Thymoquinone inhibits tumor angiogenesis and tumor growth through suppressing AKT and extracellular signal-regulated kinase signaling pathways

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Abstract

Thymoquinone, a component derived from the medial plant *Nigella sativa*, has been used for medical purposes for more than 2,000 years. Recent studies reported that thymoquinone exhibited inhibitory effects on cell proliferation of many cancer cell lines and hormone-refractory prostate cancer by suppressing androgen receptor and E2F-1. Whether thymoquinone inhibits tumor angiogenesis, the critical step of tumor growth and metastasis, is still unknown. In this study, we found that thymoquinone effectively inhibited human umbilical vein endothelial cell migration, invasion, and tube formation. Thymoquinone inhibited cell proliferation and suppressed the activation of AKT and extracellular signal-regulated kinase. Thymoquinone blocked angiogenesis *in vitro* and *in vivo*, prevented tumor angiogenesis in a xenograft human prostate cancer (PC3) model in mouse, and inhibited human prostate tumor growth at low dosage with almost no chemotoxic side effects. Furthermore, we observed that endothelial cells were more sensitive to thymoquinone-induced cell apoptosis, cell proliferation, and migration inhibition compared with PC3 cancer cells. Thymoquinone inhibited vascular endothelial growth factor–induced extracellular signal-regulated kinase activation but showed no inhibitory effects on vascular endothelial growth factor receptor 2 activation. Overall, our results indicate that thymoquinone inhibits tumor angiogenesis and tumor growth and could be used as a potential drug candidate for cancer therapy. [Mol Cancer Ther 2008;7(7):1789–96]

Introduction

Thymoquinone ($C_{10}H_{12}O_2$; molecular weight: 164.2), the main bioactive component of the volatile oil of the black seed (*Nigella sativa*, Ranunculaceae family), has been used as antioxidant, anti-inflammatory, and antineoplastic medicines for more than 2,000 years (1, 2). Previous studies reported that thymoquinone exhibited inhibitory effects on cell proliferation of many types of cancer cell lines, including breast adenocarcinoma, ovarian adenocarcinoma (3), colorectal cancer (4), human pancreatic adenocarcinoma, uterine sarcoma (5), neoplastic keratinocytes (6), human osteosarcoma (7), fibrosarcoma, lung carcinoma (8), etc. Recently, Ahmed et al. reported that thymoquinone targeted androgen receptor and transcription factor E2F-1 and inhibited hormone-refractory prostate cancer (8). As angiogenesis is critically important for the growth of solid tumors not only by supplying with oxygen and nutrients for the survival of tumor cells but also by providing the route for metastatic spread. Therefore, angiogenesis has been an attractive target for tumor therapy (9, 10). The accumulated evidence has confirmed the importance of angiogenesis and validated the theory that inhibition of neovascularization is a promising anticancer strategy (11–13). Although Erdurmus et al. reported that thymoquinone showed inhibitory effects on corneal neovascularization in the rat model (14), whether thymoquinone inhibits tumor angiogenesis and suppresses prostate tumor growth through tumor angiogenesis prevention is still not fully understood.

Endothelial cells play a major role in each step of tumor angiogenesis, including endothelial cell migration, proliferation, invasion, adhesion, and tube formation (15, 16). Among the endothelial cell signaling pathways that regulates endothelial cell migration, proliferation, growth, and survival, the two major pathways are phosphatidylinositol 3-kinase-AKT and Raf-MEK-extracellular signal-regulated kinase (ERK) pathways. Activation of these two pathways in endothelial cells is necessary for angiogenesis (17, 18). AKT (protein kinase B), a serine/threonine-specific protein kinase, is a pivotal node involved in essential cellular functions of endothelial cells such as migration, growth, proliferation, apoptosis, and metabolism. AKT regulates endothelial nitric oxide synthase activation (19), stimulating vasodilation, vascular remodeling, and

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Note: T. Yi, S-G. Cho, and Z. Yi contribute equally to this work.

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angiogenesis (20). AKT signaling stimulates the production of hypoxia-inducible factor-α transcription factors and thereby mediates secretion of vascular endothelial growth factor (VEGF) and other growth factors, which are important proangiogenic factors (21, 22). ERK is a critical kinase in regulating endothelial cell cycle, proliferation, growth, migration, and apoptosis (23). On the extracellular growth factor stimulation, the activated ERK regulates its many substrates, such as nuclear factor-κB and c-Jun, and thereby regulates angiogenesis. ERK is also necessary for endothelial nitric oxide synthase activation (17, 24).

