Potent inhibition of Lewis lung cancer growth by heyneanol A from the roots of *Vitis amurensis* through apoptotic and anti-angiogenic activities

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**Vitis amurensis** Rupr. (Vitaceae) has long been used in Chinese/Oriental herbal medicine for the treatment of cancer, but its active compounds and mechanisms of action have not been well studied. To this end, we isolated from its root heyneanol A (HA), which is a tetramer of resveratrol (RES), and established the *in vivo* antitumor activity of HA using the mouse Lewis lung carcinoma (LLC) model. We administered HA and RES by daily intraperitoneal injection to C57BL/6 mice that were subcutaneously inoculated with LLC cells. HA dose-dependently decreased tumor growth without any adverse effect on body weight and seemed more potent than RES. The tumor inhibitory effects were accompanied by a marked increase in tumor cell apoptosis detected by cleaved caspase-3 and TUNEL assays and decreased tumor cell proliferation index and tumor microvessel density, supporting the involvement of apoptotic and anti-angiogenic activities in the anticancer effects. We next investigated the cellular and molecular processes that mediate the apoptosis and anti-angiogenesis effects using cell culture models. Mechanistically, treatment of LLC cells *in vitro* with HA or RES significantly increased apoptotic cells. Both HA- and RES-induced cleavage of caspase-9 and caspase-3 and PARP were completely blocked by a pan caspase inhibitor, Z-VAD-FMK. In addition, HA and RES suppressed the basic fibroblast growth factor (bFGF)-induced proliferation and capillary differentiation of human umbilical vein endothelial cells, and inhibited the binding of bFGF to its receptor in a test tube assay and the bFGF-induced vascularization of Matrigel plugs *in vivo*. Remarkably, HA was fairly stable in cell culture medium and did not undergo intracellular conversion to RES. Therefore, HA is an active anticancer compound that induces caspase-mediated cancer cell apoptosis and inhibits angiogenesis rivaling the potency of RES and merits further evaluation for cancer chemoprevention.

**Introduction**

Chinese/Oriental medicinal herbs are rich sources of potential novel anticancer agents. Systematic and rigorous investigation of their active ingredients and mechanisms of action are crucial to provide evidence and rationale for their efficacy and for transforming traditional herbal practices into evidence-based medicine. The fruits, vine, roots and leaves of *Vitis amurensis* Rupr. (Vitaceae) (VAR) have long been used for the treatment of cancer in Chinese/Oriental folk medicine (1). Huang et al. (2) isolated heyneanol A (HA) from the roots of VAR and reported that it had anti-inflammatory activity by potently inhibiting the biosynthesis of leukotriene B4. Initially isolated from root of *Vitis heynana* in 1996 (3), HA is structurally a tetramer of resveratrol (RES). Other constituents identified from the VAR root include various RES oligomers such as amurensins, 7a, 8a-cis-ε-viniferin, (+)-hopeaphenol, isohopeaphenol, vitisin A and (+)-vitisfuran A (2,4,5).

RES (trans-3,5,4'-trihydroxystilbene) is a major stilbene compound in grapes. RES has recently attracted much attention as a potential therapeutic agent for several pathological conditions (6–9). It has been reported to act as an antioxidant (10), and to prevent vascular diseases (11) and platelet aggregation (12). RES has also been reported to induce growth inhibition and apoptosis in various human cancer cell lines (9,13–16). A number of investigations have indicated that the antitumor activity of RES is related to apoptosis (17–21). Several novel naturally occurring RES derivatives have been identified (5,22,23). Ito *et al.* (23) have reported that seven RES oligomers from *Vatica rassak* show *in vitro* cytotoxicity against human cancer cell lines. Although the effects of RES in the various fields have been well established, little is known of the effects of RES oligomers, especially HA, in terms of the anticancer efficacy and mechanisms of action. We have isolated HA from the roots of VAR by a cytotoxicity-based fractionation approach (Figure 1A) and have demonstrated that HA exerts apoptotic effects on leukemic U937 cells in cell culture (24). Our study plus data reviewed above suggest that HA may be a broad spectrum anticancer agent.

