Published OnlineFirst June 30, 2009; DOI: 10.1158/0008-5472.CAN-09-0755

Acetyl-11-Keto-β-Boswellic Acid Inhibits Prostate Tumor Growth by Suppressing Vascular Endothelial Growth Factor Receptor 2–Mediated Angiogenesis

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Abstract

The role of angiogenesis in tumor growth and metastasis is well established. Identification of a small molecule that blocks tumor angiogenesis and is safe and affordable has been a challenge in drug development. In this study, we showed that acetyl-11-keto-β-boswellic acid (AKBA), an active component from an Ayurvedic medicinal plant (*Boswellia serrata*), could strongly inhibit tumor angiogenesis. AKBA suppressed tumor growth in the human prostate tumor xenograft mice treated daily (10 mg/kg AKBA) after solid tumors reached ~100 mm^3 (n = 5). The inhibitory effect of AKBA on tumor growth was well correlated with suppression of angiogenesis. When examined for the molecular mechanism, we found that AKBA significantly inhibited blood vessel formation in the Matrigel plug assay in mice and effectively suppressed vascular endothelial growth factor (VEGF)–induced microvessel sprouting in rat aortic ring assay *ex vivo*. Furthermore, AKBA inhibited VEGF-induced cell proliferation, chemotactic motility, and the formation of capillary-like structures from primary cultured human umbilical vascular endothelial cells in a dose-dependent manner. Western blot analysis and *in vitro* kinase assay revealed that AKBA suppressed VEGF-induced phosphorylation of VEGF receptor 2 (VEGFR2) kinase (KDR/Flk-1) with IC_{50} of 1.68 μmol/L. Specifically, AKBA suppressed the downstream protein kinases of VEGFR2, including Src family kinase, focal adhesion kinase, extracellular signal-related kinase, AKT, mammalian target of rapamycin, and ribosomal protein S6 kinase. Our findings suggest that AKBA potently inhibits human prostate tumor growth through inhibition of angiogenesis induced by VEGFR2 signaling pathways. [Cancer Res 2009;69(14):5893–900]

Introduction

Angiogenesis plays an essential role in tumor growth, invasiveness, and metastasis (1–7). Vascular endothelial growth factor (VEGF), a glycoprotein that has mitogenic activity on vascular endothelial cells, is widely expressed in most cancers and is a critical component of tumor angiogenesis (2). VEGF exerts its biological actions by binding to its receptor tyrosine kinase. However, the VEGF signaling involved in angiogenesis is mainly mediated by VEGF receptor 2 (VEGFR2; ref. 8). Specifically, VEGFR2 activation results in the activation of diverse intracellular signaling molecules, such as Src family kinase/focal adhesion kinase (FAK; ref. 9), phosphoinositide 3-kinase/AKT kinase, mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase (p70S6K; ref. 10), protein kinase C/protein kinase D (11), and mitogen extracellular kinase/extracellular signal-related kinase (ERK; ref. 12), which promote the growth, migration, differentiation, and survival of endothelial cells in preexisting vasculature. The U.S. Food and Drug Administration has recently approved Avastin, an antibody against the VEGF, for the treatment of cancer (3, 5, 6). Thus, agents that are small, safe, affordable, and efficacious in suppressing tumor angiogenesis will have great potential in treating tumor angiogenesis.

Acetyl-11-keto-β-boswellic acid (AKBA) is a derivative of boswellic acid, which is the main component of gum resin from *Boswellia serrata* and *Boswellia carterii Birdw*. Traditionally, boswellic acid has been used as an anti-inflammatory agent to treat arthritis, rheumatic disorders, and respiratory diseases. As an anti-inflammatory agent, boswellic acid directly interacts with IκB kinases (13) and suppresses nuclear factor-κB–regulated gene expression (14). Boswellic acid has been reported to noncompetitively inhibit 5-lipoxygenase (15–17), topoisomerase (18), and leukocyte elastase (19). In addition, boswellic acid has been shown to potentiate apoptosis in several types of tumor cells, including colon cancer (20), prostate cancer (21), fibrosarcoma (22), hepatoma (23), and malignant glioma (24, 25), through caspase-8 activation (20) and death receptor 5–mediated signaling (21). However, whether AKBA can modulate prostate tumor growth and tumor angiogenesis in general is not understood. In this study, we investigated the functional roles of AKBA in suppressing human prostate cancer growth and angiogenesis. We show that AKBA potently inhibits angiogenesis and tumor growth by suppressing the VEGFR2 and the mTOR signaling pathways.

Materials and Methods

Reagents. Purified AKBA was supplied by Sabinsa Corp. A 50-mmol/L stock solution of AKBA was prepared and then stored at −20°C as small aliquots until needed. Bacteria-derived recombinant human VEGF ($VEGF_{165}$) was a gift from the Experimental Branch of the NIH (Bethesda, MD). Growth factor–reduced Matrigel was purchased from BD Biosciences. Antibodies against FAK, Src, AKT, ERK1, β-actin, and poly(ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology. Caspase-3, mTOR, and phospho-specific anti-Src (Tyr^{416}), anti-FAK ($Tyr^{576/577}$), anti-AKT (Ser^{473}), anti-pERK1/2 (Thr^{202}/Tyr^{204}), anti-mTOR (Ser^{2448}), anti-p70S6K (Thr^{389}), and anti-VEGFR2 (Tyr^{1175}) were purchased from Cell Signaling Technology.

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doi:10.1158/0008-5472.CAN-09-0755

Published OnlineFirst June 30, 2009; DOI: 10.1158/0008-5472.CAN-09-0755

Cell lines and cell culture. Primary human umbilical vascular endothelial cells (HUVEC) were kindly provided by Dr. Xinli Wang (Baylor College of Medicine, Houston, TX). HUVECs were cultured in endothelial cell growth medium (ECGM) as described previously (26). Human prostate cancer (PC-3) cells were purchased from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). HUVECs and PC-3 cells were cultured at 37°C under a humidified 95%:5% (v/v) mixture of air and CO_2.

