Therapeutic Discovery

(−)-Gossypol Suppresses the Growth of Human Prostate Cancer Xenografts via Modulating VEGF Signaling–Mediated Angiogenesis

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Abstract (−)-Gossypol, a natural BH3-mimetic and small-molecule Bcl-2 inhibitor, shows promise in ongoing phase II clinical trials for human cancers. However, whether (−)-gossypol plays functional roles in tumor angiogenesis has not been directly elucidated yet. In this study, we showed that (−)-gossypol dose dependently inhibited the expression of VEGF, Bcl-2, and Bcl-xL in human prostate cancer cells (PC-3 and DU 145) and primary cultured human umbilical vascular endothelial cells (HUVEC) in vitro. Notably, the growth of human prostate tumor PC-3 xenografts in mice was significantly suppressed by (−)-gossypol at a dosage of 15 mg/kg/d. This inhibitory action of (−)-gossypol in vivo was largely dependent on suppression of angiogenesis in the solid tumors, where VEGF expression and microvessel density were remarkably decreased. Furthermore, (−)-gossypol inhibited VEGF-induced chemotactic motility and tubulogenesis in HUVECs and human microvascular endothelial cells and suppressed microvessel sprouting from rat aortic rings ex vivo. When examined for the mechanism, we found that (−)-gossypol blocked the activation of VEGF receptor 2 kinase with the half maximal inhibitory concentration of 2.38 μmol/L in endothelial cells. Consequently, the phosphorylation of key intracellular proangiogenic kinases induced by VEGF was all suppressed by the treatment, such as Src family kinase, focal adhesion kinase, extracellular signal-related kinase, and AKT kinase. Taken together, the present study shows that (−)-gossypol potently inhibits human prostate tumor growth through modulating VEGF signaling pathway, which further validates its great potential in clinical practice.

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Introduction

Solid tumors recruit new blood vessels for their growth, maintenance, and metastasis (1, 2). Discovering drugs that suppress tumor-induced development of new blood vessels (angiogenesis) is an important strategy for cancer treatment. So far, angiogenesis inhibition has come off the bench and entered into clinical application. Many targets of endogenous angiogenesis inhibitors reflect the complexity of the process; however, current clinical therapies mainly target the VEGF system (3). Different agents including antibodies, aptamers, peptides, and small molecules have been extensively investigated to block VEGF and its proangiogenic functions (4). The VEGF signaling events relevant to tumor angiogenesis is mainly mediated by VEGF receptor 2 (VEGFR2, KDR/Flk-1; refs. 5, 6). Mechanistically, activating VEGFR2 at specific tyrosine sites results in the phosphorylation of various intracellular signaling molecules, such as Src family kinase (7), focal adhesion kinase (FAK; ref. 8), phosphatidylinositol 3-kinases/AKT kinase (9, 10), extracellular signal–related kinase (ERK1/2; ref. 11), mTOR kinase (12), and signal transducer and activator of transcription (13) in endothelial cells. All of these pivotal molecules collaboratively promote proliferation, migration, invasion, and differentiation to capillary-like structure of endothelial cells in the preexisting vasculature.

(−)-Gossypol, a bioactive phytochemical produced by cotton plants, has been considered as a natural BH3 mimic (Fig. 1A). Through potent inhibition of Bcl-2/Bcl-xL/Mcl-1, gossypol potentiates apoptosis in numerous human cancer cells including prostate (14), colon, breast, lung, pancreatic, hepatoma, and head and neck cancers (15). In addition, (−)-gossypol can radiosensitize prostate cancer in vitro and in vivo without augmenting toxicity (16). Multiple molecular investigations reveal that gossypol and its derivatives modulate TGF-β/Akt
signaling (17), activate p53 (18) and SAPK/JUK pathway (19), suppress the c-Myc signaling (20), inhibit NF-κB activity and NF-κB-mediated gene expression (21), regulate ROS-dependent mitochondria and death receptor 5 pathway (22, 23) and intracellular Ca²⁺ (24). Recent studies showed that (−)-gossypol and its enantiomer (AT-101) could affect proangiogenic molecules released from cancer cells at mRNA and protein levels either alone or in combination (25–27), suggesting the potential role of (−)-gossypol in antiangiogenesis. In addition, it has been shown that Bcl-2 gene expression is significantly higher in the tumor-associated endothelial cells as compared with normal endothelial cells (28), and upregulated Bcl-2 expression in microvascular endothelial cells was sufficient to enhance intratumoral angiogenesis and to accelerate tumor growth (29, 30). However, whether (−)-gossypol, known as a potent Bcl-2 inhibitor, can directly modulate the biological functions of endothelial cells remains obscure.

