

# z-Guggulsterone, a constituent of Ayurvedic medicinal plant *Commiphora mukul*, inhibits angiogenesis *in vitro* and *in vivo*

Dong Xiao and Shivendra V. Singh

Department of Pharmacology and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

## Abstract

Our previous studies have shown that z-guggulsterone, a constituent of Indian Ayurvedic medicinal plant *Commiphora mukul*, inhibits the growth of human prostate cancer cells by causing apoptosis. We now report a novel response to z-guggulsterone involving the inhibition of angiogenesis *in vitro* and *in vivo*. The z-guggulsterone treatment inhibited capillary-like tube formation (*in vitro* neovascularization) by human umbilical vein endothelial cells (HUVEC) and migration by HUVEC and DU145 human prostate cancer cells in a concentration- and time-dependent manner. The z- and E-isomers of guggulsterone seemed equipotent as inhibitors of HUVEC tube formation. The z-guggulsterone-mediated inhibition of angiogenesis *in vitro* correlated with the suppression of secretion of proangiogenic growth factors [e.g., vascular endothelial growth factor (VEGF) and granulocyte colony-stimulating factor], down-regulation of VEGF receptor 2 (VEGF-R2) protein level, and inactivation of Akt. The z-guggulsterone-mediated suppression of DU145 cell migration was increased by knockdown of VEGF-R2 protein level. Ectopic expression of constitutively active Akt in DU145 cells conferred protection against z-guggulsterone-mediated inhibition of cell migration. Oral gavage of 1 mg z-guggulsterone/d (five times/wk) to male nude mice inhibited *in vivo* angiogenesis in DU145-Matrigel plug assay as evidenced by a statistically significant decrease in tumor burden, microvessel area (staining for angiogenic markers factor VIII and CD31), and VEGF-R2 protein expression. In conclusion, the present study reveals that z-guggulsterone inhibits angiogenesis by suppressing the VEGF-VEGF-R2-Akt signaling axis. Together, our results provide compelling

rationale for further preclinical and clinical investigation of z-guggulsterone for its efficacy against prostate cancer. [Mol Cancer Ther 2008;7(1):171–80]

## Introduction

Angiogenesis (the formation of new blood vessels) is implicated in the pathogenesis of many chronic diseases including cancer (1, 2). Angiogenesis in the tumor mass permits growth and invasiveness of the cancer cells (3, 4). Accumulating evidence suggests that angiogenesis is a critical event in the progression of solid tumors because tumor growth beyond 2 to 3 mm is often preceded by an increase in the formation of new blood vessels (2–4). Angiogenesis is believed to be essential for the delivery of essential nutrients and oxygen to the tumor microenvironment (2–4). Identification and preclinical/clinical development of novel antiangiogenic agents continues to be a topic of intense research (5–7). It is interesting to note that several promising antiangiogenic agents are natural products (5–7).

Guggulsterone [4,17(20)-pregnadien-3,16-dione] is a plant sterol derived from the gum resin (guggulu) of the Indian Ayurvedic medicinal plant *Commiphora mukul* that has been safely used for thousands of years for the treatment of different disorders including bone fracture, arthritis, and inflammation (8–10). Guggulsterone is an antagonist of farnesoid X receptor (11, 12). In addition, guggulsterone increases the transcription of bile salt export pump and regulates cholesterol homeostasis (11–14). We have shown previously that the *trans*-(z)-isomer of guggulsterone inhibits the growth of PC-3, DU145, and LNCaP human prostate cancer cells in culture by causing apoptosis (15, 16). Interestingly, a normal prostate epithelial cell line (PrEC) is significantly more resistant to growth inhibition and apoptosis induction by z-guggulsterone compared with prostate cancer cells (15). The z-guggulsterone-induced cell death in PC-3 cells was not influenced by Bcl-2 protein level but correlated with the induction of proapoptotic multidomain Bcl-2 family members Bax and Bak (15). More recent studies from our laboratory have revealed that the z-guggulsterone-induced apoptosis in human prostate cancer cells is initiated by reactive oxygen intermediate-mediated activation of *c-Jun* NH<sub>2</sub>-terminal kinase (16). Antiproliferative and/or apoptosis-inducing effects for guggulsterone have also been documented in other cell types including human lung, acute myeloid leukemia, and breast cancer cells (17, 18).