In the current work, we sought to establish the *in vivo* antitumor activity of HA using the mouse Lewis lung carcinoma (LLC) model and provide evidence of *in vivo* involvement of apoptotic and anti-angiogenic activities in the inhibition of tumor growth. We chose the LLC model because of its rapid growth in syngeneic mice and its strong dependence on angiogenesis. We have used this model to study the *in vivo* effects of novel anti-angiogenic and anticancer agents from Oriental herbs (25). Besides, lung cancer is the number one cause of cancer mortality in the United States and Western countries, and the efficacy of current cancer therapeutic drugs is very dismal; therefore, novel agents targeting multiple pathways such as angiogenesis and apoptosis may hold the key to the effective reduction of lung cancer risk through chemoprevention.
In addition, we used cell culture models to delineate the cellular and molecular processes and mechanisms that account for the apoptosis and anti-angiogenesis effects. Throughout the work, we used RES as a reference compound for comparison. Our data support HA as an active anticancer compound of VAR root that induces caspase-mediated cancer cell apoptosis and inhibits tumor angiogenesis.

Materials and methods

Isolation of HA and identification of its structure

The V.amurensis roots were purchased from Koryo Company of Traditional Crude Drugs (Seoul, Korea) and kindly authenticated by Nam-In Baek, Professor of Department of Oriental Herbal Materials, Kyung Hee University. HA was isolated from V.amurensis roots as described previously (24). In brief, the dried roots were extracted three times with 95% methanol (Figure 1A).
Heyneanol A inhibits tumor by apoptosis and anti-angiogenesis

The resultant extract was fractionated by a series of solvents including hexane, methylene chloride, ethyl acetate and butanol. The ethyl acetate fraction, which was most effective in cytotoxicity screening, was subjected to silica gel column chromatography eluted by chloroform and methanol (5 : 1–8 : 1) and was analyzed by various analytical and activity assays. The effective fraction was purified by HPLC (J’sphere ODS-HP80, 250 × 20 mm I.D., S-4um, 80A, methanol : H2O = 4 : 6–6 : 4). Effective compound was identified as HA by NMR and FAB–MS analysis (Figure 5).

Chemical reagents
Dulbecco’s modified eagle medium (DMEM), M199 medium and antibiotic-antimycotics were purchased from Gibco (Grand Island, NY). Basic fibroblast growth factor (bFGF) was from R & D (Minneapolis, MN). Fetal bovine serum (FBS) was from JRH (Lenexa, KS). Heparin, gelatin, BSA, RES, sodium bicarbonate, 2.3-bis-[methoxy-4-nito-sulfo]-2H-tetrazolium-5-carboxanilide (XTT), PMS, propidium iodide (PI) and RNase A are from Sigma Chemical (St. Louis, MO). Dead End® fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit was from Promega (Madison, WI). Protease inhibitor cocktail was from Boehringer Mannheim (Indianapolis, IN) and DC protein assay kit II was from Bio-Rad ( Hercules, CA). Rabbit anti-human/mouse PARP (#9542), rabbit anti-human/mouse cleaved caspase-3 (#9661) and rabbit anti-mouse caspase-9 (#9504) were from Cell Signaling Technology (Beverly, MA). Mouse anti-human/mouse β-actin (A5316) was from Sigma. Goat anti-mouse IgG HRP conjugated secondary antibody and goat anti-rabbit IgG HRP conjugated secondary antibody were from Zymed (San Francisco, CA). 4× NuPAGE LDS sample buffer, 4–12% NuPAGE Bis–Tris gels and NuPAGE MES SDS running buffer were from Invitrogen (Carlsbad, CA). Hybond ECL transfer membrane and ECL western blotting detection kit were from Amersham Pharmacia (Arlington Heights, IL). X-ray films were from Agfa-Gevaert (CP-BU, Belgium). Z-VAD-FMK (Z-Val-Ala-Asp(Ome)CH2F) was from Enzyme Systems Products (Livermore, CA). Annexin V–FITC apoptosis detection kit I was from BD Biosciences (San Diego, CA). Polyester membranes (12 μm pores) were purchased from Nuero Probe (Cabin John, MD). The resultant extract was fractionated by a series of solvents including hexane, methylene chloride, ethyl acetate and butanol. The ethyl acetate fraction, which was most effective in cytotoxicity screening, was subjected to silica gel column chromatography eluted by chloroform and methanol (5 : 1–8 : 1) and was analyzed by various analytical and activity assays. The effective fraction was purified by HPLC (J’sphere ODS-HP80, 250 × 20 mm I.D., S-4um, 80A, methanol : H2O = 4 : 6–6 : 4). Effective compound was identified as HA by NMR and FAB–MS analysis (Figure 5).