Therefore, in the present study, we investigated the biological roles of (−)-gossypol in tumor angiogenesis, and our results revealed that (−)-gossypol significantly inhibited angiogenesis and the growth of prostate tumor xenografts by targeting VEGF signaling pathway.

Materials and Methods

Reagents

(−)-Gossypol was supplied by Tocris Bioscience. A 100-mmol/L stock solution was prepared in dimethyl sulfoxide (DMSO) and then stored at −20°C as small aliquots until needed. Bacteria-derived recombinant human VEGF (rhVEGF) was a gift from NIH (Bethesda, MD). Growth factor–reduced Matrigel was purchased from BD Biosciences. Antibodies against ERK1, Bcl-2, VEGF, and β-actin were obtained from Santa Cruz Biotechnology. PARP, Bcl-xL, VEGFR2, AKT, Src, FAK, phospho-specific anti-VEGFR2 (Tyr¹¹⁷⁵) and anti-VEGFR2 (Tyr²⁰⁶), anti-Src (Tyr⁴¹⁶), anti-FAK (Tyr³⁹⁷), anti-AKT (Ser⁴⁷³), and anti-pERK1/2 (Thr²⁰²/Tyr²⁰⁴) were purchased from Cell Signaling Technology. Antibody against CD31 was bought from Epitomics.
Cell lines and cell culture

Primary human umbilical vascular endothelial cells (HUVEC) kindly gifted from Dr. Xinli Wang (Cardiothoracic Surgery Division of Michael E. DeBakey Department of Surgery at Baylor College of Medicine, Houston, TX) in 2008 were cultured in endothelial cell culture medium (ECM) as described previously (31). Human microvascular endothelial cells (HMEC-1), human prostate cancer PC-3 cells, and human prostate cancer DU 145 cells were obtained from American Type Culture Collection in 2009. HMEC-1 was cultured with MCDB 131 medium (Sigma) supplemented with 10% FBS (Hyclone), 2 mmol/L l-glutamine, 100 IU/mL of penicillin, 100 mg/mL of streptomycin, 10 ng/mL of epidermal growth factor, and 1 mg/mL of hydrocortisone (Sigma). HUVECs and HMEC-1 were confirmed by their typical microscopic morphology: homogeneous, large, polygonal, and cobblestone-like. PC-3 cells were cultured in RPMI 1640 medium (Hyclone) supplemented with 10% FBS and DU 145 cells were cultured in Dulbecco’s Modified Eagle’s Medium (Hyclone) supplemented with 10% FBS. Western blotting using epithelial markers authenticated that they were of epithelial origin before experiments. All these cells were freed of mycoplasma by PCR methods before use and maintained at 37°C under a humidified 95%:5% (v/v) mixture of air and CO₂.

Animal studies

Animals used in the present study were maintained according to the NIH standards established in the Guidelines for the Care and Use of Experimental Animals. All of the experimental protocols were approved by the Animal Investigation Committee of East China Normal University.

Xenograft human prostate tumor mouse model

Xenograft mouse model was conducted as previously described (31). Five- to 6-week-old male Balb/cA nude mice (National Rodent Laboratory Animal Resources, Shanghai, China) were randomly divided into each group of 6 to 7 mice. PC-3 cells were grown to 80% to 90% confluence, harvested, prepared at 5 × 10⁶ cells/100 μL cell suspensions, and inoculated on the flank region of nude mice. After tumors grew to about 50 mm³, mice were treated with or without (-)-gossypol for 48 hours. HUVECs (6 × 10⁴ to 7 × 10⁵ cells/well) were treated with or without VEGF (50 ng/mL) and various concentrations of (-)-gossypol for 48 hours. To determine cell viability, we used a CellTiter 96 AQueous One Solution Cell Proliferation kit (Promega) and a VERSAmax microplate reader (Molecular Devices).