Shishodia and Aggarwal (17) have shown that guggulsterone suppresses the activation of constitutive and inducible nuclear factor- $\kappa$ B (NF- $\kappa$ B), a transcription factor, induced by cigarette smoke condensate, tumor promoters (phorbol myristate acetate and okadaic acid), hydrogen

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**Requests for reprints:** Shivendra V. Singh, 2.32A Hillman Cancer Center Research Pavilion, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-623-3263; Fax: 412-623-7828. E-mail: singhs@upmc.edu

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peroxide and cytokines (interleukin-1 $\beta$  and tumor necrosis factor). Subsequently, the same group of investigators showed inhibition of receptor activator of NF- $\kappa$ B ligand- and tumor cell-induced osteoclastogenesis by this agent (19). Guggulsterone treatment blocked the NF- $\kappa$ B signaling pathway by targeting I $\kappa$ B kinase in intestinal epithelial cells and ameliorated acute murine colitis (20).

The NF- $\kappa$ B regulates the expression of various genes including inflammatory cytokines, chemokines, cell adhesion molecules, and growth factors including vascular endothelial growth factor (VEGF; ref. 21). Because the expression of VEGF, which plays a critical role in angiogenesis (22), is regulated by NF- $\kappa$ B (17, 21), we postulated that guggulsterone might inhibit angiogenesis. The present study shows that *z*-guggulsterone inhibits angiogenic features (capillary-like tube formation and/or migration) of human umbilical vein endothelial cells (HUVEC) and DU145 human prostate cancer cells *in vitro*.

Furthermore, oral gavage of *z*-guggulsterone to male nude mice (five times/wk) inhibits *in vivo* angiogenesis in DU145-Matrigel plug assay.

## Materials and Methods

### Reagents

The *z*-(*trans*) and *E*-(*cis*) isomers of guggulsterone were purchased from Steraloids. Cell culture reagents and fetal bovine serum were procured from Life Technologies. An antibody specific for the detection of phosphorylated (Ser<sup>473</sup>)-Akt was from Cell Signaling Technology, and the antibodies against total Akt and VEGF receptor R2 (VEGF-R2) were from Santa Cruz Biotechnology. Matrigel was from BD PharMingen. The ELISA kits for measurements of VEGF, epidermal growth factor (EGF), granulocyte colony-stimulating factor (G-CSF), fibroblast growth factor (FGF), interleukin (IL)-12, IL-17, and matrix metalloproteinase (MMP)-2, and MMP-9 were from R&D Systems or Biosource. Akt-1/2 inhibitor [1,3-dihydro-1-1(1-((4-(6-phenyl-1H-imidazo(4,5-g)quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one] was purchased from Calbiochem.

### Cell Culture and Cell Viability Assay

HUVEC were purchased from Clonetics and maintained in endothelial cell growth medium-2 (EGM2 MV Single-Quots; Clonetics) supplemented with 5% fetal bovine serum. Monolayer cultures of DU145 cells were maintained as we have previously described (16). Stock solutions of each isomer of guggulsterone were prepared in DMSO and diluted with complete medium. An equal volume of DMSO (final concentration <0.2%) was added to the controls. The effects of *z*- and *E*-guggulsterone treatments on HUVEC viability was determined by sulforhodamine B assay as we have previously described (23).

### *In vitro* Capillary-Like Tube Structure Formation and Migration Assays

The effects of *z*- and *E*-guggulsterone treatments on *in vitro* angiogenesis were determined by tube formation assay as we have previously reported (24). The HUVEC seeded on Matrigel differentiate and form capillary-like

tube structures. In some tube formation experiments, the HUVEC were exposed to 20  $\mu$ mol/L of *z*-guggulsterone for 24 h in the absence or presence of 1  $\mu$ mol/L of the Akt-1/2 inhibitor. The effect of *z*-guggulsterone treatment on *in vitro* migration by HUVEC or DU145 cells was determined using a Transwell Boyden Chamber (Corning) containing a polycarbonate filter (pore size 8  $\mu$ m) as we have previously described (24). In some migration assays, HUVEC or DU145 cells were treated with 20  $\mu$ mol/L of *z*-guggulsterone for 24 h in the absence or presence of 1  $\mu$ mol/L of Akt-1/2 inhibitor.