Cell culture
Mouse LLC cells were kindly provided by Dr I. Saiki (Toyama Medical and Pharmaceutical Univ., Toyama, Japan) and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml antibiotic-antimycotics and 2.2 g/l sodium bicarbonate. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord veins according to a published protocol (26) and cultured in M199 supplemented with 20% heat-inactivated FBS, 3 mg/ml bFGF, 5 U/ml heparin and 100 U/ml antibiotic-antimycotics in 0.1% gelatin-coated flasks. HUVECs were used in passages three to six. All other procedures were performed as described previously (31). Cells treated with HA or RES were harvested, washed twice with cold PBS and fixed in 75% ethanol at −20°C. After washing twice with cold PBS, dispersed cells were resuspended in 100 μl of PBS containing 10 μl of RNase A (10 mg/ml) and incubated for 1 h at 37°C. The cells were stained by adding 400 μl of PI (50 μg/ml) for 30 min at RT in the dark. After filtering with nylon mesh (40 μm pore), the DNA contents of stained cells were analyzed using CellQuest Software with a FACScan Vantage SE flow cytometry system (Becton Dickinson, Heidelberg, Germany).

Cell cycle analysis
To identify apoptosis, cells were stained with PI according to a published protocol (31). Cells treated with HA or RES were harvested, washed twice with cold PBS and fixed in 75% ethanol at −20°C. After washing twice with cold PBS, dispersed cells were resuspended in 100 μl of PBS containing 10 μl of RNase A (10 mg/ml) and incubated for 1 h at 37°C. The cells were stained by adding 400 μl of PI (50 μg/ml) for 30 min at RT in the dark. After filtering with nylon mesh (40 μm pore), the DNA contents of stained cells were analyzed using CellQuest Software with a FACScan Vantage SE flow cytometry system (Becton Dickinson, Heidelberg, Germany).

Western blotting
Cells treated with HA or RES were harvested and washed with cold PBS. Cell pellets were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, protease inhibitor cocktail) for 20 min on ice. Lysates were centrifuged at 14 000 × g for 20 min at 4°C. The supernatants containing 20 μg of protein were mixed with same volume of sample buffer and boiled for 5 min. The proteins were separated on 4–12% NuPAGE Bis–Tris gels and then electrotransferred onto a Hybond ECL transfer membrane at 300 mA for 90 min. The blocked membranes were then immunoblotted with specific antibodies and the proteins were visualized using enhanced chemiluminescence. For normalization of loading, the same membrane was soaked in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 150 mM NaCl, 2% SDS, 100 mM β-mercaptoethanol) at 65°C for 30 min and hybridized with β-actin antibody.

HUVECs’ proliferation assay
HUVECs (5 × 104) were seeded onto 0.1% gelatin-coated 96-well microplates and incubated in a humidified incubator for 24 h. Cells were starved for 6 h in M199 containing 5% heat-inactivated FBS and then treated with various concentrations of HA or RES in M199 containing 5% heat-inactivated FBS, 5 μg/ml bFGF and 5 U/ml heparin. After 48 h incubation, XTT working solution was added and then the optical density was measured using microplate reader (Molecular Devices) at 450 nm.

HUVECs’ tube formation assay
In vitro differentiation assay
HUVECs on Matrigel into capillary-like tubes was performed as described previously (32). HUVECs (3 × 104) were seeded onto Matrigel-coated 24-well plates, and HA or RES were added in M199 with 1% FBS, 5 μg/ml bFGF and 5 U/ml heparin. After 7 h, cells were fixed with 4% paraformaldehyde, and randomly chosen fields were photographed under an Axiovert S 100 light microscope (Carl Zeiss, USA) at ×400 magnification. The results were expressed as a percentage of positive cells for each biomarker by the following equation (%): (number of positive cells/total number of cells) × 100.