Here, we have investigated whether thymoquinone inhibited angiogenesis through suppression of intracellular signaling pathways. We found that thymoquinone can inhibit human umbilical vein endothelial cell (HUVEC) migration, invasion, proliferation, and tube formation by decreasing AKT/ERK activation. Thymoquinone also blocks angiogenic properties in both *in vitro* aortic ring assay and *in vivo* Matrigel plug assays. Furthermore, thymoquinone inhibits tumor angiogenesis and thereby prevents human prostate tumor growth at low dosage in xenograft mouse models.

Materials and Methods

Animals, Cell Lines, and Reagents

Severe combined immunodeficient male mice (5-6 weeks old) were purchased from the National Cancer Institute. Thymoquinone was ordered from Sigma-Aldrich. HUVEC were kindly gifted from Dr. Xinli Wang (Cardiothoracic Surgery Division, Michael E. DeBakey Department of Surgery, Baylor College of Medicine Hospital). The human prostate cancer cell line (PC3) was purchased from the American Type Culture Collection and maintained in a mixture of RPMI 1640 and 10% fetal bovine serum. Matrigel was ordered from BD Biosciences. HTScan VEGF receptor 2 (VEGFR2) kinase assay kit was ordered from Cell Signaling Technology. Horseradish peroxidase–labeled secondary antibody, TMB substrate, and stop solution were kindly gifted by Cell Signaling Technology. Streptavidin-coated yellow 96-well plates were kindly gifted by Perkin-Elmer Life Sciences.

Proliferation Assay

Cell proliferation assay with different concentration of thymoquinone was done as following the manual (Promega; CellTiter 96 Aqueous One Solution Cell Proliferation Assay).

Flow Cytometry Fluorescence-Activated Cell Sorting Analysis

Either HUVEC or PC3 cells ($\sim 2 \times 10^6$) were treated with different concentrations of thymoquinone at 37°C, 5% CO_2 incubator for 24 h. The cells were collected, stained with propidium iodide, and subjected to the flow cytometry analysis. The percentage at sub-G_1 was defined as the apoptotic population.

Migration Assay

Migration assay was done as described previously (25). HUVEC were allowed to grow to confluence on six-well plates precoated with 0.1% gelatin and inactivated by 0.1% mitomycin C as described previously. Monolayer cells were wounded by scratching with 1 mL pipette tips and washed twice with 1× PBS. Fresh endothelial cell growth medium was added with 4 nmol/L VEGF, which was received from the NIH Experimental Branch, and with different concentrations of thymoquinone. Images were taken after 7 to 10 h of incubation at 37°C, 5% CO_2 by Nikon digital camera. The migrated HUVEC were qualified by manual counting. Similar patterns of the inhibition effects were observed in three independent experiments.

Transwell Invasion Assay

The Transwell (Corning) was coated with Matrigel (BD Biosciences) and incubated at 37°C for 45 min. The bottom chambers (600 μL) were filled with endothelial cell growth medium with 20% fetal bovine serum supplemented with 4 nmol/L VEGF and the top chambers were seeded with 100 μL endothelial cell growth medium and HUVEC (4×10^4 per well). The top and bottom chambers contained the same series of concentration of thymoquinone. HUVEC were allowed to migrate for 4 h at 37°C, 5%CO_2. After the incubation, cells on the top surface of the membrane (nonmigrated) were scraped with a cotton swab. Cells on the bottom side of the membrane (migrated cells) were fixed with 4% paraformaldehyde for 20 min and washed three times with 1× PBS. The cells were stained by H&E staining and then destained with 1× PBS (pH 7.4). The membranes were left to air dry at room temperature for 30 min. Images were taken using Olympus inverted microscope. Three independent areas per well were counted and the mean number of migrated cells was calculated.

Tube Formation Assay

Matrigel was dissolved at 4°C for overnight, and each well of prechilled 24-well plates was coated with 100 μL Matrigel and incubated at 37°C for 45 min. HUVEC (4×10^4) were added in 1 mL endothelial cell growth medium with various concentration of thymoquinone. After 12 to 16 h of incubation at 37°C, 5% CO_2, endothelial cell tube formation was assessed with an inverted photomicroscope. Tubular structures were quantified by manual counting of low-power fields and percent inhibition was expressed using untreated wells as 100%.

Aortic Ring Assay

The aortic ring assay was done as described previously (25).