### Immunoblotting

The immunoblotting of total Akt, phosphorylated Akt, and VEGF-R2 was done as we have previously described (23). Briefly, HUVEC or DU145 cells were treated with desired concentrations of *z*-guggulsterone for specified time periods, and both floating and attached cells were collected. The cell lysates were prepared as we have previously described (23, 24). The lysate proteins were resolved by 6% to 10% SDS-PAGE and transferred onto polyvinylidene fluoride membrane. After treatment with the desired primary and secondary antibodies, the immunoreactive bands were visualized using an enhanced chemiluminescence method. The blots were stripped and reprobed with antiactin antibody to correct for differences in protein loading. Changes in protein levels were determined by densitometric scanning of the immunoreactive bands. The immunoblotting for each protein was done at least twice using independently prepared lysates.

### Analyses of Growth Factors, Interleukins, and MMPs

HUVEC or DU145 cells ( $2 \times 10^5$ ) were seeded in 24-well plates and allowed to attach by overnight incubation. Cells were treated with the desired concentrations of *z*-guggulsterone or DMSO (control) for 24 and 48 h. Subsequently, the culture medium was collected and used to determine the secretion of VEGF, EGF, G-CSF, FGF, IL-12, IL-17, MMP-2, and MMP-9 using commercially available ELISA kits as we have previously described (25).

### RNA Interference of VEGF-R2

RNA interference of VEGF-R2 was done using a VEGF-R2-targeted short interfering RNA (siRNA; Upstate Biotechnology). A nonspecific control siRNA was purchased from Qiagen. For transfection, DU145 cells ( $5 \times 10^4$ ) were seeded in six-well plates and allowed to attach overnight. Cells were transfected with 200 nmol/L of control nonspecific siRNA or VEGF-R2-targeted siRNA using OligofectAMINE (Invitrogen) according to the manufacturer's recommendations. Twenty-four hours after transfection, the cells were treated with DMSO (control) or 20  $\mu$ mol/L of *z*-guggulsterone for 24 h. The cells were collected and processed for analysis of migration and immunoblotting as described above.

### Ectopic Expression of Constitutively Active Akt

DU145 cells were transiently transfected with pCMV6 vector encoding constitutively active Akt-1 (Myr-Akt1-HA; kindly provided by Dr. Daniel Altschuler, University of Pittsburgh, PA) or empty vector using Fugene 6 transfection reagent (Roche Applied Science). Briefly, DU145 cells

were seeded in six-well plates at a density of  $2 \times 10^5$  cells/mL and allowed to attach by overnight incubation. Cells were transfected with the expression vector encoding constitutively active Akt or empty vector. Twenty-four hours after transfection, the cells were treated with 20  $\mu\text{mol/L}$  of z-guggulsterone or DMSO (control) for 24 h and processed for immunoblotting of total or phosphorylated Akt levels and migration assay.

