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(−)-Gossypol, a Natural BH3 Mimetic, 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 demonstrated that (−)-gossypol dose-dependently inhibited the expression of vascular endothelial growth factor (VEGF), Bcl-2 and Bcl-xL in human prostate cancer cells (PC-3 and DU 145) and human primary cultured umbilical vein endothelial cells (HUVECs) *in vitro*. Notably, the growth of human prostate tumor PC-3 xenografts in mice was significantly suppressed by (−)-gossypol at 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, VEGF-triggered phosphorylation of key intracellular proangiogenic molecules, such as Src family kinase, focal adhesion kinase, extracellular signal-related kinase and AKT kinase, were all suppressed by the treatment. Taken together, the present study demonstrates that (−)-gossypol potently inhibits human prostate tumor growth through modulating VEGF signaling pathway, which further validates its great potential in clinical practice.

Keywords

(−)-Gossypol; tumor angiogenesis; VEGF; VEGF receptor 2 (KDR/Flk-1); Bcl-2

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

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(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 vascular endothelial growth factor (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) (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) (8), phosphatidylinositol 3-kinases/AKT kinase (9, 10), extracellular signal-related kinase (ERK1/2) (11), mammalian target of rapamycin 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 mimetic (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-beta/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 Ca^{2+} (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. Additionally, 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 up-regulated 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 (St. Louis, MO). 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 National Institutes of Health (NIH; Bethesda, MD). Growth factor–reduced Matrigel was purchased from BD Biosciences (San Jose, CA). Antibodies against ERK1, Bcl-2, VEGF and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Poly (ADP-ribose) polymerase (PARP), Bcl-xL, VEGFR2, AKT, Src, FAK and phospho-specific anti-VEGFR2 (Tyr^{1175}) and anti-VEGFR2 (Tyr^{996}), anti-Src (Tyr^{416}), anti-FAK (Tyr^{397}), anti-AKT (Ser^{473}), anti-pERK1/2 (Thr^{202}/Tyr^{204}) were purchased from Cell Signaling Technology (Danvers, MA). Antibody against CD31was bought from Epitomics (Burlingame, CA).

Cell lines and cell culture

Primary human umbilical vascular endothelial cells (HUVECs) kindly gifted from Dr. Xinli Wang (Cardiothoracic Surgery Division of Michael E. DeBakey Department of Surgery at Baylor College of Medicine in Houston) 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 got from American Type Culture Collection (ATCC; Manassas, VA) in 2009. HMEC-1 was cultured with MCDB 131 medium (Sigma; St Louis, MO) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 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 Medium (Hyclone) supplemented with 10% FBS. Western blotting using epithelial markers authenticated that they were of epithelial origin before experiments. All these cells were tested for mycoplasma-free by PCR methods before use and maintained at 37°C under a humidified 95%:5% (v/v) mixture of air and CO_2.

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 6~7 mice. PC-3 cells were grown to 80–90% confluence, harvested, prepared at 5×10^6 cells/100 μL cell suspensions, and inoculated on the flank region of nude mice. After tumors grew to about 50 mm^3, mice were treated with or without (−)-gossypol (15 mg/kg) by daily intralesional injections for consecutive 50 days. (−)-Gossypol (dissolved in DMSO) was delivered through one or two injection sites around the tumors, depending on tumor size at the time of injection. The control mouse group was administrated with the control solution containing the same amount of DMSO without the drug. The body weight of each mouse was recorded every 5 days. The volume of solid tumors were determined using Vernier caliper measurement and calculated according to the formula of $A\times B^2\times 0.52$, where A is the longest diameter of the tumor and B is the shortest. After 50 d, mice were sacrificed.

Histology and immunohistochemistry

Solid tumors were fixed with 10% formaldehyde and embedded in paraffin. Antibodies against CD31, VEGFR2 and VEGF were applied to indicate infiltrating blood vessel and detect VEGF expression on 5-μm tumor sections. Images were taken using a Leica DM 4000B photo microscope (Solms, Germany; magnification, 400×). The microvessel density was calculated statistically by using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD) according to CD31 immunohistochemistry (n=5).