Endothelial cell migration assay

Transwell migration assay was done as described previously (32). Briefly, HUVECs (2 × 10⁵ cells/well) or HMEC-1 (2 × 10⁵ cells/well) along with the indicated concentrations of (-)-gossypol were seeded into the wells. Fresh medium was replaced every 2 days. After a week, microvessel formation, tubulogenesis, and a VERSAmax microplate reader (Molecular Devices).

Endothelial cell capillary-like tube formation assay

Tube formation was assessed as described previously (32). Briefly, HUVECs or HMEC-1 were pretreated with various dilutions of (-)-gossypol for 2 hours and then seeded onto the Matrigel layer in 24-well plates at a density of 4 × 10⁴ to 7 × 10⁶ cells. ECM (0.5% FBS) with or without 30 ng/mL of VEGF was added into wells. After 4 to 6 hours, tubulogenesis was fixed and photographed using an inverted microscope (Olympus; original magnification, ×100). Three independent experiments were done.

Rat aortic ring assay

In brief, 48-well plates were coated with 120 μL of Matrigel per well and polymerized in an incubator. Aorta isolated from 5-week-old male Sprague Dawley rats was cut into rings of 1 to 1.5 mm in circumference, randomized into wells, and sealed with a 100 μL-overlay of Matrigel. VEGF in 500 μL ECM (0.5% FBS) with or without (-)-gossypol was added into the wells. Fresh medium was replaced every 2 days. After a week, microvessel sprouting was fixed and photographed using an inverted microscope (Olympus; original magnification, ×100). The assay
was scored from 0 (least positive) to 5 (most positive) in a double-blind manner.

**Western blotting analysis**

To examine the apoptotic effects of (-)-gossypol on prostate cancer cells, PC-3 and DU 145 were directly treated with various concentrations of (-)-gossypol for 24 hours. PC-3, DU 145, and HUVECs were incubated with (-)-gossypol for 24 hours to detect the expression of VEGF, Bcl-2, and Bcl-xL in treated cancer cells and endothelial cells. To determine the molecular basis of (-)-gossypol in angiogenesis signaling, HUVECs were first starved in serum-free ECM for 4 to 6 hours and then pretreated with or without various concentrations of (-)-gossypol for 30 minutes, followed by stimulation with 50 ng/mL of VEGF for 2 to 20 minutes. The whole-cell extracts were prepared in RIPA (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 Na3P2O7, and 1 mmol/L phenylmethylsulfonylfluoride) supplemented with proteinase inhibitor cocktail (Calbiochem). About 40 to 50 μg of cellular protein from each sample was applied to 6% to 12% SDS-PAGE 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 concentration was determined using bicinchoninic acid assay and equalized before loading. Relative optical density of blotting bands was qualified by Image J software (NIH).

**In vitro VEGFR2 kinase inhibition assay**

VEGFR2 kinase assay was done using an HTScan VEGFR2 kinase kit from Cell Signaling Technology combined with colorimetric ELISA detection as described previously (33). The final reaction system contained 60 mmol/L HEPES (pH 7.5), 5 mmol/L MgCl2, 5 mmol/L MnCl2, 3 μmol/L Na3VO4, 1.25 mmol/L DTT (dithiothreitol), 20 μmol/L ATP, 1.5 μmol/L substrate peptide, 100 ng of VEGF receptor kinase and different concentrations of (-)-gossypol.

**Statistical analysis**

Statistical comparisons between groups were performed using 1-way ANOVA followed by Student’s t test. Data were presented as means ± standard error (SE). Values of P ≤ 0.05 were considered statistically significant.