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**Results**

HA is more active than RES in suppressing growth of LLC cells

To compare the direct growth-suppressing effect of HA on LLC cells with RES, we treated exponentially growing cells with various concentrations of each agent for 6, 12, 24 and 48 h and estimated the number of viable cells by XTT assay. HA significantly decreased viable cell number in a time- and concentration-dependent manner as compared with 0.12% bFGF (300 ng/mouse) and heparin (5 IU). After 7 days, mice were killed, and the Matrigel plugs were removed and photographed. To quantify the formation of functional blood vessels, the amount of hemoglobin (Hb) was measured using the Drabkin reagent kit 525.

Matrigel plug assay

Matrigel plug assay was performed as described by Drabkin et al. (33). Briefly, 6-week-old C57BL/6 mice were each given an s.c. injection of 0.5 ml of PBS per well was immobilized in 96-well plates. The wells were washed and blocked with 3% BSA in PBS for 2 h. HA or RES and bFGF receptor fragment FGFRI(Fc) were added to each well. After 2 h incubation, the wells were washed and the bound FGF R1(Fc) was incubated with anti-human IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) detected by chemiluminescent substrate using a luminometer (Wallac 1420, Perkin Elmer, Turku, Finland). All experiments were carried out at RT.

**Statistical analysis**

All data are presented as means ± standard deviation (SD) or SE. The statistically significant differences between control and sample groups were calculated by the Student’s t-test.

HA and RES inhibit tumor growth in LLC-bearing mice

To compare the antitumor efficacy of HA and RES in vivo, we inoculated C57BL/6 mice with an s.c. injection of LLC cells, and starting 4 days after tumor inoculation we administered HA or RES by daily i.p. injection (Figure 2A). Tumor growth was monitored every other day for 3 weeks. There was no significant difference of body weight between vehicle control group and HA- or RES-treated groups: the final body weight was 20.0 ± 0.5, 20.2 ± 0.9, 20 ± 0.8 and 19.9 ± 0.7 g for control mice and for HA at doses of 5 and 20 mg/kg and RES at 20 mg/kg, respectively. Side effects such as lethargy and mortality were not observed in response to HA or RES treatment. The tumor volumes in HA-treated groups were decreased in a dose-dependent manner (Figure 2B). At necropsy, the tumor weight was decreased to 67.5 and 37.5% of control mice by 5 and 20 mg/kg of HA and to 46.3% by 20 mg/kg of RES (Figure 2C).

HA and RES induce apoptosis and caspase 3 activation in vivo

To verify that the antitumor activity of HA was associated with tumor cell apoptosis in vivo, we stained tumor sections with...
TUNEL assay (Figure 3A). Quantification of TUNEL positive staining showed that 15.2, 19.6 and 16.8% of apoptotic cells were induced by HA (5 and 20 mg/kg) and RES (20 mg/kg) compared with 8.7% of apoptotic cells in vehicle control mice, respectively (Figure 3E). We detected increased cleaved caspase 3 on tumor sections, confirming its involvement in in vivo apoptosis (Figure 3B). Compared with the control group, there were a 2-fold, a 2.7-fold and a 2.2-fold increase of caspase 3 positive cells in the HA- (5 and 20 mg/kg) or RES-treated group, respectively (Figure 3F). These results indicated that caspase activation was involved in HA- and RES-induced apoptosis in vivo.

**HA and RES decrease PCNA index in vivo**

To assess the anti-proliferative effects of HA in vivo, we immunostained tumor sections for PCNA, a well-accepted endogenous cell proliferation marker (Figure 3C). The PCNA staining was decreased by 15.8 and 35.3% of control group in the HA-treated groups at doses of 5 and 20 mg/kg, respectively (Figure 3G). RES treatment at 20 mg/kg decreased the PCNA index to a comparable extent as did the same dose of HA.