Cell viability assay

PC-3 and DU 145 ($5\sim6\times10^3$ cells/well) cells were directly incubated with indicated concentrations of (−)-gossypol for 48 h. HUVECs ($6\sim7\times10^3$ cells/well) were treated with or

without VEGF (50 ng/mL) and various concentrations of (−)-gossypol for 48 h. To determine cell viability, we used a CellTiter 96 AQueous One Solution Cell Proliferation kit (Promega; Madison, WI) and a VERSAmax microplate reader (Molecular Devices; Sunnyvale, CA).

Endothelial cell migration assay

Transwell migration assay was performed as described previously (32). Briefly, HUVECs (2×10^4 cells/well) or HMEC-1 (2×10^4 cells/well) along with the indicated concentrations of (−)-gossypol were seeded into the upper chambers. The bottom chambers were filled with 500 μL basal endothelial cell culture medium supplemented with 0.5% FBS and 30 ng/mL VEGF. After 6–8 h incubation, migrated cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an OLYMPUS inverted microscope (Olympus; magnification, 160×). Three independent experiments were performed.

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 h and then seeded onto the Matrigel layer in 24-well plates at a density of $4\sim7\times10^4$ cells. ECM (0.5% FBS) with or without 30 ng/mL of VEGF was added into wells. After 4~6 h, tubulogenesis was fixed and photographed using an inverted microscope (Olympus; original magnification, 100×). Three independent experiments were performed.

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 1~1.5 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 d. After a week, microvessel sprouting was fixed and photographed using an inverted microscope (Olympus, Center Valley, PA; 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 h. To detect the expression of VEGF, Bcl-2 and Bcl-xL in treated cancer cells and endothelial cells, PC-3, DU 145 and HUVECs were incubated with (−)-gossypol for 24 h. To determine the molecular basis of (−)-gossypol in angiogenesis signaling, HUVECs were first starved in serum-free ECM for 4–6 h and then pretreated with or without various concentrations of (−)-gossypol for 30 min, followed by stimulation with 50 ng/mL of VEGF for 2~20 min. The whole-cell extracts were prepared in RIPA 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 PMSF) supplemented with proteinase inhibitor cocktail (Calbiochem, San Diego, CA). About 40~50 μg of cellular protein from each sample was applied to 6%–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 concentration was determined using bicinchoninic acid assay and equalized before loading. Relative optical density of blotting bands was qualified by Image J software (NIH; Bethesda, MD).

In vitro VEGFR2 kinase Inhibition assay

VEGFR2 kinase assay was performed using an HTScan VEGFR2 kinase kit from Cell Signaling Technology (Danvers, MA) combined with colorimetric ELISA detection as described previously (33). The final reaction system contained 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 different concentrations of (−)-gossypol.

Statistical analysis

Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Student's *t*-test. Data were presented as means ± standard error. *P* values ≤ 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 ~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 poly (ADP-ribose) polymerase (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 active nuclear factor-κB (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 conducted a prostate tumor (PC-3) xenograft mouse model. We found that administration of (−)-gossypol (15 mg/kg/d) for 50 d substantially suppressed tumor volume (Fig. 2A) and reduced tumor weight (Fig. 2B) compared to 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 mm^3 to 829.83 ±187.91 mm^3 at the end of the experiments, whereas that in the (−)-gossypol-treated group decreased from 58.46 ± 3.25 mm^3 to 19.74 ± 7.71 mm^3. Additionally, the average tumor weight of the control group was 397.12 ± 99.69 mg, whereas that in the (−)-gossypol-treated group was only 43.10 ± 16.83 mg (Fig. 1B), 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 *in vitro*