Animal studies. Animals were maintained in a laminar airflow cabinet under specific pathogen-free conditions and a 12-h light-dark cycle. Mice were maintained according to the NIH standards established in the Guidelines for the Care and Use of Experimental Animals, and all of the experimental protocols were approved by the Animal Investigation Committee of the Institute of Biosciences and Technology, Texas A&M University Health Science Center.

Xenograft human prostate tumor mouse model. Xenograft mouse model assay was performed as previously described (27). Five-week-old male BALB/cA nude mice (National Rodent Laboratory Animal Resources, Shanghai, China) weighing ~25 g each were randomly divided into each group of five. PC-3 cells were s.c. injected into the mice (2×10^6 per mouse). After tumors grew to ~100 mm^3, mice were treated s.c. with or without AKBA (10 mg/kg/d) for a month. The body weight of each mouse was recorded every 5 d. At the same time, solid tumor volume was determined using Vernier caliper measurements and the formula of $A \times B^2 \times 0.52$, where A is the longest diameter of the tumor and B is the shortest diameter of the tumor. After 30 d, mice with tumors no greater than 1.5 cm in diameter were sacrificed.

Histology and immunohistochemistry. Solid tumors were removed, fixed with 10% formaldehyde, and embedded in paraffin. A blood vessel staining kit (von Willebrand factor; Millipore) was used to stain blood vessels in 5-μm tumor sections. Images of the stained blood vessels were taken using a Leica DM 4000B photomicroscope. Blood vessels were counted ($n = 5$).

***In vivo* Matrigel plug assay.** Matrigel plug assay was performed and modified as described previously (28). Briefly, we s.c. injected 0.5 mL of Matrigel containing indicated amounts of AKBA, 80 ng of VEGF, and 20 units of heparin into the ventral area of 6-wk-old C57BL/6 mice (NIH). Five mice were used for each group. After 6 d, the skin of each mouse was pulled back to expose an intact Matrigel plug. H&E staining was performed to identify the formation and infiltration of new, functional microvessels. Functional microvessels with intact RBCs were quantified manually using a microscope [high-power field (HPF) × 200].

Rat aortic ring assay. Rat aortic ring assay was performed as described previously (28). In brief, 48-well plates were coated with 120 μL of Matrigel per well and polymerized in an incubator. Aortas isolated from 6-wk-old male Sprague-Dawley rats were cleaned of periadventitial fat and connective tissues in cold PBS and cut into rings of 1 to 1.5 mm in circumference. The aortic rings were randomized into wells and sealed with a 100-μL overlay of Matrigel. VEGF in 500 μL of serum-free ECGM with or without AKBA was added into the wells. As a control, ECGM alone was assayed, and the fresh medium was exchanged for every 2 d. After 6 d, microvessel sprouting was fixed and photographed using an inverted microscope (magnification, ×100; Olympus). The assay was scored from 0 (least positive) to 5 (most positive) in a double-blind manner. Each data point was assayed six times.

Cell viability assay. HUVECs or PC-3 cells (2×10^4 per well) were treated with or without VEGF (10 ng/mL) and various concentrations of AKBA for 24 h. To determine cell viability, we used a CellTiter 96

