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

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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) (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-µm 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 biphenyl was 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-
formed rat epithelial cells (RIEpZip and RIEpZipK-Ras12V) and fibroblasts (NIH3T3 pZip and NIH3T3 pZip-Ras12V) were maintained at 37 °C, 10% CO₂, in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum (R) or 10% fetal calf serum (NIH3T3) (14, 15). Cells were plated at 10⁵/well in six-well plates. Vector and trans-
formed NIH3T3 and RIE cells were treated with either vehicle (20 μl of Me₂SO) or increasing concentrations (5, 10, 20, and 40 μg/ml) of hono-
kiol (from a 2 mg/ml Me₂SO 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 processed. Adherent cells were washed with 500 μl of PBS, 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 (Clon	
tech, 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 (from a 2 mg/ml Me₂SO stock) and observed for morphology changes after 24 h.

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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-32P]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, 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 × 10⁶) 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 (width² x length) x 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. 1A). 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. 1B). 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 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 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. 3A).

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

The oncoprotein Src can activate the phosphoinositide 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. 3A). Treatment with lower concentrations of honokiol for extended times did not cause inhibition of Src phosphorylation (Fig. 3A 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 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 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.
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/ml). 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).

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 HUVECs. 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. 6A) 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). Because honokiol inhibited VEGF-induced KDR autophosphorylation, we next examined whether this effect is mediated through the inhibition of Rac1. As shown in Fig. 6B, 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.
We have found that honokiol, unlike curcumin, can inhibit angiogenesis inhibitors trinitrophenylnucleotide 1470 and 2-deoxy-D-glucose (39, 40), the blockade of VEGFR2 autophosphorylation and subsequent Rac activation indicate potential for honokiol as an anti-angiogenic agent that functions in human endothelial cells. The preferential inhibition of PI 3-kinase signaling over MAPK signaling resulting from honokiol treatment implies that polyphenols, including curcumin and epicatechin gallate, both target proteasomes, we examined its effect on expression and phosphorylation of key signal transduction pathways. Activation of both 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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