#### *In vivo* Matrigel Plug Assay

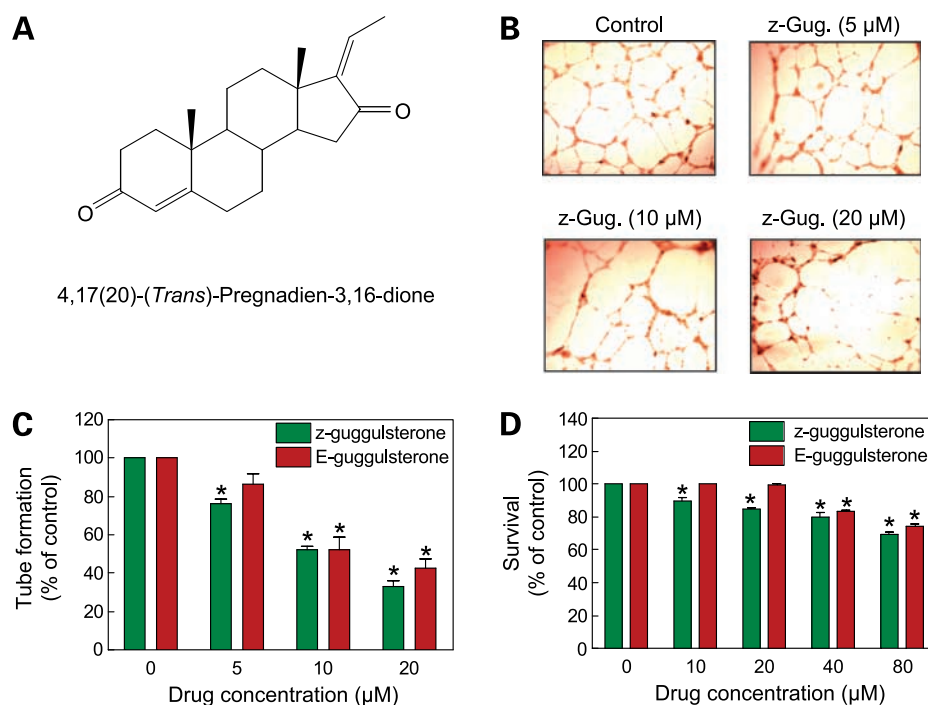
The effect of z-guggulsterone administration on *in vivo* angiogenesis was determined by DU145-Matrigel plug assay. Male nude mice (5–6 weeks old) were purchased from Taconic and randomized into two groups of five mice per group. The mice were orally gavaged with 0.1 mL of vehicle (PBS) or 1 mg of z-guggulsterone/mouse in 0.1 mL of PBS (corresponding to  $\sim 40$  mg z-guggulsterone/kg body weight) five times per week for 2 weeks prior to Matrigel plug implantation. The Matrigel plugs containing  $3 \times 10^6$  DU145 cells were implanted s.c. into the flank of each mouse. The z-guggulsterone and vehicle administration was continued for two more weeks. Tumor volume was determined by using a caliper as we have previously described (26). Body weights of the vehicle-treated control and z-guggulsterone-treated mice were recorded weekly. Mice from each group were also monitored for other symptoms of side effects, including food and water withdrawal and impaired posture or movement. Animals were sacrificed 14 days after Matrigel plug implantation. At the termination of the experiment, the Matrigel plugs from control and z-guggulsterone-treated mice were removed and fixed in 10% neutral-buffered formalin. The fixed Matrigel plugs from control and z-guggulsterone adminis-

tered mice were dehydrated, embedded in paraffin, and sectioned at 4  $\mu\text{m}$  of thickness. Sections from control and z-guggulsterone administered mice were used for immunohistochemical analysis of CD31, factor VIII, and VEGF-R2. Quantitative image analysis of the microvessel area based on CD31 and factor VIII immunostaining was done using Image Analysis software (Media Cybernetics).