Matrigel Plug Assay

Matrigel (0.5 mL/plug) containing no VEGF, 4 nmol/L VEGF, or a mixture of VEGF (4 nmol/L) and 1 (or 10) μmol/L thymoquinone was injected s.c. in the midventral abdominal region of 5- to 6-week-old C57BL/6 mice (five mice for each group). After 7 days, the plugs were removed from the sacrificed mice. The Matrigel plugs were fixed with formalin and embedded with paraffin. The 5 μm sections were stained with H&E staining. The number of erythrocyte-filled blood vessels was counted (×200 fold magnification).

Western Blotting

Total protein (200 μg) of the cells of each sample was done for the immunoprecipitation with anti-c-Src antibody

(Santa Cruz Biotechnology) and then followed by Western blotting. The pTyr antibody (Santa Cruz Biotechnology) was used for detecting c-Src phosphorylation, and the pFAK397 antibody (Cell Signaling) was blotted for FAK phosphorylation. AKT phosphorylation was examined using $pSer^{473}$-AKT antibody (Cell Signaling). Anti-cleaved caspase-3 (Santa Cruz Biotechnology) was used for detecting apoptosis. Poly(ADP-ribose) polymerase cleavage was detected by anti–poly(ADP-ribose) polymerase antibody (Zymed Laboratory).

Xenograft Mouse Model

The 5- to 6-week-old severe combined immunodeficient male mice weighing ~20 g were divided into groups (five mice each group). PC3 cancer cells were s.c. injected (2×10^6 per mouse) into the mice. After the tumors had become established (~50 mm^3), the mice were s.c. injected with or without 6 mg/kg thymoquinone every day. The body weights and tumor sizes were recorded every day, and the tumor size was determined by a Vernier caliper measurement. After 15 days, mice with s.c. tumors were sacrificed.

Histology and Immunohistochemistry

The tumors were removed, fixed with Histochoice MB (Molecular Biology) tissue fixative (Amresco), and embedded with parafilm. The 5 μm sections were done for the blood vessel staining (Chemicon International; blood vessel staining kit, peroxidase system). The number of blood vessel in ×200 magnification was counted.

VEGFR2 Inhibition Assay

A 4× reaction cocktail containing 100 ng VEGFR2 (12.5 μL; supplied from the HTScan VEGFR2 kinase assay kit; Cell Signaling Technology) was incubated with 12.5 μL/tube of series of concentration of thymoquinone for 5 min at room temperature. A 2× ATP/substrate peptide cocktail (25 μL) was added to the preincubated reaction cocktail/thymoquinone compound. After incubation at room temperature for 30 min, a 50 μL stop buffer (50 mmol/L EDTA; pH 8) was added per tube to stop the reaction. Then, 25 μL of each reaction were transferred with 75 μL H_2O/well to a 96-well streptavidin-coated plate (Perkin-Elmer Life Sciences) and incubated at room temperature for 60 min. After washing the wells three times with 200 μL/well PBS-T (0.05% Tween 20 in 1× PBS), a 100 μL primary antibody [phosphotyrosine monoclonal antibody (PTyr100), 1:1,000 in PBS-T with 1% bovine serum albumin] was added per well. After being incubated at room temperature for 60 min, the wells were washed three times with 200 μL PBS-T. A 100 μL diluted horseradish peroxidase–labeled anti-mouse IgG (1:500 in PBS-T with 1% bovine serum albumin) was added per well. After incubation at room temperature for 30 min, the wells were washed five times with 200 μL PBS-T/well. Then, a 100 μL/well TMB substrate was added per well and the plate was incubated at room temperature for 15 min. The stop solution (100 μL/well) was added and mixed followed by incubation at room temperature 15 min. The plate was then detected at 405 nm with