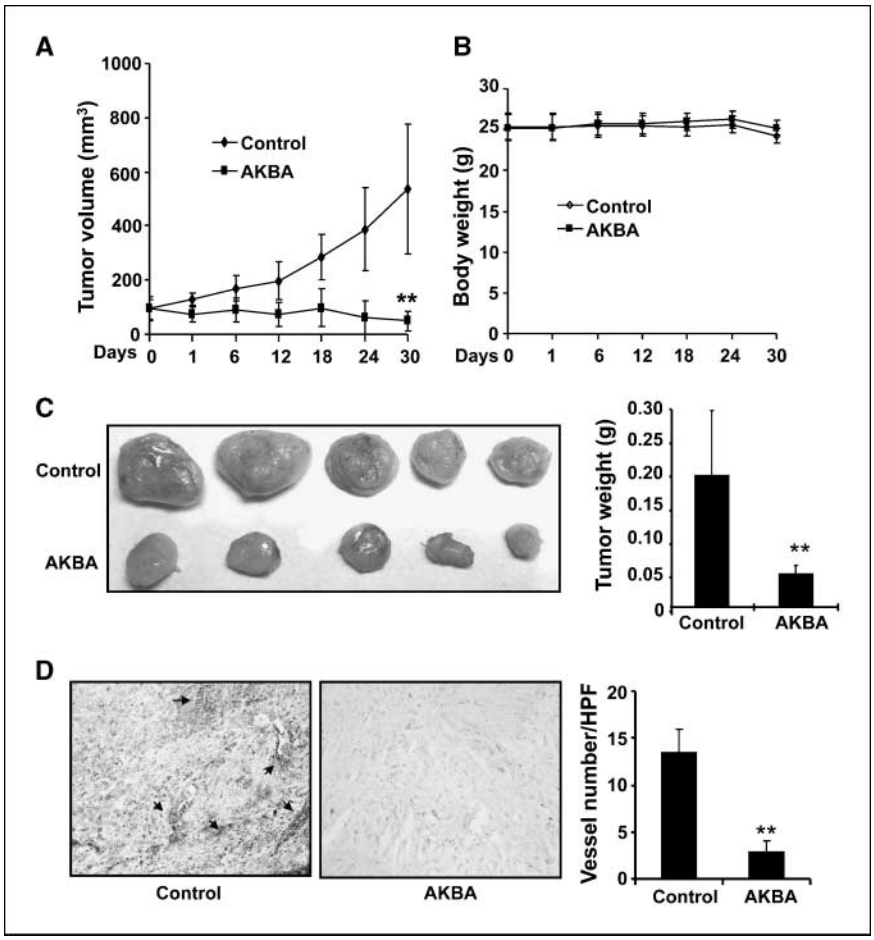

Figure 1. AKBA inhibits tumor growth and tumor angiogenesis in a xenograft mouse model. PC-3 cells were injected into 5-wk-old BALB/cA nude mice (2×10^6 per mouse). After solid tumors grew to ~100 mm^3, the mice were s.c. treated with or without AKBA (10 mg/kg/d). *A,* AKBA inhibited tumor growth as measured by tumor volume. *B,* as shown by the body weight change in mice, AKBA had little toxicity in the amount tested. There was no significant difference in body weight between the treated group and the control group. *C,* solid tumors in the AKBA-treated mice were significantly smaller than those in the control mice. *D,* blood vessel staining revealed that AKBA inhibited tumor angiogenesis. The 5-μm tumor sections from the control and AKBA-treated groups were stained using von Willebrand factor blood vessel staining, and the number of blood vessels in a HPF was counted. *Arrows,* blood vessels. *Columns,* mean; *bars,* SD. **, $P < 0.01$ versus control.

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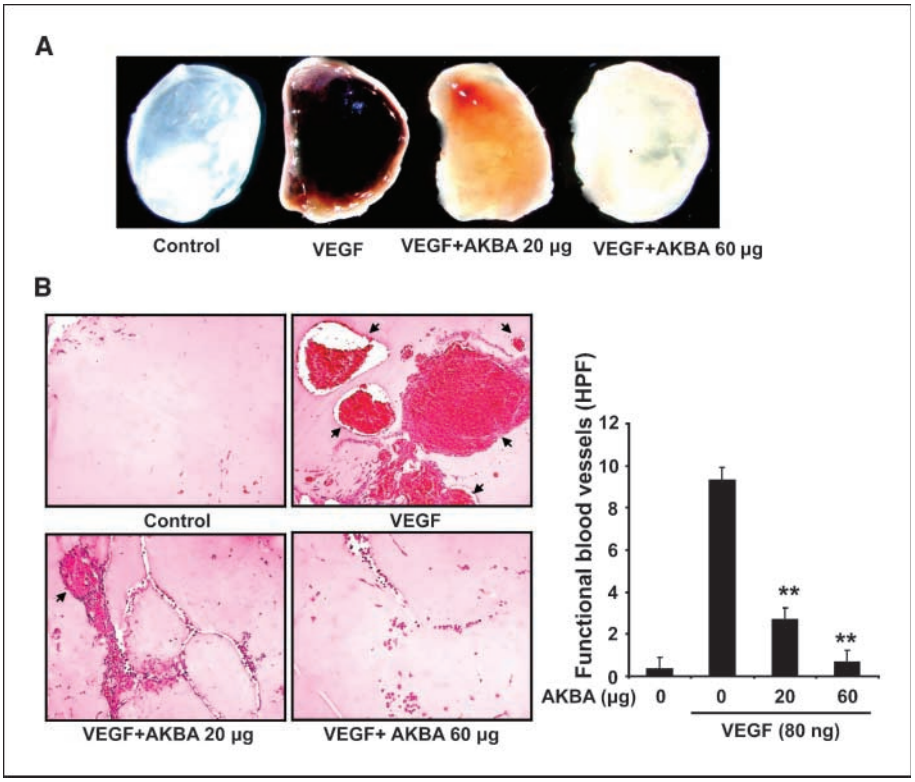

Figure 2. AKBA inhibits VEGF-induced angiogenesis *in vivo*. Six-week-old C57BL/6 mice were injected with 0.5 mL of Matrigel containing indicated amounts of AKBA, 80 ng of VEGF, and 20 units of heparin into the ventral area (five mice per group). After 6 d, the skin of mice was pulled back to expose the intact Matrigel plugs. *A,* representative Matrigel plugs were photographed. *B,* AKBA inhibited blood vessel formation. The Matrigel plugs were fixed, sectioned, and stained with H&E. Infiltrating microvessels with intact RBCs were qualified by manual counting. Magnification, ×200. *Columns,* mean; *bars,* SD. **, $P < 0.01$ versus VEGF alone.

AQueous One Solution Cell Proliferation Assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS); Promega] and a VERSAmax microplate reader (Molecular Devices).

Endothelial cell migration assay. HUVECs were allowed to grow to full confluence in six-well plates precoated with 0.1% gelatin (Sigma) and then starved with ECGM containing 0.5% FBS for 6 h to inactivate cell proliferation. The cells were then wounded with pipette tips and washed with PBS. ECGM containing 0.5% FBS was added into the wells with or without 10 ng/mL VEGF and various concentrations of AKBA. Images of the cells were taken after 8 to 10 h of incubation at 37°C in a 95%:5% (v/v) mixture of air and CO_2. The migrated cells were counted manually, and the percentage of inhibition was expressed using untreated wells at 100%. Three independent experiments were performed.

Endothelial cell Transwell migration assay. The chemotactic motility of HUVECs was determined using a Transwell migration assay (BD Biosciences) with 6.5-mm-diameter polycarbonate filters (8-μm pore size) as described previously (26). In brief, the filter of the Transwell plate was coated with 0.1% gelatin. The bottom chambers were filled with 500 μL of ECGM containing 0.5% FBS supplemented with 10 ng/mL VEGF. Inactivated HUVECs (4×10^4) suspended in 100 μL of ECGM containing 0.5% FBS plus various concentrations of AKBA were seeded in the top chambers. Cells were allowed to migrate for 8 to 10 h. Nonmigrated cells were removed with cotton swabs, and migrated cells were fixed with cold 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an inverted microscope (Olympus), and migrated cells were quantified by manual counting. The percentage of migrated cells inhibited by AKBA was expressed based on untreated control wells.