**Results**

**(-)-Gossypol decreases cell viability and induces apoptosis in human prostate cancer cells**

Prostate cancer continues to represent a burgeoning medical problem in the United States. In our study, the cytotoxic effects of (-)-gossypol were first examined on PC-3 and DU 145 cancer cells. The MTS results showed that (-)-gossypol inhibited cell viability in a dose-dependent manner, with the half maximal inhibitory concentrations of about 20 μmol/L (Fig. 1B). Western blotting analysis further revealed that (-)-gossypol induced potent apoptosis in PC-3 and DU 145 cells, where the full length of nuclear PARP were cleaved from the intact form (116 kD) into cleaved from (89 kD; Fig. 1C). These results were consistent with previous finding that (-)-gossypol suppressed the proliferation of prostate cancer cells *in vitro* (34).

**(-)-Gossypol suppresses the expression of VEGF, Bcl-2, and Bcl-xL in human prostate cancer cells and endothelial cells**

VEGF is a major tumor-associated growth factor that potently stimulates endothelial cell proliferation, chemotaxis, angiogenesis, and vascular permeability. Bcl-2 has been shown to activate NF-κB in cancer cells, which regulates expression of chemokines and proangiogenic factors involved in inflammation and angiogenesis (35, 36). Thus, we examined whether (-)-gossypol could downregulate the expression of VEGF while blocking Bcl-2. As shown in Fig. 1D, treatment with (-)-gossypol resulted in a dose-dependent inhibition of VEGF and Bcl-2/Bcl-xL in both cancer cells and endothelial cells, indicating its great function in tumor angiogenesis.

**(-)-Gossypol suppresses tumor growth and angiogenesis in a human prostate tumor xenograft mouse model**

To investigate the effect of (-)-gossypol on tumor growth and tumor angiogenesis *in vivo*, we studied a prostate tumor (PC-3) xenograft mouse model. We found that administration of (-)-gossypol (15 mg/kg/d) for 50 days substantially suppressed tumor volume (Fig. 2A) and reduced tumor weight (Fig. 2B) compared with the control groups injected with the same solution without the drug. The average tumor volume of the control group increased from 61.19 ± 6.96 mm3 to 829.83 ± 187.91 mm3 at the end of the experiments, whereas that in the (-)-gossypol–treated group decreased from 58.46 ± 3.25 mm3 to 19.74 ± 7.71 mm3. In addition, the average tumor weight of the control group was 397.12 ± 99.69 mg, whereas that in the (-)-gossypol–treated group decreased to 43.10 ± 3.25 mg, suggesting a significant inhibition of tumor growth by (-)-gossypol. In our experimental system, low dosage of (-)-gossypol at 5 mg/kg/d was also tested; however, little effect was observed in mice. During treatment, the (-)-gossypol–treated mice appeared healthy and (-)-gossypol had little effect on the body weight of mice (data not shown). In addition, pathologic analysis at autopsy revealed no (-)-gossypol-induced tissue changes in the organs, suggesting that (-)-gossypol had little toxicity at the tested dosage.

To further examine whether (-)-gossypol inhibited angiogenesis (new blood vessel formation), we carried out immunohistochemistry with anti-CD31, anti-VEGFR2, and anti-VEGF antibodies on tumor sections.
with or without the treatment of \((-\)gossypol. The results showed that VEGF expression was remarkably inhibited by \((-\)gossypol. The microvessel density in \((-\)gossypol-treated group was 42.84% of the control group (Fig. 2C), indicating that addition of \((-\)gossypol significantly inhibited neovascularization besides its direct cytotoxic effect on prostate tumor cells.

\((-\)Gossypol inhibits VEGF-induced endothelial cell migration and differentiation to capillary-like structure \textit{in vitro}

To assess the detailed activities of \((-\)gossypol on angiogenesis \textit{in vitro}, we examined whether \((-\)gossypol modulated the VEGF-induced proliferation of endothelial cells by MTS assay. As shown in Fig. 3A, about 2 \(\mu\)mol/L of \((-\)gossypol significantly decreased VEGF-induced cell viability in HUVECs after 48 hours of incubation. We further examined its inhibitory function on the chemotactic motility by the Boyden chamber assay in 2 kinds of endothelial cells, HUVECs and HMEC-1. Our results showed that invasive endothelial cells in the \((-\)gossypol-treated group were dramatically less than that of the VEGF alone group, suggesting a potent inhibitory effect of \((-\)gossypol on VEGF-induced endothelial cell motility (Fig. 3B).