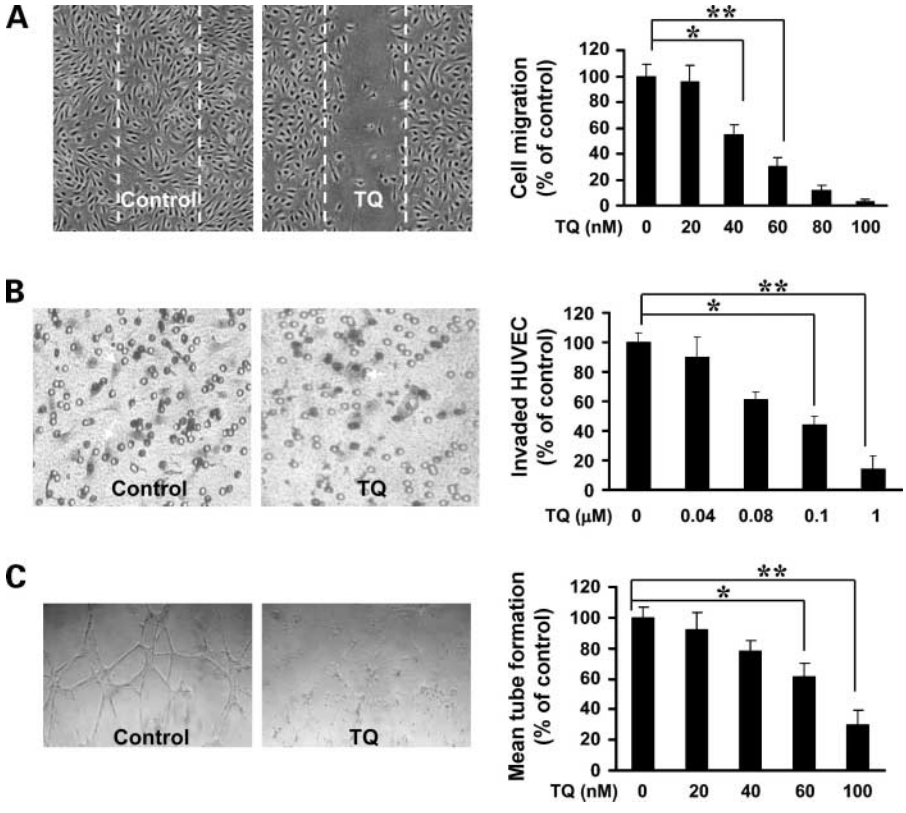

Figure 1. Thymoquinone inhibits HUVEC migration, invasion, and tube formation. **A,** inhibitory effect of thymoquinone on HUVEC migration. Inactivated HUVEC were subjected to wound-healing migration assays and the migrated cells were counted. **B,** effect of thymoquinone on HUVEC invasion assay using Transwell. Red cells with irregular shape were invaded cells attached on the outside surface of the top chamber. **C,** inhibitory effect of thymoquinone on HUVEC tubule-like structure formation. Tubule-like structure was quantified by manual counting of low-power fields (×25). Percent inhibition was expressed using untreated wells as 100% ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

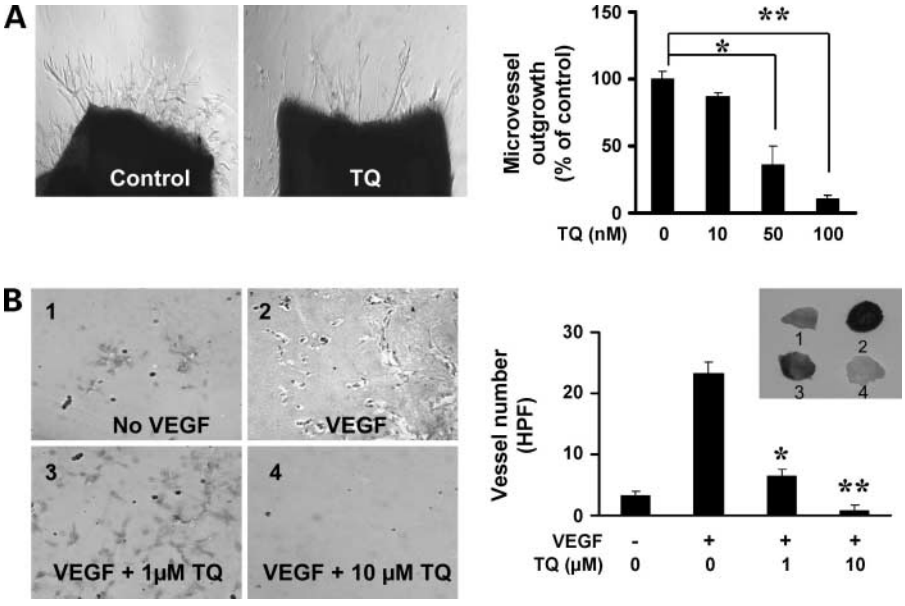

Figure 2. Thymoquinone inhibits angiogenesis *in vitro* and *in vivo*. **A,** effects of thymoquinone on angiogenesis *in vitro*. Images were taken using Olympus IX 70 invert microscope and microvessel outgrowth was counted as methods described previously (aortic ring number = 4). *, $P < 0.05$. **B,** inhibitory effects of thymoquinone on VEGF-induced angiogenesis *in vivo* (plug number = 5). *, $P < 0.05$; **, $P < 0.01$.

VERSAmax microplate reader (Molecular Devices) and the data were repeated three times.

Statistical Analysis

Three independent experiments were done, and data were presented as mean ± SE. Statistical significance of differences in control and sample groups was determined by using t test (maximum $P < 0.05$).