**HA and RES inhibit tumor angiogenesis in vivo**

Angiogenesis is obligatory for solid tumor growth and progression (35,36). We, therefore, looked for in vivo evidence of anti-angiogenic effects of HA and RES treatment by immunostaining of tumor sections for an endothelial cell marker, Factor VIII. Numerous Factor VIII-positive cells were observed in control tumors, but fewer were observed in HA- and RES-treated tumors (Figure 3D). The number of microvessels in HA- and RES-treated tumors was significantly decreased by
2- to 2.4-fold compared with control group (Figure 3H). These results indicated that HA and RES inhibited tumor angiogenesis in vivo.

**HA and RES induce apoptosis in LLC cells in vitro**

To delineate the pathways and mechanisms through which HA inhibits tumor cell growth and induces apoptosis, we performed a series of experiments in cell culture with LLC cells. Flow cytometric analysis showed that HA or RES significantly increased apoptotic sub-G1 cells in a concentration- (Figure 4A) and time-dependent manner (Figure 4B). For example, after treatment with various concentrations of HA (6, 9 and 12 μM) for 12 h, apoptotic cells were increased by 7.4-, 24.1- and 26.7-fold compared with control (Figure 4A). Temporally, HA exposure within the first 6 h did not increase sub-G1 cell number, but at 12 and 24 h, this parameter was significantly increased by 7.4- and 15.8-fold (Figure 4B). HA was ~10-fold more potent than RES for apoptosis induction (Figure 4A). The kinetics of death onset was the same for both agents (Figure 4B).

In addition to apoptosis, HA affected cell cycle distribution in dissimilar patterns in comparison with RES. For example, in dose–response experiments, HA exposure that increased apoptosis also decreased G1 population (Figure 4A, left panel), whereas RES did not result in such a change (Figure 4A, right panel). Temporally, RES induced a rapid enrichment of G1 population and corresponding depletion of both S and G2/M cells (Figure 4B, right panel), but HA only affected S-phase cells by 24 h.

**HA and RES activate caspases 9 and 3 and PARP cleavage in LLC cells**

Because caspase activation plays a central role in the induction of apoptosis (37,38), and because we detected caspase-3 activation in HA- and RES-treated tumors (Figure 3B and F), we further investigated the role of caspases in HA-induced apoptosis in LLC cells in cell culture model. Western blot analyses detected a concentration- (Figure 4C) and time-dependent (Figure 4D) increase of the cleavage of pro-caspase 9 from its full-length form (49 kDa) into the active form (39 kDa) in HA- and RES-treated cells. Procaspase 3 and its classic substrate PARP were cleaved in similar concentration-dependent fashions as was caspase-9 (Figure 4C and D), and temporally these events appeared to be slightly behind caspase-9 peak cleavage in HA- and RES-treated cells (Figure 4D), suggesting a hierarchical activation cascade from caspase-9 to caspase-3, and then to PARP. HA was ~8.3-fold more potent than RES for eliciting caspase activation and PARP cleavage (i.e. 6 μM HA ≈ 50 μM RES) (Figure 4C and D).

**HA- and RES-induced apoptosis is caspase-dependent**

To confirm that apoptosis induced by HA or RES was dependent on caspases, LLC cells were pre-incubated with 100 μM of Z-VAD-FMK, a pan caspase inhibitor, for 1 h before being treated with 6 μM HA or 50 μM RES for 12 h. The HA- and RES-induced cleavage of caspase-9, caspase-3 and PARP were significantly inhibited by Z-VAD-FMK (Figure 4E). Inhibition of caspases blocked HA- and RES-induced apoptosis (Figure 4F).

**HA and RES inhibit bFGF-induced proliferation of HUVECs**

Because angiogenesis is required for tumor growth and progression (35,36), and because of the observed anti-angiogenic effect in HA- and RES-treated tumors (Figure 3D and H), we performed the next series of in vitro experiments using HUVECs to identify the specific processes that were targeted by HA or RES to inhibit angiogenesis. Mitogen-stimulated endothelial proliferation is a key angiogenic response (39). In terms of angiogenesis activators, bFGF has potent angiogenesis stimulatory activities and is widely used in both in vitro and in vivo models of angiogenesis (25,40). HA inhibited bFGF-induced proliferation of HUVECs in a concentration-dependent manner above an exposure concentration of 10 μM (Figure 5A). Similarly, RES was only growth inhibitory at 50 μM or higher (Figure 5A). In contrast to LLC cells, treatment of HUVECs for 48 h did not induce apoptosis <30 μM HA, or 100 μM RES by flow cytometric analysis (data not shown). These results indicated that HA or RES inhibited mitogen-stimulated endothelial proliferation at high level of exposure without apparent apoptosis.