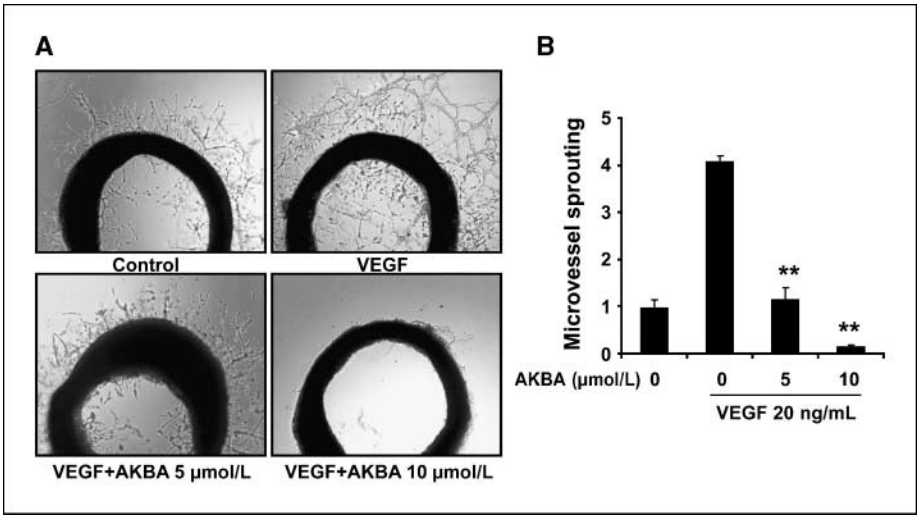

Figure 3. AKBA inhibits VEGF-induced microvessel sprouting *ex vivo.* Aortic segments isolated from Sprague-Dawley rats were placed in growth factor–reduced Matrigel-covered wells and treated with VEGF (20 ng/mL) in the presence or absence of AKBA for 6 d. *A,* representative photographs of endothelial cell sprouts forming branching cords from the margins of aortic rings. *B,* sprouts were scored from 0 (least positive) to 5 (most positive) in a double-blinded manner. *Bars,* SD. **, $P < 0.01$ versus VEGF alone.

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Endothelial cell capillary-like tube formation assay. Tube formation was assessed as previously described (26). Growth factor–reduced Matrigel was pipetted into prechilled 24-well plates (100 μL Matrigel per well) and polymerized for 45 min at 37°C. HUVECs were first incubated in ECGM containing 0.5% FBS for 6 h and then treated with various concentrations of AKBA for 30 min before seeding. HUVECs were collected and placed onto the layer of Matrigel (1×10^5 per well) in 1 mL of ECGM containing 0.5% FBS followed by the addition of 10 ng/mL of VEGF. After 6 to 8 h of incubating at 37°C in a 95%:5% (v/v) mixture of air and CO_2, the endothelial cells were photographed using an inverted microscope (magnification, ×100; Olympus). Three independent experiments were performed.

Western blot analysis. To determine the effects of AKBA on VEGFR2-dependent signaling cascade, HUVECs were first starved in serum-free ECGM for 6 h and then pretreated with or without various concentrations of AKBA for 30 min followed by stimulation with 50 ng/mL of VEGF. Different stimulation time is responsible for different substrate phosphorylation by VEGF. The whole-cell extracts were prepared in radioimmunoprecipitation assay buffer (20 mmol/L Tris, 2.5 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, and 1 mmol/L phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor cocktail (Calbiochem). Forty micrograms of cellular protein from each sample were applied to 8% to 12% SDS-polyacrylamide gels and probed with specific antibodies followed by exposure to a horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit antibody (Cell Signaling Technology). Protein concentrations were determined using bicinchoninic acid assay and equalized before loading.

***In vitro* VEGFR2 kinase inhibition assay.** VEGFR2 kinase assay was performed using an HTScan VEGFR2 kinase kit (Cell Signaling Technology) combined with colorimetric ELISA detection. The final reaction system included 60 mmol/L HEPES (pH 7.5), 5 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, 3 μmol/L Na_3VO_4, 1.25 mmol/L DTT, 20 μmol/L ATP, 1.5 μmol/L substrate peptide, 100 ng of VEGF receptor kinase, and indicated concentrations of AKBA. The kinase assay was repeated three times independently.

Statistical analysis. Statistical comparisons between drug-treated group and untreated control group were performed using one-way ANOVA followed by Dunnett's test. Data were presented as means ± SDs. P values of ≤0.05 were considered statistically significant.

Results

AKBA inhibits tumor growth and tumor angiogenesis in the xenograft mouse model. We used a xenograft prostate tumor model to investigate the effect of AKBA on tumor growth and angiogenesis and found that 10 mg/kg/d of AKBA significantly suppressed tumor volume (Fig. 1*A*) and tumor weight (Fig. 1*C*) but had no effect on the body weight of mice (Fig. 1*B*). In our present tumor inhibition model, the average tumor volume in the control mice increased from 95.83 ± 43.37 mm^3 to 536.58 ± 241.801 mm^3 after 30 days, whereas the average tumor volume in the AKBA-treated mice decreased from 92.43 ± 39.70 mm^3 to 47.99 ± 35.49 mm^3, indicating that proliferation rate of tumor cells in treated mice was greatly inhibited by AKBA. Additionally, the average tumor weight in the control group was 0.194 ± 0.097 g, whereas the average tumor weight in the AKBA-treated group was only 0.049 ± 0.012 g