Results

Thymoquinone Inhibits HUVEC Migration, Invasion, and Tube Formation

As endothelial cell migration is an important step of angiogenesis (26), we did wound-healing migration assay to determine the effects of thymoquinone on HUVEC migration and found that thymoquinone inhibited HUVEC migration in a concentration-dependent manner (Fig. 1A). Then, in the following Transwell assay in Fig. 1B, thymoquinone significantly inhibited HUVEC invasion at 80 to 100 nmol/L. In tube formation assay, we found that thymoquinone significantly blocked HEVEC tube formation at 100 nmol/L (Fig. 1C).

Thymoquinone Inhibits Angiogenesis *In vitro* and *In vivo*

To ascertain the inhibitory effect of thymoquinone on angiogenesis, we did aortic ring assay. As shown in Fig. 2A, thymoquinone inhibited microvessel growth *in vitro* from 50 to 100 nmol/L (Fig. 2A) after 4 days of incubation, suggesting that thymoquinone inhibits angiogenesis *in vitro*. To confirm the antiangiogenesis effects of thymoquinone *in vivo*, Matrigel plug assays were done with different concentrations. As shown in Fig. 2B, 1 μmol/L thymoquinone significantly inhibited VEGF-induced angiogenesis, whereas 10 μmol/L thymoquinone almost completely abolished angiogenesis in the Matrigel plug assays (Fig. 2B), indicating that thymoquinone effectively inhibited angiogenesis *in vivo*.

Thymoquinone Inhibits Tumor Angiogenesis and Arrests Prostate Tumor Growth

To investigate thymoquinone-induced inhibition of tumor angiogenesis *in vivo*, we used the xenograft mouse model with human prostate cancer cells (PC3). PC3 cancer cells (2×10^6 per mouse) were injected s.c. into the mice (five mice for each group). After the tumors have become established (~50 mm^3), the mice were s.c. injected with or without thymoquinone at a dosage of 6 mg/kg/d. After 15 days, mice were sacrificed and the tumors were removed. As shown in Fig. 3A and B, thymoquinone effectively prevented the tumor growth in both size (thymoquinone-treated group 184.2 ± 25.8 mm^3 versus control group 1,143.98 ± 169.14 mm^3) and weight (thymoquinone-treated group 0.013 ± 0.002 g versus control group 0.3 ± 0.07 g). In further analyses of the tumor sections treated and untreated with thymoquinone, respectively, we found that thymoquinone significantly inhibited tumor angiogenesis as it decreased the number of blood vessels in the tumor (thymoquinone-treated group 2.8 ± 0.8 per high-field performance versus control group 17 ± 2.4 per high-field performance; Fig. 3C). At the concentration used in the xenograft mouse model, thymoquinone shows no toxicity as measured by mouse body weights in control and thymoquinone-treated groups. The average mouse body weight of control group decreased from 22.28 ± 1.22 to 21.24 ± 1.32 g, whereas that of thymoquinone-treated group increased from 22 ± 1.5 to 24.4 ± 1.2 g (Fig. 3D). The slight decrease of the control group is due to the growth of tumors in the xenograft mice.

HUVEC Are More Sensitive to Thymoquinone-Induced Apoptosis and Inhibition in Cell Migration and Proliferation Than PC3 Cancer Cells

To analyze whether the inhibition of tumor growth by thymoquinone primarily resulted from antiangiogenesis,

we evaluate whether thymoquinone has different effects on endothelial cells compared with cancer cells. We first compared the inhibition of thymoquinone on HUVEC and PC3 cell migration induced by VEGF. As shown in Fig. 4A, HUVEC were 10-fold more sensitive to thymoquinone inhibition on cell migration compared with PC3 cancer cells and the inhibitory effect of 0.1 μmol/L in HUVEC is equivalent to 1 μmol/L in PC3 tumor cells (Fig. 4A and B). Furthermore, we found that HUVEC were more sensitive than PC3 cancer cells to thymoquinone-induced inhibition in cell proliferation (Fig. 4C and D) and promotion in cell apoptosis assays (Table 1). These data indicate that HUVEC are more sensitive to thymoquinone-induced apoptosis and inhibition in cell migration and proliferation compared with PC3 cancer cells, suggesting that thymoquinone may target tumor angiogenesis at a lower dosage and then effectively inhibit tumor growth.