**HA and RES inhibit bFGF-induced capillary differentiation of HUVECs**

Stimulation of HUVECs seeded on Matrigel by angiogenic factors such as VEGF and bFGF promotes differentiation to form capillary-like tubes (33). We performed the tube-formation assay to assess any specific effect of HA or RES on this process. Treatment with HA (10 and 30 μM) and RES (100 μM) for 7 h resulted in 46, 64 and 50% inhibition of bFGF-induced tube formation, respectively (Figure 5B and C).

**HA and RES interfere with the binding of bFGF to its receptor**

Because binding of bFGF to its receptor is crucial to initiate angiogenesis signaling, we tested the possibility of a direct interference by HA or RES with this process. Various concentrations of HA or RES along with a defined amount of FGF R1α (IIIc)/Fc were added to bFGF-coated wells. After 2 h incubation at RT, HA decreased the binding of bFGF to its receptor up to 58, 62.5 and 87% at 10, 30 and 50 μM, respectively (Figure 6A). RES showed a similar inhibitory pattern; however, the potency was ~1/5 of HA (Figure 6B).

**HA inhibits bFGF-induced angiogenesis in Matrigel plugs**

The molecular and cellular effects observed above with bFGF receptor binding and in HUVECs predict a strong anti-angiogenic activity of HA and RES. This was confirmed in the Matrigel plug assay (Figure 6C). The bFGF-only plugs exhibited dark-red color, indicating abundant red blood cells in the newly formed vasculature, whereas the HA and RES-treated plugs showed light yellowish color (see representative photographs). HA significantly reduced in a dose-dependent manner the Hb content, which is an indicator of functional angiogenesis, compared with bFGF control.

**HA does not readily convert to RES in DMEM and in LLC cells**

Since structurally HA is a tetramer of RES, we were interested in finding out whether the aforementioned molecular and cellular actions of HA required conversion to RES. Figure 7 showed HPLC detection of the HA and RES extracted from DMEM or LLC cells. In the cell culture medium, HA degraded ~1% per hour, but did not convert to RES (Table I). Under the same culture condition, RES was more stable than HA, degrading ~0.5% per hour.

Upon exposure to LLC cells, HA accumulated very rapidly initially, reaching >15% of total dosage after 10 min (Table II). The rate of accumulation gradually slowed, achieving ~27% and ~31% after 2 and 6 h, respectively. There was no
Fig. 4. (A and B) Concentration- (A) and time-dependent (B) induction of LLC cell apoptosis by HA and RES as detected by sub-G1 DNA content. Data are presented as means ± SD, n = 5. The statistically significant differences between control and sample groups were calculated by the Student’s t-test. **P < 0.01, ***P < 0.001. (C and D) Concentration- (C) and time-dependent (D) induction of cleavage of caspase-9, caspase-3 and PARP in LLC cells by HA and RES as detected by western blot. (E and F) Effect of a pan caspase inhibitor Z-VAD-FMK (100 μM) on HA- and RES-induced cleavage of caspase-9 and 3 and PARP (E) and apoptosis detected by sub-G1 DNA content (F). Z-VAD-FMK was pre-treated for 1 h before the initiation of HA or RES treatment for 12 h. Data in F are presented as means ± SD, n = 5. The statistically significant differences between control and sample groups were calculated by the Student’s t-test. **P < 0.01, ***P < 0.001.
intracellular conversion of HA to RES during the exposure period examined. In comparison, RES exposure resulted in cellular retention of 6% after 10 min, which then subsided to 4% after 2 and 6 h.

**Discussion**

Oriental medicinal herbs are rich sources of anticancer agents that deserve more rigorous and systematic scientific investigations (41). Identification of their active anticancer compounds and characterization of their anticancer mechanisms will be an essential and necessary first step to tap these rich sources of new drugs for cancer chemoprevention and treatment. This paper reported our efforts with HA from the VAR roots.