Tubulogenesis is the event mimicking one of the last steps of angiogenesis. Therefore, we examined whether \((-\)gossypol regulated capillary tube formation in HUVECs and HMEC-1. As shown in Fig. 3C, when endothelial cells were seeded on 2-dimensional Matrigel, robust tubular-like structures were formed. However, treatment with \((-\)gossypol (5–10 \(\mu\)mol/L) could abolish this process to a great extent. The number, length, and area of capillary-like structures were significantly decreased. Together, these results indicated that \((-\)gossypol could block angiogenesis \textit{in vitro} by inhibiting VEGF-induced cell proliferation, chemotaxis, and capillary-like structure formation of endothelial cells.
To study whether (-)-gossypol could inhibit angiogenesis ex vivo, we examined the sprouting of microvessels from aortic rings in the presence or absence of (-)-gossypol. As shown in Fig. 4A, the presence of VEGF significantly triggered the microvessel sprouting around the aortic rings. Addition of different concentrations of (-)-gossypol antagonized the VEGF-induced sprouting in a dose-dependent manner and 10 μmol/L of (-)-gossypol completely abolished those microvessel sprouts (Fig. 4B).

(-)-Gossypol blocks VEGFR2 kinase in vascular endothelial cells
To understand the molecular basis of (-)-gossypol-mediated antiangiogenesis, we examined whether (-)-gossypol could inhibit the activation of VEGFR2, a critical receptor tyrosine kinase on the cell surface of endothelial cells. As shown in Fig. 5A and B, (-)-gossypol (5 μmol/L) strongly inhibited VEGF-activated VEGFR2 phosphorylation at both Tyr1175 and Tyr996 sites. To confirm this result, we did an in vitro kinase assay using a kinase kit. Our data showed that (-)-gossypol inhibited VEGFR2 kinase activity in a dose-dependent manner with the half maximal inhibitory concentration of 2.38 μmol/L (Fig. 5C).

(-)-Gossypol inhibits the activation of key intracellular proangiogenic kinases
VEGFR2 activation induced by VEGF leads to the phosphorylation of various downstream signaling molecules that are responsible for endothelial cell migration, proliferation, and survival. To determine
whether (-)-gossypol inhibited the intracellular angiogenic signaling, we examined several key kinases involved in the process of VEGFR2-mediated angiogenesis. We found that 5 to 10 μmol/L of (-)-gossypol significantly suppressed the phosphorylation of Src, FAK, AKT, and ERK induced by VEGF (50 ng/mL) in HUVECs (Fig. 6A), suggesting that (-)-gossypol exerted its antiangiogenic function through blockade of VEGF/VEGFR2 signaling cascade in endothelial cells.

Figure 4. (-)-Gossypol inhibits VEGF-induced microvessel sprouting ex vivo. Aortic segments isolated from Sprague Dawley rats were placed in Matrigel-covered wells and treated with VEGF in the presence or absence of (-)-gossypol for 6 days. A, representative photographs of endothelial cell sprouts from aortic rings. B, sprouts were scored from 0 (least positive) to 5 (most positive) in a double-blinded manner. Columns, mean; bars, SE; **, P < 0.01 versus VEGF alone group.