Thymoquinone Suppresses VEGF-Dependent ERK Activation but Is Not a VEGFR2 Inhibitor

VEGFR2 plays a major role in VEGF-dependent angiogenesis. To investigate the molecular mechanism of thymoquinone-induced inhibition on VEGF-dependent angiogenesis, we examined the thymoquinone effects on VEGFR2 activation with a VEGFR2-specific activation assay. We found that thymoquinone showed very little inhibitory effects on VEGFR2 (Fig. 5A), suggesting that thymoquinone was not a direct VEGFR2 inhibitor. As shown in Fig. 5B, thymoquinone could effectively suppress VEGF-dependent ERK activation at 10 nmol/L. Taken together, thymoquinone inhibit angiogenesis by suppressing VEGF-induced ERK activation but has no direct effect on VEGFR2 activation.

Thymoquinone Induces Cell Apoptosis Pathways

We have shown that thymoquinone significantly induced HUVEC apoptosis in a dose-dependent manner (Table 1).

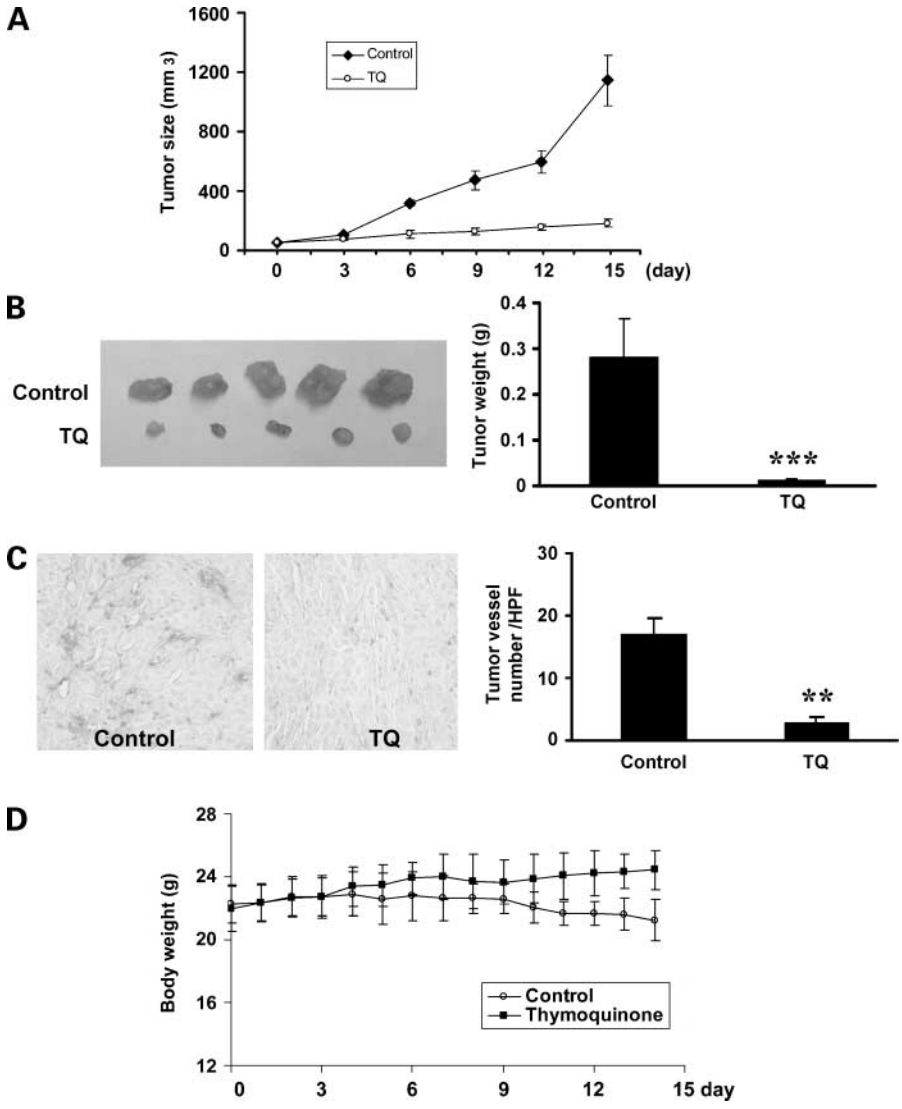

Figure 3. Thymoquinone inhibits tumor angiogenesis and prevents tumor growth *in vivo*. **A,** effects of thymoquinone on tumor volume growth. **B,** effects of thymoquinone on the increase of tumor weight. **C,** inhibition of thymoquinone on tumor angiogenesis. The number of microvessels was significantly decreased. **D,** effects of thymoquinone on mouse body growth (mouse number = 5). **, $P < 0.01$; ***, $P < 0.001$.