To assess the detailed activities of (−)-gossypol on angiogenesis *in vitro*, we examined whether (−)-gossypol modulated the VEGF-induced proliferation of endothelial cells by MTS assay. As shown in figure 3A, about 2 μmol/L of (−)-gossypol significantly decreased VEGF-induced cell viability in HUVECs after 48 h incubation. We further examined its inhibitory function on the chemotactic motility by the Boyden chamber assay in two 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 figure 3C, when endothelial cells were seeded on two-dimensional Matrigel, robust tubular-like structures were formed. However, treatment with (−)-gossypol (5~10 μmol/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 *in vitro* by inhibiting VEGF-induced cell proliferation, chemotaxis, and capillary-like structure formation of endothelial cells.

(−)-Gossypol inhibits VEGF-induced microvessel sprouting *ex vivo*

To study whether (−)-gossypol could affect angiogenesis *ex vivo*, we examined the sprouting of microvessels from aortic rings in the presence or absence of (−)-gossypol. As shown in figure 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 figure 5A and 5B, (−)-gossypol (5 μmol/L) strongly inhibited VEGF-activated VEGFR2 phosphorylation at both Tyr 1175 site and Tyr 996 site. To confirm this result, we performed *in vitro* kinase assay using a kinase kit. Our data demonstrated 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 5~10 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.

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 *in vivo* medicated by (−)-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-8 levels after treatment with 10 μmol/L of (−)-gossypol in human prostate or ovarian cancer cells (26, 27), indicating (−)-gossypol could affect the profile of proangiogenic factors released from tumors. This information provide us significant clue to study the direct antiangiogenic role of (−)-gossypol *in vitro* and *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 *in vitro* (Fig. 1C) and *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). Up-regulated 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 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 anti-angiogenic strategies. And nowadays, short interfering

RNA and small molecule inhibitors of Bcl-2 are being developed to inhibit solid tumors (46–48). In our present investigation, we demonstrated 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 (−)-gossypol's treatment (Fig. 3). Previous study revealed (−)-gossypol inhibited NF-κB activity and NF-κB-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.

Further, 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–derived 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 three tyrosine receptor kinases, VEGFR-1, -2, and -3, 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 interleukin 8 (50). As evidenced by previously 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 target 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* (Fig. 3, 5 and 6) and *ex vivo* angiogenesis assays (Fig. 4) while 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 1C). These data suggest that (−)-gossypol's antiangiogenic activity *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.

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Abbreviations

VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
HUVECs	human umbilical vein endothelial cells
HMEC-1	human microvascular endothelial cells
ERK	extracellular signal-regulated protein kinase
FAK	focal adhesion kinase
NF-κB	nuclear factor-kappa B
PARP	Poly (ADP-ribose) polymerase
ECM	endothelial cell culture medium
DMSO	dimethyl sulfoxide

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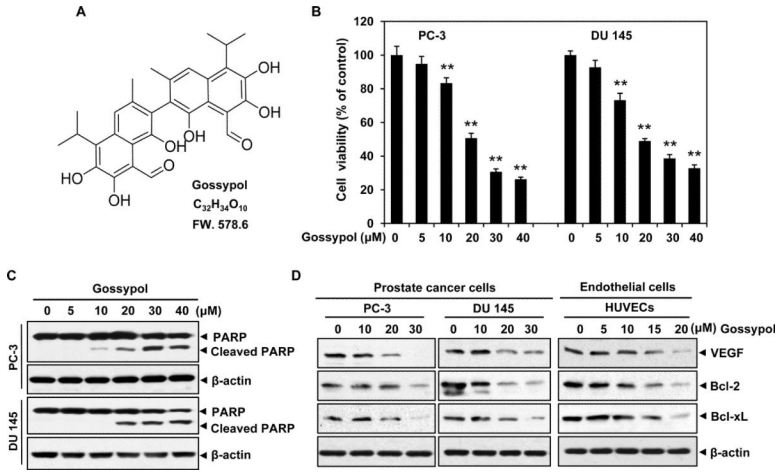