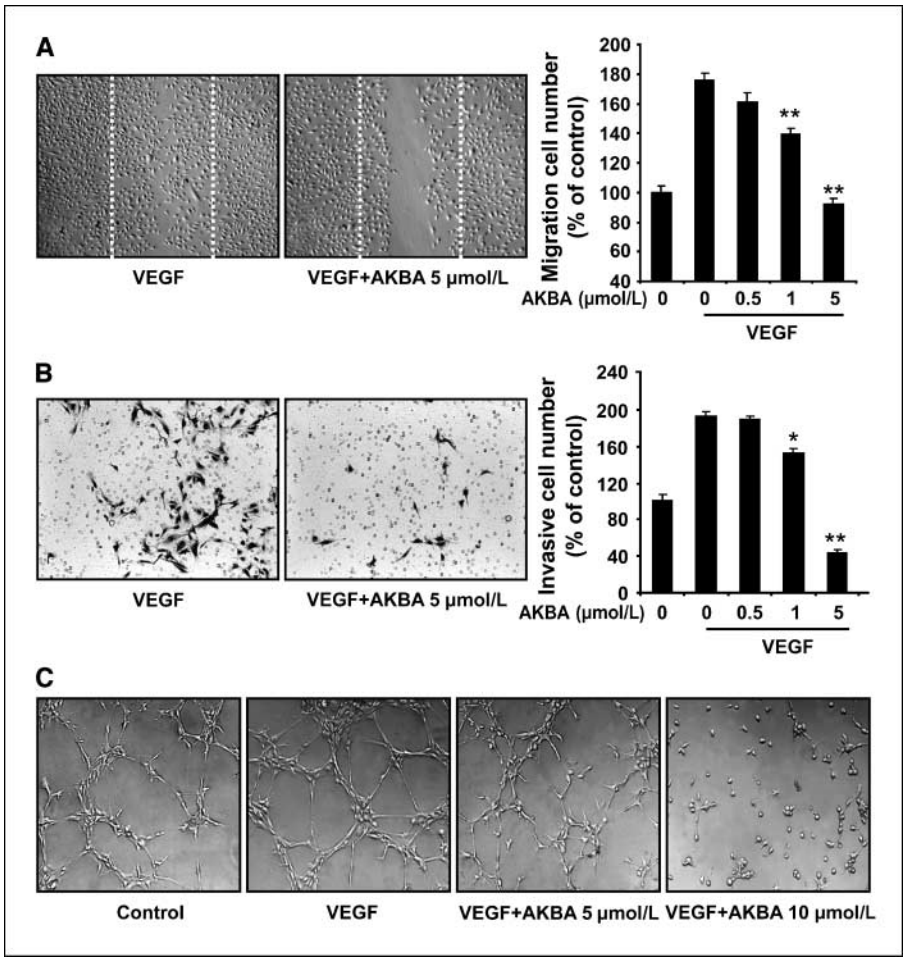

Figure 4. AKBA inhibits VEGF-induced chemotactic motility and capillary-structure formation of endothelial cells. *A,* AKBA inhibited HUVEC migration. HUVECs were grown into full confluence in six-well plates and then starved with 0.5% FBS ECGM for 6 h to inactivate cell proliferation. Cells were scratched by pipette and treated with or without 10 ng/mL VEGF and indicated concentrations of AKBA. The migrated cells were quantified by manual counting. *B,* AKBA inhibited HUVEC invasion. HUVECs were seeded in the upper chamber of a Transwell and treated with different concentrations of AKBA. The bottom chamber was filled with ECGM supplemented with VEGF. After 8 to 10 h, the HUVECs that migrated through the membrane were quantified. *C,* AKBA inhibited the VEGF-induced tube formation of HUVEC tubes. HUVECs were placed in 24-well plates coated with Matrigel (4×10^4 per well). After 6 to 8 h, cells were fixed, and tubular structures were photographed. Magnification, ×100. *Columns,* mean from three different experiments with duplicates; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$ versus VEGF alone.

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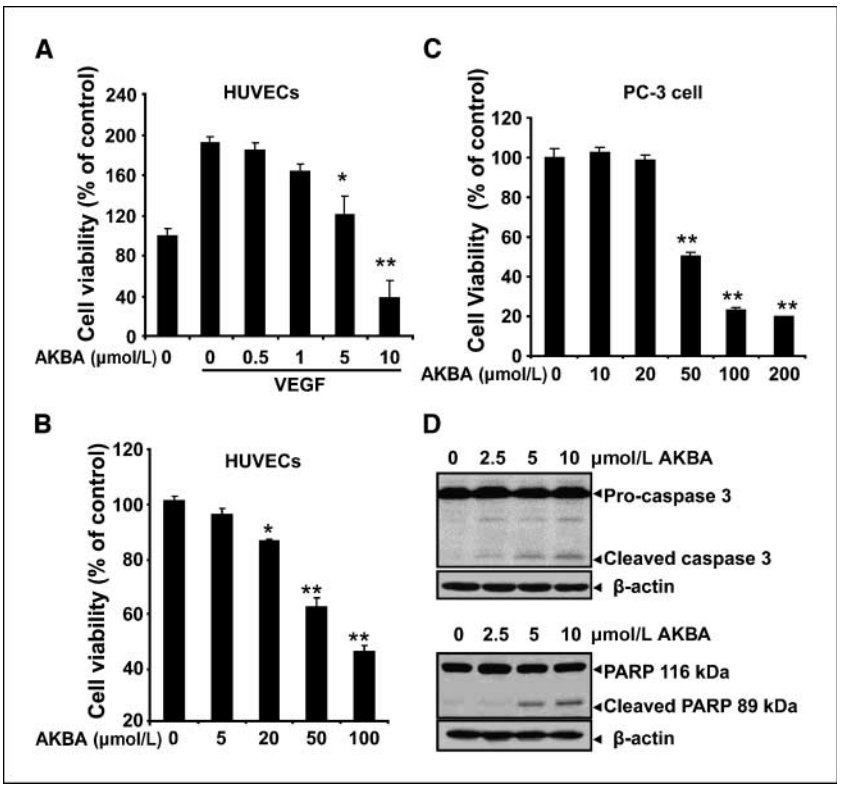