In this study, we established the antitumor effect of HA in the LLC tumor model (Figure 2). HA decreased LLC tumor growth in a dose-dependent manner. We further identified three processes that were associated with the observed anticancer activities: apoptosis effect measured by TUNEL and cleaved caspase-3 (Figure 3A and E, 3B and F); antiproliferative effect as measured by PCNA index (Figure 3C and G); and anti-angiogenic effect as indicated by Factor VIII staining of tumor vascular endothelial cells (Figure 3D and H). On an equal weight basis, HA appeared to be slightly more potent than RES on all these parameters (Figures 2 and 3). These data established the in vivo occurrence of these effects, which led us to perform mechanistic investigations using in vitro cell culture models.

Apoptosis can be induced by two caspase-activation pathways: the extrinsic death receptors cascade or the intrinsic...
mitochondria cascade (37,38,42). In both pathways, caspases are critical components of the apoptotic machinery (37,38,42). As shown in Figure 4, we detected a close association of caspase 9 and 3, and PARP cleavage induced by HA with apoptosis. A mediator effect of caspases was confirmed by

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the inhibition of these biochemical events and apoptosis with the general caspase inhibitor Z-VAD-FMK. These findings support caspase-mediated apoptosis induction by HA in LLC cells both in vitro and in vivo. The temporal course and patterns of caspase cleavage by HA were similar to those of RES, in spite of the >8-fold difference in their minimal molar concentrations required to induce apoptosis. Because each HA contains four molecules of RES, the tetramer would, therefore, still be ~2-fold more effective on an equal weight basis. It should be noted that HA remained fairly stable in the aqueous medium of cell culture (Table I) and it did not convert to RES inside the LLC cells in spite of efficient retention (Table II). These data, therefore, suggest that the apoptogenic activities of HA observed in the LLC cell culture model did not require conversion to its monomeric form. The greater retention of HA, possibly due to greater lipophilicity than RES, might, therefore, in part, account for the greater apoptotic efficacy of HA. In spite of their similarities of caspase-mediated apoptosis execution, a difference between HA and RES on LLC cell cycle further attested to the unique actions of HA without conversion to RES. Currently, we are investigating the pharmacokinetics of HA in animals to shed more light on this issue in vivo.

In addition to the apoptotic effect of HA on tumor cells, we demonstrated its in vitro anti-angiogenic effect as shown by decreased microvessel density in the HA-treated tumors (Figure 3D and H) and delineated likely target cellular processes for the anti-angiogenic action in cell culture model (Figures 5 and 6). It is noteworthy that HA inhibited the bFGF-induced HUVEC proliferation (Figure 5A) and capillary tube formation on Matrigel (Figure 5B and C). HA and RES also directly interfered with the binding of bFGF to its receptor in a test tube binding assay (Figure 6A), revealing one possible direct inactivation of angiogenic signaling without the need of cellular metabolism of HA. Taken together, the data support the notion that HA and RES affect tumor angiogenesis by targeting multiple molecular and cellular processes.

In summary, we have shown that HA is an active antitumor compound from V. amurensis through, at least in parts, a strong induction of tumor cell apoptosis, an inhibition of tumor cell proliferation and anti-angiogenic effects. The results indicate that HA has comparable or better anticancer efficacy than RES in the LLC-bearing mice. Although these two agents have similar actions on a number of cellular and molecular processes involved in apoptosis and angiogenesis, HA can exert its actions without first converting to RES. The greater retention of HA by tumor cells compared with RES may make HA more desirable as an anticancer agent. On the basis of the results from our study, HA merits further investigation in appropriate primary carcinogenesis models for lung cancer chemoprevention.

### Table II. Cellular retention of HA and RES in LLC cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Resveratrol (50 μM)</th>
<th>Heyneanol A (6 μM)</th>
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<tbody>
<tr>
<td>Cellular retention (%)</td>
<td>10 min</td>
<td>2 h</td>
</tr>
<tr>
<td>Resveratrol</td>
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<td>4.0</td>
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<tr>
<td>Heyneanol A</td>
<td>ND</td>
<td>ND</td>
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Cellular retention was calculated as % of total dosage. ND—not detectable.

### References


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**Heyneanol A inhibits tumor by apoptosis and anti-angiogenesis**