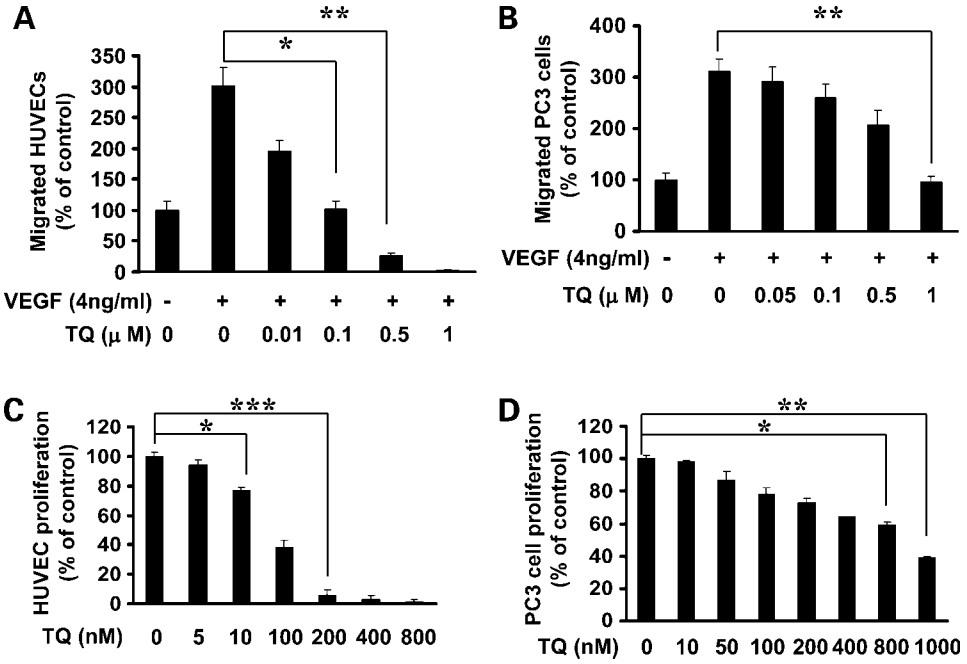

Figure 4. Sensitivity of HUVEC and tumor cells to thymoquinone. Effects of thymoquinone on cell migration of VEGF-induced HUVEC (**A**) and PC3 cancer cells (**B**). Inhibitory effects of thymoquinone on HUVEC (**C**) and PC3 (**D**) cancer cell proliferation ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

As shown in Fig. 5C, thymoquinone induced the activation of caspase-3 cleavage at 40 nmol/L and the data were confirmed by the increased cleavage of poly(ADP-ribose) polymerase in the absence or presence of VEGF. Furthermore, one of the key kinases involved in cell survival and apoptosis, AKT, was also significantly inhibited by thymoquinone at 20 to 40 nmol/L (Fig. 5D).

Discussion

Based on our present results, we conclude that thymoquinone effectively inhibits endothelial cell migration, invasion, proliferation, and tube formation, prevents angiogenesis *in vitro* and *in vivo*, and suppresses tumor angiogenesis and tumor growth *in vivo*. Thymoquinone inhibits angiogenesis by suppressing the activation of VEGF-induced ERK and AKT but is not a VEGFR2 inhibitor. This is the first report to comprehensively show that thymoquinone inhibits angiogenesis and tumor growth at low dosages by blocking tumor angiogenesis.

The known angiogenesis inhibitors always inhibit one or several steps of angiogenesis by targeting endothelial cells (27). Here, we not only showed that thymoquinone effectively inhibited endothelial cell migration, invasion, proliferation, and tube formation but also identified that thymoquinone inhibited angiogenesis *in vitro* and *in vivo*, suggesting that thymoquinone is an angiogenesis inhibitor. Furthermore, we found that thymoquinone inhibited tumor angiogenesis and prevented prostate tumor growth *in vivo* at low dosage of 6 mg/kg/d in xenograft mouse model. We also elucidated that endothelial cells were more sensitive to thymoquinone-caused apoptosis (Table 1) and inhibition in cell migration and proliferation compared with PC3 cancer cells. As prostate cancer is predominantly a tumor of old men with limited treatment options for the coexisting illnesses, the lower dosage used, the less chemotoxic side effects (11). Our data indicate that thymoquinone is a potential drug candidate for treating human prostate tumor.