Figure 5. AKBA potentiates viability and apoptosis in endothelial and tumor cells. *A,* AKBA inhibited VEGF-induced cell viability in a dose-dependent manner. HUVECs (2×10^4 per well) were starved with serum-free ECGM and then treated with or without VEGF (10 ng/mL) and various concentrations of AKBA for 24 h. Cell viability was quantified by MTS assay. *, $P < 0.05$; **, $P < 0.01$ versus VEGF alone. *B,* AKBA inhibited HUVEC viability and induced cell apoptosis in normal culture condition. HUVECs (2×10^4 per well) were treated with different concentrations of AKBA in ECGM with 20% FBS for 24 h followed by MTS measurement. *, $P < 0.05$; **, $P < 0.01$ versus medium alone. *C,* AKBA inhibited PC-3 cell proliferation as measured by MTS. *Columns,* mean from three different experiments with six duplicates; *bars,* SD. *D,* AKBA induced caspase-3 activation and cell apoptosis. Proteins from HUVECs treated with indicated concentrations of AKBA for 24 h were analyzed by Western blot analysis for cleaved caspase-3 and cleaved PARP determination.

(Fig. 1*C*), suggesting that AKBA strongly inhibited tumor growth in the xenograft prostate tumor mouse model.

To further investigate whether AKBA inhibited tumor growth by suppressing tumor angiogenesis, we used a blood vessel staining kit to stain solid tumor sections. Our data show that the average number of blood vessels in AKBA-treated group is 2.83 ± 1.17 blood vessels/HPF (Fig. 1*D*) compared with 13.50 ± 2.43 blood vessels/HPF in the control group, indicating that AKBA significantly inhibited tumor angiogenesis.

AKBA inhibits VEGF-induced angiogenesis *in vivo* and vessel sprouting *ex vivo*. We further explored the antiangiogenic activity of AKBA using *in vivo* and *ex vivo* angiogenesis models. First, we used Matrigel plug assay to determine the effects of AKBA on VEGF-induced angiogenesis *in vivo*. After 6 days, Matrigel plugs containing VEGF alone appeared dark (Fig. 2*A*) and were filled with intact RBCs, indicating that functional vasculatures had formed inside the Matrigel via angiogenesis triggered by VEGF. In contrast, addition of different concentrations of AKBA in the Matrigel plugs containing VEGF dramatically inhibited vascular formation (Fig. 2*A*). The color of the Matrigel plugs becomes pale due to the lack of RBCs. AKBA (60 μg) totally blocked vasculature formation in the assays (Fig. 2*A*).

We used H&E staining to quantify the amount of functional vasculature in Matrigel plugs (Fig. 2*B*) and found that 20 μg AKBA dramatically inhibited VEGF-induced vessel formation *in vivo*.

To further investigate whether AKBA inhibited VEGF-induced angiogenesis *ex vivo*, we examined the sprouting of vessels from aortic rings in the presence or absence of AKBA. VEGF (20 ng/mL) significantly stimulated microvessel sprouting, leading to the formation of a network of vessels around the aortic rings (Fig. 3). Treatment of AKBA antagonized the VEGF-induced sprouting in a dose-dependent manner (Fig. 3*A*). AKBA (10 μmol/L) can completely block the VEGF-induced microvessel sprouting of rat aortic rings (Fig. 3*B*).

AKBA inhibits VEGF-induced chemotactic motility and capillary structure formation of HUVECs. Endothelial cell migration is essential for blood vessel formation in angiogenesis and also critical for tumor growth. To assess the antiangiogenic action of AKBA *in vitro*, we investigated the inhibitory effects of AKBA on the chemotactic motility of endothelial cells by using wound-healing migration assay (Fig. 4*A*) and Transwell assay (Fig. 4*B*), respectively. We found that AKBA inhibited VEGF-induced HUVEC migration (Fig. 4*A*) and cell invasion (Fig. 4*B*) in a dose-dependent manner, with significant inhibition at 1 to 5 μmol/L of AKBA.

Although angiogenesis is a very complex process involving several kinds of cells, tube formation of endothelial cells is one of the key steps of angiogenesis (29). We used two-dimensional Matrigel assays to examine the potential effects of AKBA on the tubular structure formation of endothelial cells. When HUVECs were seeded on the growth factor–reduced Matrigel, robust tubular-like structures were formed in the presence of VEGF. However, treatment with 5 or 10 μmol/L of AKBA significantly suppressed or terminated VEGF-induced tubular formation of endothelial cells (Fig. 4*C*). These results indicated that AKBA could block VEGF-induced angiogenesis *in vitro* by inhibiting cell motility and tubular structure formation of endothelial cells.

AKBA potentiates viability and apoptosis in endothelial and tumor cells. To further elucidate the effects of AKBA on endothelial cells, we used MTS assays to examine cell proliferation

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and survival. Because angiogenesis is primarily initiated by growth factors, we first tested whether AKBA decreased VEGF-mediated HUVEC proliferation and viability. We found that a concentration of 5 μmol/L AKBA significantly inhibited VEGF-mediated HUVEC survival (Fig. 5*A*). Under normal culture conditions (i.e., no VEGF), AKBA (20–50 μmol/L) also inhibited HUVEC proliferation (Fig. 5*B*). We also examined the inhibitory effects of AKBA on PC-3 cell proliferation and found that a much higher concentration of AKBA (~50 μmol/L) was required to suppress PC-3 cell proliferation compared with the concentration of AKBA required to suppress endothelial cell proliferation (~5 μmol/L), indicating that endothelial cells were more sensitive to AKBA than PC-3 cells (Fig. 5*C*).