Figure 5. (-)-Gossypol is a VEGFR2 kinase inhibitor. A, (-)-gossypol suppressed the activation of VEGFR2 triggered by VEGF in endothelial cell. HUVECs were starved in serum-free medium for 4 to 6 hours, pretreated with (-)-gossypol for 30 minutes, and then stimulated with 50 ng/mL VEGF for 2 minutes. The activation of VEGFR2 from different treatments was analyzed by Western blotting and probed with anti-phospho-VEGFR2 antibody at Tyr1175 and Tyr996 sites. B, the relative optical density was qualified by Image J software. C, (-)-gossypol inhibited VEGFR2 kinase activity in vitro. Dots, mean; bars, SE.
Discussion

Prostate cancer continues to represent a burgeoning medical problem in males. Recent studies show that (−)-gossypol treatment induces DNA damage in metastatic (37), hormone-resistant, drug-resistant, and castrate-resistant prostate cancer cells (38, 39) and prostate tumor–initiating cells (18). Notably, there are a number of clinical trials that (−)-gossypol and its derivatives show promising efficacy against some refractory human cancers (38). And recently, (−)-gossypol has also been selected as an adjuvant agent for human prostate cancer (14). In the present study, we show for the first time that the suppression of prostate tumor \textit{in vivo} treated with (−)-gossypol is partially dependent on angiogenesis inhibition, and our results further reveal that (−)-gossypol modulates multiple steps of VEGF signaling–mediated angiogenesis.

It was shown that different hormone- and drug-resistant prostate cancers constitutively express some important angiogenic cytokines, which are known to regulate tumorigenicity and angiogenesis. Previous studies on (−)-gossypol had shown that there were 1.6- and 1.8-fold decreases in VEGF and interleukin (IL) 8 levels after treatment with 10 μmol/L of (−)-gossypol in human prostate or ovarian cancer cells (26, 27), indicating that (−)-gossypol could affect the profile of proangiogenic factors released from tumors. This information provides us significant clue to study the direct antiangiogenic role of (−)-gossypol \textit{in vitro} and \textit{in vivo}. In the present study, we found that (−)-gossypol functioned as a potent angiogenesis inhibitor. It not only inhibited VEGF expression of prostate cancer cells and endothelial cells \textit{in vitro} (Fig. 1C) and \textit{in vivo} (Fig. 2C), but blocked multiple steps in VEGF-activated biological events of endothelial cells including endothelial cell proliferation, migration, and differentiation (Fig. 3). As evidenced by the human prostate tumor xenograft mouse model, tumor growth was significantly inhibited when (−)-gossypol antagonized angiogenesis (Fig. 2).

It has already been validated that racemic (−)-gossypol and its enantiomer (AT-101) are natural BH3 mimetics that bind to the BH3 binding pocket of Bcl-2 and Bcl-xL to inhibit antiapoptotic functions (40–42) or induce autophagic cell death in apoptosis-resistant cancer cells (43). In agreement, we also found that treatment with (−)-gossypol led to inhibition of cell viability (Fig. 1B) and induction of apoptosis in different kinds of prostate cancer cells (Fig. 1C). However, recent work identifies a new function for Bcl-2 in cancer biology that is beyond its classic role in cell survival by its close relationship with VEGF (28, 44). VEGF from paracrine/autocrine of tumor cells and endothelial cells induces expression of Bcl-2 in tumor-associated microvascular endothelial cells (45). Upregulated Bcl-2 expression in microvascular endothelial cells is sufficient to enhance intratumoral angiogenesis and to accelerate tumor growth (29). Interestingly, Bcl-2 in turn functions as a proangiogenic molecule through its ability to activate the NF-κB signaling pathway and to induce expression of the proangiogenic CXCL8.