AKT is a critical regulator generally involved in cell cycle, proliferation, and apoptosis through regulating gene expression, protein synthesis, and transcription procedure (20). ERK is an important factor in mediating cell proliferation, survival, and cell migration (17). Both AKT and ERK activation are necessary for essential cellular procedures of endothelial cells and angiogenesis (17). Here, we showed that thymoquinone inhibited the activation of both AKT and ERK (VEGF dependent or VEGF independent) in endothelial cells. However, thymoquinone had no inhibitory effects on VEGFR2 activation in a specific VEGFR2 assay. Other than VEGF, there are many other proangiogenic growth factors, such as fibroblast growth factors, placental growth factor, and platelet-derived growth factor (28). Almost all of these proangiogenic growth factors regulate angiogenesis through AKT and ERK signaling pathways (28–30). Therefore, thymoquinone

Table 1. Thymoquinone activates apoptosis in HUVEC and PC3 cancer cells

Thymoquinone (nmol/L)	Apoptotic population (% total cells)			
	0	25	50	100
PC3	2.3 ± 0.6	3.9 ± 0.5	3.5 ± 0.2	4.8 ± 1.8
HUVEC	2.7 ± 1.5	24.5 ± 1.2	28.4 ± 1.6	45 ± 2.6

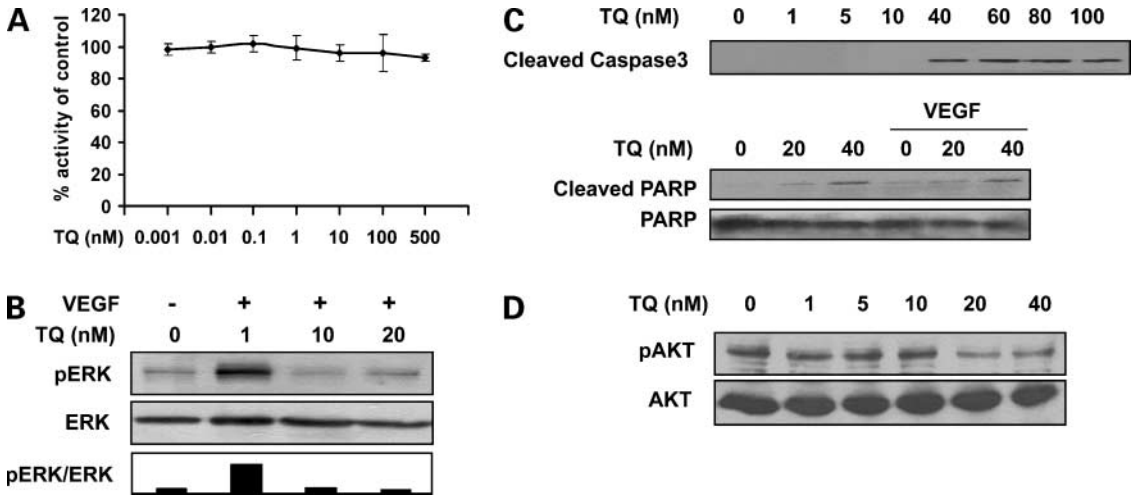

Figure 5. Effects of thymoquinone on VEGFR2 activation and signaling pathways. **A,** thymoquinone has little effect of the activation of VEGFR2. **B,** effects of thymoquinone on VEGF-dependent ERK activation. **C,** thymoquinone induces the levels of cleaved caspase-3 and the increased levels of cleaved poly(ADP-ribose) polymerase by thymoquinone are independent of VEGF in HUVEC. HUVEC were treated with thymoquinone at the indicated concentrations for 24 h and whole-cell proteins were assessed by Western blotting. VEGF (4 ng/mL) was used in the poly(ADP-ribose) polymerase cleavage assay. **D,** thymoquinone inhibited the activation of AKT at 20 to 40 nmol/L. HUVEC were treated with the indicated amount of thymoquinone for 5 min.

may inhibit angiogenesis/tumor angiogenesis through suppressing AKT/ERK signaling pathways but not directly inhibit VEGFR2 activation.

The efficacy of angiogenesis inhibitors depend on the tumor stage: premalignant, small tumor, and large tumor (31, 32). We concluded that thymoquinone can effectively inhibit prostate tumor growth at small tumor stage (50 mm^3) at the dose of 6 mg/kg/d.

In summary, we systemically showed that thymoquinone, the major biologically active component of the natural medicine of *N. sativa*, inhibited endothelial cell migration, invasion, proliferation, and tube formation, effectively inhibited angiogenesis *in vitro* and *in vivo*, and prevented tumor growth in a xenograft mouse model with a low dosage by blocking tumor angiogenesis. We identified that thymoquinone inhibited angiogenesis by suppressing AKT/ERK signaling pathway activation. Together, these data suggest that thymoquinone is a potential drug candidate for cancer chemotherapies with low chemotoxic side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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