To determine whether AKBA regulates endothelial cell death, we used Western blot analysis to examine caspase-3 activation and PARP cleavage in cells treated with different concentrations of AKBA. We found that addition of AKBA (2–5 μmol/L) led to the activation of caspase-3 and the cleavage of PARP from its intact form (116 kDa) to its cleaved form (89 kDa) in endothelial cells (Fig. 5*D*), suggesting that AKBA decreases cell viability and induces apoptosis through a caspase-dependent pathway.

AKBA is a potent VEGFR2 kinase inhibitor. To determine the molecular basis of AKBA-mediated antiangiogenesis, we examined whether AKBA could inhibit VEGFR2 activation. As shown in Fig. 6*A*, AKBA (5 μmol/L) strongly inhibited VEGF-activated VEGFR2 phosphorylation. To confirm our Western blot analysis data and determine whether AKBA directly inhibits VEGFR2 kinase activity, we performed *in vitro* kinase assays with different concentrations of AKBA using HTScan VEGFR2 kinase assay kit according to the manufacturer's suggested methods (Cell Signaling Technology and Perkin-Elmer Life Sciences). Our data showed that AKBA directly inhibited VEGFR2 kinase activity in a dose-dependent manner with an IC_{50} of 1.68 μmol/L (Fig. 6*B*). These findings suggest that AKBA inhibits tumor angiogenesis by blocking VEGFR2 activation.

AKBA inhibits the activation of VEGFR2-mediated signaling pathways. VEGFR2 activation leads to the activation of various downstream signaling substrates that are responsible for endothelial cell migration, proliferation, and survival. To determine whether AKBA inhibited downstream signaling of VEGFR2, we screened some key kinases involved in VEGFR2 signaling pathway. We found that 5 μmol/L of AKBA significantly suppressed the activation of c-Src/FAK (Fig. 6*C, c-1*) and AKT, ERK, mTOR, and p70S6K (Fig. 6*C, c-2*), which suggested that AKBA exerted its antiangiogenic function through direct inhibiting VEGFR2 activation on the surface of endothelial cells and further suppressing VEGFR2-mediated downstream signaling cascade (Fig. 6*D*).