Figure 6. (−)-Gossypol inhibits activation of key proangiogenic molecules involved in VEGF signaling. A, (−)-gossypol inhibited VEGF-induced activation of Src, FAK, AKT, and ERK kinase in endothelial cells. Several key signaling molecules that mediate angiogenesis were analyzed by Western blotting assay. HUVECs were starved in serum-free medium for 4 to 6 hours, pretreated with (−)-gossypol for 30 minutes, and then stimulated with 50 ng/mL VEGF for 5 to 20 minutes. Protein was harvested for the analysis. B, schematic model depicted the (−)-gossypol-mediated antiangiogenic signaling pathway. (−)-Gossypol potentially affected the production of VEGF released from tumor cells and endothelial cells by inhibition of Bcl-2 family protein, and further it blocked the activation of VEGFR2 and intracellular kinases in vascular endothelial cells.
and CXCL1 chemokines from endothelial cells to affect nearby tumor cells (30). Therefore, the VEGF/Bcl-2/CXCL8 pathway suggests new targets for the development of antiangiogenic strategies. And nowadays, siRNA and small molecule inhibitors of Bcl-2 are being developed to inhibit solid tumors (46–48). In our present investigation, we showed that treatment of (-)-gossypol led to obvious downregulation of VEGF in both cancer cells and endothelial cells (Fig. 1C), which help to significantly decrease VEGF concentration in tumor microenvironment in vivo. As shown in Fig. 1D, the suppression on Bcl-2/Bcl-XL by (-)-gossypol paralleled with its inhibition on VEGF, which partially suggested that the Bcl-2/VEGF signaling pathway could be blocked by (-)-gossypol. Consequently, the biological dysfunctions of activated endothelial cells with higher Bcl-2 expression can be rectified by treatment of (-)-gossypol (Fig. 3). Previous study revealed that (-)-gossypol inhibited NF-kB activity and NF-kB-mediated gene expression (21). Although we did not examine the CXC chemokine production in the treated cells, combination of these observations confirmed the antiangiogenic effect of (-)-gossypol in cancer treatment.

Furthermore, we investigated the molecular events associated with the antiangiogenic activity of (-)-gossypol in endothelial cells. It is shown that Bcl-2 gene expression is significantly higher in the tumor-associated endothelial cells as compared with normal endothelial cells (28, 45). Tumor cell- or endothelial cell-derived VEGF signals to modulate endothelial cell proliferation, migration, and differentiation in a pathway that requires its binding to VEGFR2 and activation of downstream signaling (9, 45). In the present study, we found that (-)-gossypol dose dependently inhibited VEGFR2 kinase activity, with the half maximal inhibitory concentration of 2.38 μmol/L (Fig. 5C). Although there are 3 tyrosine kinase receptors, VEGFR1, VEGFR2, and VEGFR3, expressed in endothelial cells, the VEGF signaling events relevant to tumor angiogenesis are mainly mediated by VEGFR2 (49). Conversely, VEGFR1 (Flt-1) is a dual regulator of angiogenesis with very low activity in endothelial cells, even in VEGFR1-overexpressing primary endothelial cells in culture, and VEGFR3 is the critical modulator of lymphangiogenesis. With a specific pattern, VEGFR2 activation results in activation of diverse intracellular substrates in endothelial cells. Our data revealed that the phosphorylation of Src, FAK, AKT, and ERK kinases induced by VEGF were all suppressed by (-)-gossypol (Fig. 6A). Src kinase has been reported to participate in tumor angiogenesis via regulating gene expression of proangiogenic growth factors and cytokines, especially VEGF and IL-8 (50). As evidenced by a previous report that (-)-gossypol had ability to decrease VEGF and IL-8 expression in cancer cells (27), we reason that this effect is partially due to the inhibition of (-)-gossypol on activation of these intracellular kinases.

When compared with the inhibitory effect of (-)-gossypol in endothelial cells and prostate cancer cells, we found that the effective concentration in activated endothelial cells was much lower than that in cancer cells, suggesting that biological alterations of endothelial cells (angiogenesis) might be primary targets of (-)-gossypol in tumor inhibition in vivo at relative low dosage. It is noteworthy that 5 μmol/L of (-)-gossypol is sufficient to inhibit VEGF-induced angiogenic responses in vitro (Figs. 3, 5, and 6) and ex vivo angiogenesis assays (Fig. 4), whereas 10 μmol/L of (-)-gossypol completely blocks microvessel sprouting (Fig. 4). However, higher concentrations of gossypol are required to inhibit cancer cell viability and to induce cancer cell apoptosis (Fig. 1B and C). These data suggest that antiangiogenic activity of (-)-gossypol in vivo is probably much earlier than its toxic effects on tumor cells.

In conclusion, we found that (-)-gossypol potently inhibited angiogenesis-mediated tumor growth by modulating VEGF signaling (Fig. 6B), which suggested the therapeutic potential in the treatment of human cancers in clinical settings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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