possibilities may be true as increased levels of reactive oxygen have been shown previously to augment phosphorylation of receptors. This may be due in part to oxidative inactivation of protein tyrosine phosphatases, which exhibit an active cysteine residue that can be inactivated by reactive oxygen (27, 28).

Honokiol Exhibits Anti-tumor Activity in Mice—To determine whether honokiol exhibited anti-tumor activity *in vivo*, mice were inoculated with 1×10^6 SVR angiosarcoma cells
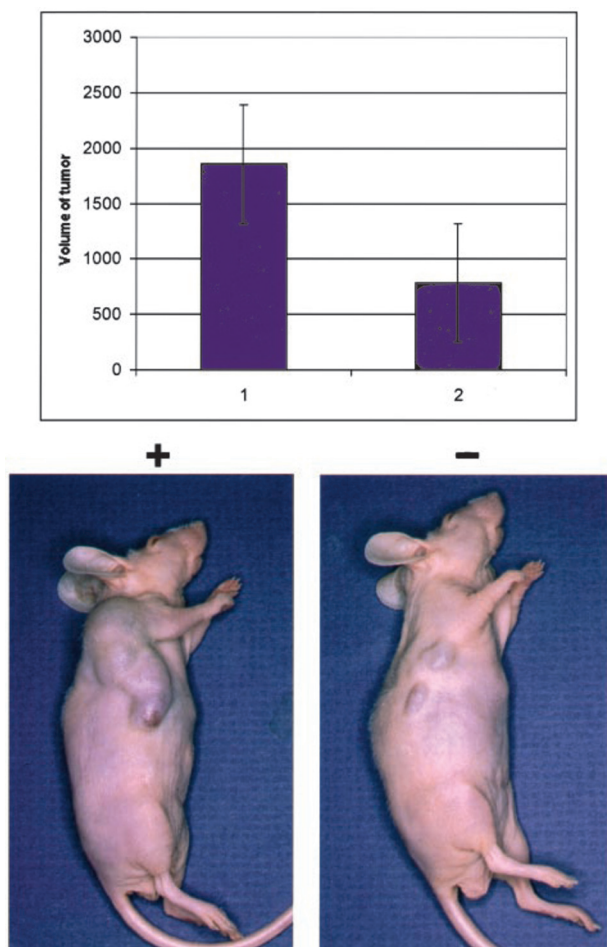
FIG. 7. **Effect of honokiol on *in vivo* growth of SVR angiosarcoma in nude mice.** The *y* axis represents tumor volume. *Column 1* represents vehicle-treated mice ($n = 3$), and *column 2* represents honokiol-treated mice ($n = 3$).

subcutaneously and treated with honokiol or vehicle when tumors became clinically evident. Honokiol treatment led to ~50% inhibition of tumor growth compared with vehicle control ($p < 0.05$) (Fig. 7).

DISCUSSION

We describe the isolation of a systemically active inhibitor of tumor growth through a rapid bioassay of proliferation of SVR-transformed endothelium. We have previously shown that SVR cells accurately predict *in vivo* responses of two known angiogenesis inhibitors, trinitrophenylnucleotide 1470 and 2-methoxyestradiol (12, 29). In addition, curcumin, a natural product not previously known to be anti-angiogenic, was demonstrated to have anti-angiogenic activity on both immortalized and primary endothelial cultures (3, 30).

A number of chemopreventive agents have been isolated and characterized primarily through activities against skin and colon cancer promotion protocols. These include tea polyphenols, curcumin, and caffeic acid phenethyl ester (6, 31–33). Although many of these agents have potent chemopreventive activities, their activity against established tumors is not potent. One possible explanation is that high concentrations of drug are achievable at the skin and colon while sustained systemic concentrations cannot be achieved. This is clearly the case with curcumin in which previous studies have shown that it is poorly absorbed from the gastrointestinal tract and, even when systemically administered, is rapidly cleared by hepatic metabolism (1). We have found that honokiol, unlike curcumin, can inhibit

growth of an established tumor when administered systemically.

Our fractionation process using inhibition of transformed SVR endothelial cell proliferation as a bioassay yielded a fraction that contains magnolol and honokiol, which are substituted biphenols. Magnolol and honokiol are closely related, but honokiol appeared to have enhanced activity in the SVR inhibition assay. Substitution of the hydroxylated biphenyl is required for activity, because the 2,2′- and 4,4′-dihydroxybiphenyl had no effect on proliferation of SVR cells *in vitro*. Consistent with increased activity of honokiol against SVR cells, honokiol is more effective in the induction of apoptosis than magnolol.

To determine the mechanisms of activity of honokiol, we examined its effect on expression and phosphorylation of key signal transduction pathways. Activation of both MAPK and Akt was inhibited, indicating that a mechanism of activity is probably upstream of these pathways and possibly at the level of Src. Inhibition of Akt phosphorylation occurred at an earlier time point and at lower doses than inhibition of MAPK phosphorylation, indicating a preferential inhibition of PI 3-kinase signaling. A potential explanation for the diverging regulation of MAPK and PI 3-kinase resulting from honokiol treatment may stem from the involvement of TRAIL as demonstrated in human endothelial cells. The preferential inhibition of PI 3-kinase signaling over MAPK signaling coupled with preferential activity of honokiol against multiple neoplastic cells and endothelium while having little effect on primary fibroblasts is consistent with TRAIL activation (34–36). Although TRAIL promotes apoptosis, TRAIL also stimulates activation of MAPK (37). The lack of effect on MAPK may result from the combined effect of high constitutive levels of MAPK activation in SVR cells and MAPK up-regulation by TRAIL.

Honokiol demonstrated inhibition of VEGFR2/Flk/KDR autophosphorylation in human endothelial cells. In addition, honokiol treatment resulted in blockade of VEGF-induced Rac activation (38). Because Rac is required for VEGF-induced endothelial migration and proliferation (39, 40), the blockade of VEGFR2 autophosphorylation and subsequent Rac activation indicate potential for honokiol as an anti-angiogenic agent that functions in human cells in addition to anti-tumor activity. TRAIL blockade did not antagonize the effect of honokiol on phosphorylation of VEGFR2 or activation of Rac1. This may be the result of TRAIL affecting signaling downstream of VEGFR2/Rac1 or through an independent pathway.

Given that polyphenols, including curcumin and epicatechin gallate, both target proteasomes, we examined the effect of honokiol on the COP9 signalosome. Honokiol is an effective inhibitor of COP9 signalosome kinase activity.

In conclusion, we demonstrate the utility of the SVR bioassay in isolating an active principle in a natural product and characterize its mechanism of action. The active principle, honokiol, has been previously described as a component of a Japanese herbal medicine "saiboku-to" and of the Chinese medicine "houpo" and has been shown to have anxiolytic properties in mice (21–23, 41, 42). We have demonstrated for the first time that honokiol has potent anti-angiogenic and anti-tumor properties *in vitro* and is systemically active against aggressive angiosarcoma *in vivo*. In addition, honokiol is well tolerated by the host animal in therapeutically beneficial doses, making it an attractive candidate for further preclinical testing as an anti-neoplastic agent.

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