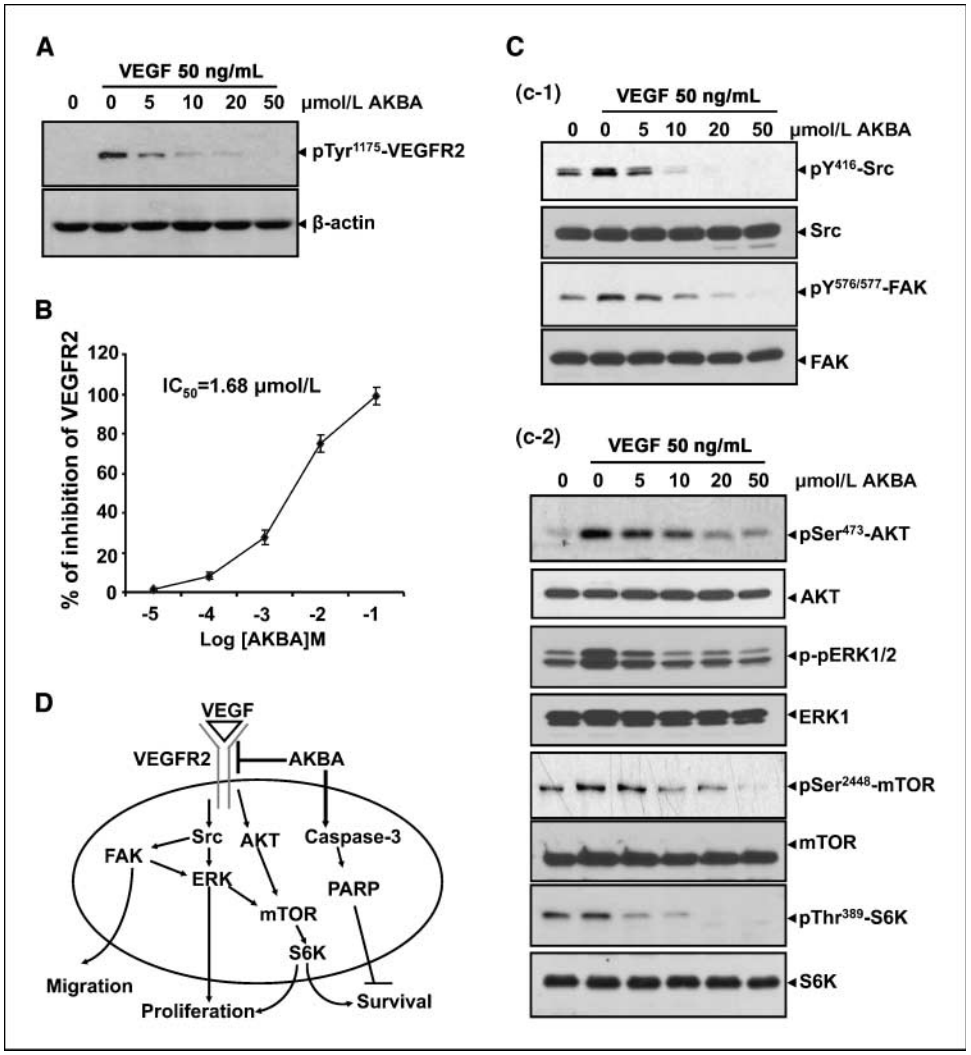

Figure 6. AKBA inhibits VEGFR2 kinase activity and its downstream signaling molecules. *A,* AKBA suppressed the activation of VEGFR2 triggered by VEGF in HUVECs. The activation of VEGFR2 from different treatments was analyzed by Western blotting and probed with anti-phospho-VEGFR2 antibody. *B,* AKBA inhibited VEGFR2 kinase activity *in vitro*. The inhibition of VEGFR2 kinase activity of AKBA was analyzed using an *in vitro* kinase assay kit according to the manufacturer's instructions. *Points,* mean from three different experiments with three duplicates; *bars,* SD. *C,* AKBA inhibited the activation of VEGFR2 downstream cascade, including mTOR pathway. Activation of Src and FAK complex (*c-1*) and AKT/mTOR/S6K signaling cascade was suppressed by AKBA (*c-2*). Proteins from different treatments were analyzed by Western blotting and probed with specific antibodies. *D,* antiangiogenic signaling pathways regulated by AKBA in HUVECs.

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Discussion

In this study, we found that AKBA was a potent angiogenesis inhibitor and inhibited multiple steps of VEGF-mediated tumor angiogenesis, including cell proliferation, migration, invasion, and tube formation. As evidenced in our xenograft human prostate tumor mouse model experiments, tumor growth was significantly inhibited when AKBA antagonized tumor angiogenesis.

VEGF receptor inhibitors are a promising class of cancer treatment drugs (6, 8). VEGF is the primary and the most potent inducer of angiogenesis. VEGF activates cellular signaling pathways by binding to its receptor tyrosine kinase, which promotes several events required for angiogenesis, including endothelial cell survival, proliferation, and migration and vascular permeability (2). Currently, >20 agents with antiangiogenic properties, including a VEGF-neutralizing antibody, soluble receptors, receptor antagonists, and tyrosine kinase inhibitors, are either already approved for cancer treatment or undergoing clinical (phase I–III) studies (2, 30). VEGF signaling events relevant to tumor growth and angiogenesis are mainly mediated by VEGFR2 (8, 31). In the present study, we found that a half-maximum inhibitory concentration of 1.68 μmol/L AKBA significantly blocked the kinase activity of VEGFR2, making AKBA a potent VEGFR2 inhibitor. Previous study also reported that AKBA inhibited basic fibroblast growth factor–induced signaling responses (32), suggesting that AKBA might be a broad receptor tyrosine kinase inhibitor.

In clinical trials, antiangiogenic therapy with VEGF antagonists has thus far produced disappointing results (33). Successful antiangiogenic therapy may require the simultaneous blockade of signaling pathways downstream from multiple proangiogenic factor receptors (34). Previous studies have shown that the Src family kinase is substantially involved in VEGF-induced angiogenesis *in vitro* and *in vivo* (35, 36). By interacting with its downstream molecule, FAK, Src regulates cell motility (37) and vascular permeability (9). In the current study, we found that a low concentration of AKBA (5 μmol/L) effectively inhibited VEGF-triggered Src and FAK activation in endothelial cells.

Recently, AKT/mTOR/p70S6K signaling has been identified as a novel, functional mediator in angiogenesis (10, 38). In the current study, we found that AKBA significantly decreased the phosphorylation of mTOR and p70S6K, and its upstream kinase, AKT and ERK, indicating that AKBA suppresses tumor angiogenesis by inhibiting VEGFR2 and blocking its multiple downstream signaling components, thereby suppressing endothelial cell migration, proliferation, and survival (Fig. 6*D*). Interestingly, the mTOR/p70S6K pathway has been found to regulate the expression of proangiogenic factors such as interleukin-8 and VEGF in various human carcinomas by regulating hypoxia-inducible factor-1α expression at the translational level (38–42), making the pathway a potential target for anticancer therapy. Therefore, AKBA may suppress tumor angiogenesis by inducing tumor cells to produce fewer proangiogenic molecules.

In the present studies, we show that 5 μmol/L AKBA was sufficient to inhibit VEGF-induced angiogenic responses in *in vitro* and *ex vivo* angiogenesis assays, whereas 10 μmol/L AKBA completely blocked capillary-like structure formation and microvessel sprouting. Our data indicate that the antiangiogenic activity of AKBA inhibits tumor growth *in vivo* much earlier than its cytotoxic effects on tumor cells. Furthermore, we found that AKBA induced endothelial cell apoptosis through a caspase-dependent pathway, which suggests that AKBA inhibits endothelial cell survival not only by blocking VEGFR2 but also by blocking certain extrinsic death receptors on the cell surface or triggering the apoptotic cascade of the intrinsic mitochondrial pathway, as suggested by previous studies that higher concentrations of AKBA (30–50 μmol/L) directly interact with IκB kinase (13) to suppress nuclear factor-κB–regulated gene expression (14).

In conclusion, we found that AKBA potently inhibited tumor growth and angiogenesis by targeting VEGFR2 activation and mTOR signaling pathways, suggesting that AKBA has a potential role in cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 2/26/09; revised 3/31/09; accepted 5/1/09; published OnlineFirst 6/30/09.

Grant support: Science and Technology Commission of Shanghai Municipality, Research Platform for Cell Signaling Networks grant 06DZ22923, and NIH (National Cancer Institute) grant 1R01CA106479.

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We thank Joseph A. Munch for carefully proofreading the manuscript and providing valuable comments.

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Published OnlineFirst June 30, 2009; DOI: 10.1158/0008-5472.CAN-09-0755

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Cancer Res 2009;69:5893-5900. Published OnlineFirst June 30, 2009.