Figure 1. (−)-Gossypol decreases cell viability via apoptosis induction and inhibits Bcl-2/Bcl-xL/VEGF signaling in prostate cancer cells and endothelial cells

A, the chemical structure of (−)-gossypol. *B*, (−)-gossypol inhibited prostate cancer cell viability in a dose-dependent manner. PC-3 and DU 145 cells ($5\sim6\times10^3$ cells) were directly treated with or without various concentrations of (−)-gossypol for 48 h. Cell viability was quantified by MTS assay. *Columns*, mean; *bars*, standard error; **, $P < 0.01$ *vs.* untreated group. *C*, (−)-gossypol induced potent apoptosis in prostate cancer cells. PC-3 and DU 145 cells were treated with (−)-gossypol for 24 h. The whole cell protein was applied to western blotting analysis. The full length of PARP was cleaved into 89 KD form as indicated. *D*, (−)-gossypol suppressed the expression of VEGF, Bcl-2 and Bcl-xL in human prostate cancer cells and endothelial cells. PC-3, DU 145 and HUVECs were incubated with (−)-gossypol for 24 h. The whole cell protein was harvested and probed with specific antibodies.

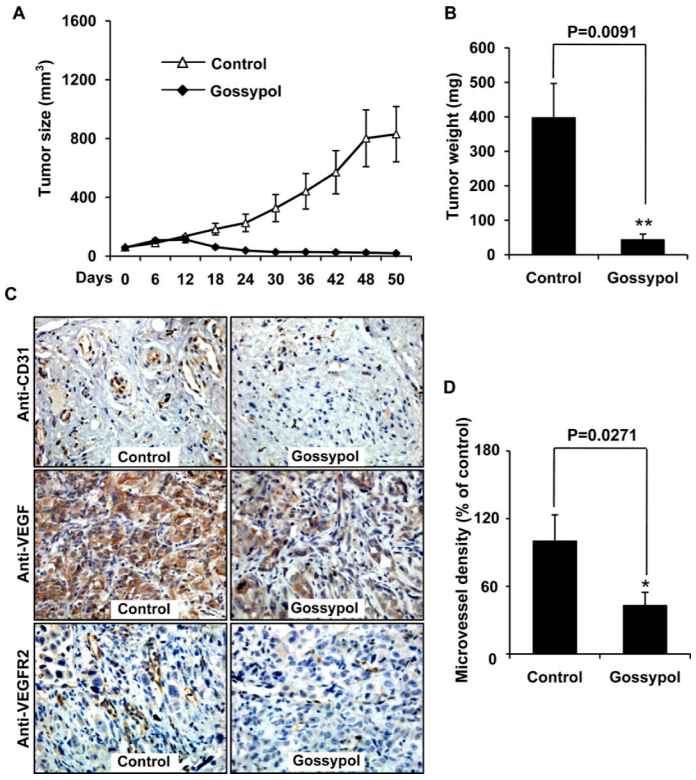

Figure 2. (−)-Gossypol suppresses tumor growth and angiogenesis of human prostate tumor xenografts

PC-3 cells were injected into 5- to 6-week-old BALB/cA nude mice (5×10^6 cells per mouse). After solid tumors established, the mice were subcutaneously treated with or without (−)-gossypol at dosage of 15 mg/kg daily. *A*, (−)-gossypol inhibited tumor growth as measured by tumor volume. *B*, the weight of solid tumors in the (−)-gossypol-treated mice was significantly lower than that of the control group. *C*, anti-CD 31, anti-VEGF and anti-VEGFR2 immunohistochemistry revealed that (−)-gossypol inhibited neovascularization and VEGF expression in solid tumors. *D*, microvessel density was analyzed by Image-Pro Plus 6.0 software. *Columns*, mean; *bars*, standard error; *, $P < 0.05$ *vs.* the control group.

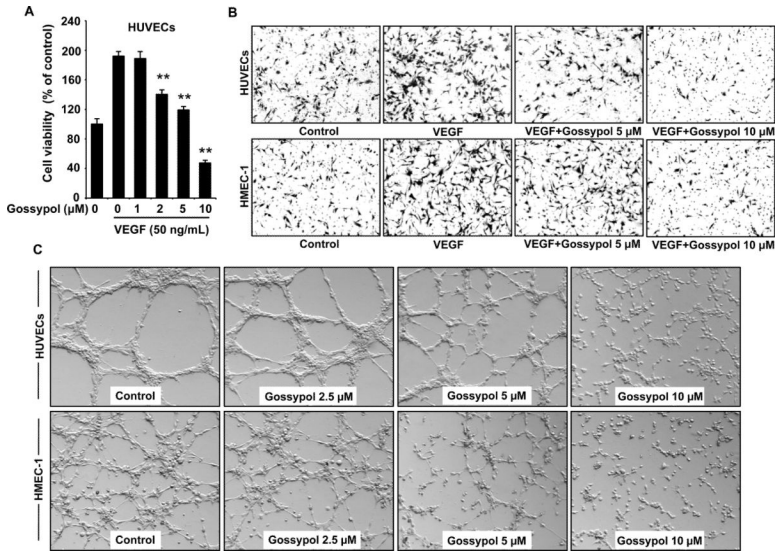

Figure 3. (−)-Gossypol inhibits VEGF-induced viability, chemotactic motility and capillary-like structure formation of endothelial cells

A, (−)-gossypol dose-dependently inhibited VEGF-induced cell viability. HUVECs ($6\sim7\times10^3$ cells/well) were starved with serum-free medium and then treated with or without VEGF (50 ng/mL) and various concentrations of (−)-gossypol for 48 h. Cell viability was quantified by MTS assay. *Columns*, mean; *bars*, standard error; **, $P < 0.01$ *vs.* VEGF alone group. *B*, (−)-gossypol inhibited cell migration in HUVECs and HMEC-1. Endothelial cells were seeded in the upper chamber of Transwells and treated with different concentrations of (−)-gossypol. The bottom chamber was filled with medium supplemented with 30 ng/mL VEGF. Cells that migrated through the membrane were photographed (magnification, 160×). *C*, (−)-gossypol inhibited the capillary-like structure formation in endothelial cells. Pretreated HUVECs and HMEC-1 were placed in 24-well plates coated with Matrigel. After 4~6 h, cells were fixed, and tubular structures were photographed (original magnification, 100×).

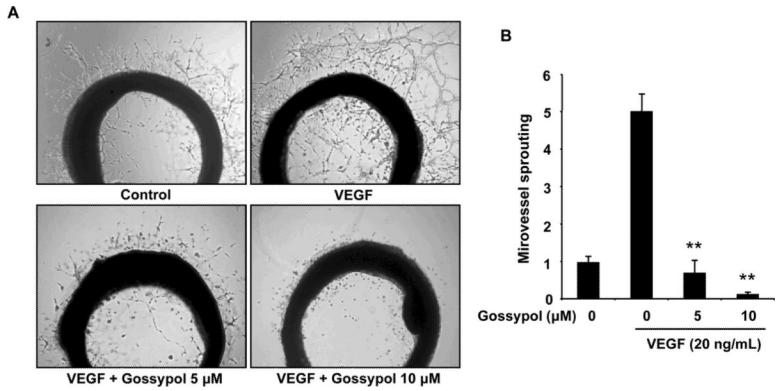

Figure 4. (−)-Gossypol inhibits VEGF-induced microvessel sprouting *ex vivo*

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 d. *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*, standard error; **, $P < 0.01$ *vs*. VEGF alone group.

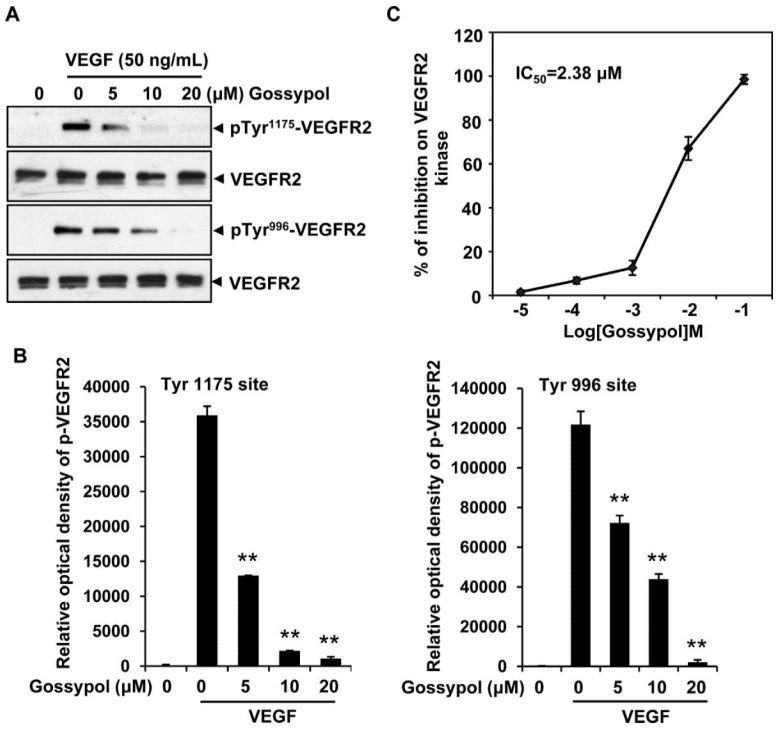

Figure 5. (−)-Gossypol is a VEGFR2 kinase inhibitor

A, (−)-gossypol suppressed the activation of VEGFR2 triggered by VEGF in endothelial cell. HUVECs were starved in serum-free medium for 4~6 h, pretreated with (−)-gossypol for 30 min, and then stimulated with 50 ng/mL VEGF for 2 min. The activation of VEGFR2 from different treatments was analyzed by western blotting and probed with anti-phospho-VEGFR2 antibody at Tyr 1175 and Tyr 996 sites. *B*, the relative optical density was qualified by Image J software. *C*, (−)-gossypol inhibited VEGFR2 kinase activity *in vitro*. *Dots*, mean; *Bars*, standard error.

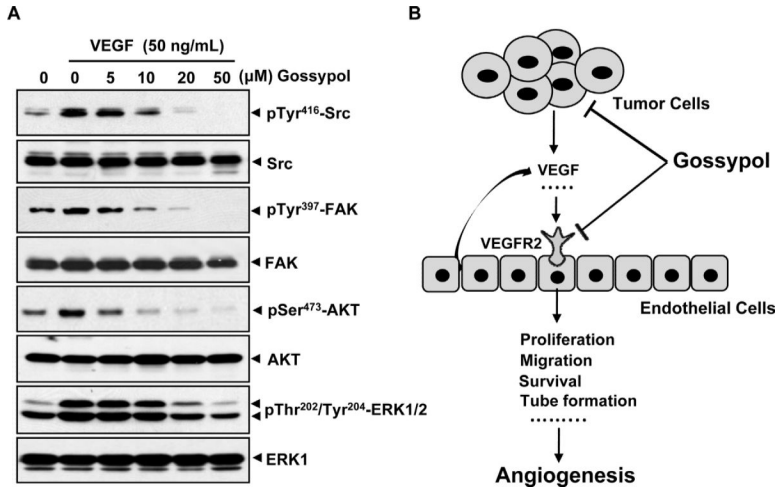

Figure 6. (−)-Gossypol inhibits activation of key proangiogenic molecules involved in VEGF signaling

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 4~6 h, pretreated with (−)-gossypol for 30 min, and then stimulated with 50 ng/mL VEGF for 5~20 min. 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.