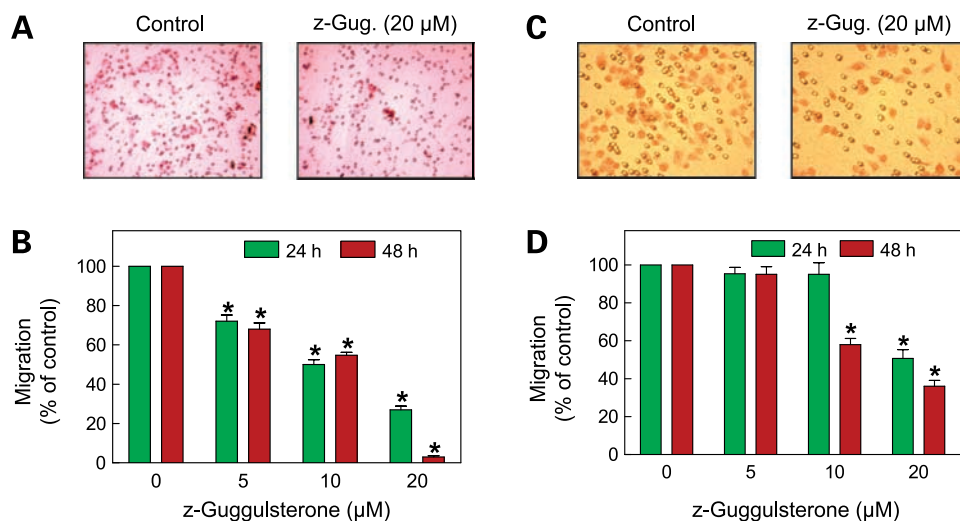
## Results

### Guggulsterone Treatment Inhibited the Formation of Capillary-Like Tube Structures by HUVEC

Initially, we determined the effects of z- (*trans*) and E- (*cis*) isomers of guggulsterone (refer to Fig. 1A for the structure of the z-isomer) on the formation of capillary-like tube structures using HUVEC, which is a well-accepted technique to measure *in vitro* angiogenesis. Figure 1B depicts capillary-like tube structures following a 24 h treatment of HUVEC with DMSO (control) or different concentrations of z-guggulsterone. The capillary-like tube structures were clearly visible in DMSO-treated control HUVEC (Fig. 1B). The z-guggulsterone treatment caused the disruption of the capillary-like tube network in a concentration-dependent manner (Fig. 1B). The capillary-like tube structures were scored from control and z-guggulsterone-treated HUVEC and the results are summarized in Fig. 1C. The formation of capillary-like tube structures was inhibited by  $\sim 24\%$ , 48%, and 67% by a 24-h treatment of HUVEC with 5-, 10- and 20  $\mu\text{mol/L}$  of z-guggulsterone, respectively, compared with DMSO-treated control (Fig. 1C). The z-guggulsterone-mediated disruption of the HUVEC capillary-like tube structures was



**Figure 1.** **A**, structure of z-guggulsterone. **B**, formation of capillary-like tube structures by HUVEC following 24 h of treatment with DMSO (control) or the indicated concentrations of z-guggulsterone. **C**, quantitation of tube formation in HUVEC treated for 24 h with the indicated concentrations of z- and E-guggulsterone. **D**, viability of HUVEC following a 24-h treatment with DMSO (control) or the indicated concentrations of z- and E-guggulsterone as judged by sulforhodamine B assay. Results relative to DMSO-treated controls. Columns, mean; bars, SE; \*,  $P < 0.05$ , significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test ( $n = 3$ ). Similar results were observed in two independent experiments.



**Figure 2.** **A**, migration by HUVEC treated for 24 h with DMSO (*control*) or 20 μmol/L of *z*-guggulsterone as determined by modified Boyden chamber assay. **B**, quantitation of HUVEC migration following 24 and 48 h of exposure to the indicated concentrations of *z*-guggulsterone. **C**, DU145 cell migration following 24 h of treatment with DMSO (*control*) or 20 μmol/L of *z*-guggulsterone. **D**, quantitation of DU145 cell migration following 24 and 48 h of exposure to the indicated concentrations of *z*-guggulsterone. Results relative to DMSO-treated control by one-way ANOVA followed by Dunnett's test ( $n = 3$ ). Similar results were observed in two independent experiments.

even more pronounced at the 48-h time point (results not shown). As can be seen in Fig. 1C, the *trans*- and *cis*-isomers of guggulsterone seemed equipotent as inhibitors of HUVEC tube formation.

Next, we considered a possibility that the suppression of tube formation in our model was due to a decrease in HUVEC viability. To explore this possibility, we determined the effects of *z*- and *E*-guggulsterone treatments on HUVEC viability following a 24-h drug treatment. As shown in Fig. 1D, the viability of HUVEC was minimally affected by either *z*- or *E*-guggulsterone at concentrations effective against tube formation (i.e., 10–20 μmol/L concentrations). Thus, the  $IC_{50}$  of *z*- and *E*-guggulsterone for suppression of HUVEC viability (>80 μmol/L; Fig. 1D) was markedly higher compared with the concentrations needed to achieve 50% inhibition of tube formation (~10 μmol/L; Fig. 1C). These results indicated that guggulsterone treatment inhibited angiogenesis *in vitro*. Because the *z*- and *E*-isomers of guggulsterone were more or less equally effective in suppressing HUVEC tube formation, we used only the *z*-isomer for further experimentation, as described below.

#### ***z*-Guggulsterone Treatment Inhibited Migration by HUVEC and DU145 Human Prostate Cancer Cells**

We proceeded to determine the effects of *z*-guggulsterone treatment on migration by HUVEC using a modified Boyden chamber assay (Fig. 2A). In DMSO-treated controls, a large fraction of HUVEC migrated to the bottom face of the membrane. The HUVEC migration was decreased significantly in the presence of *z*-guggulsterone in a concentration- and time-dependent manner (Fig. 2B). For instance, compared with DMSO-treated control, HUVEC migration was inhibited by ~28%, 50%, and 77% upon a 24-h treatment with 5-, 10- and 20 μmol/L of *z*-guggulsterone, respectively (Fig. 2B). Because cell migration and invasion are fundamental to tumor metastasis (27), we raised the question of whether *z*-guggulsterone-mediated suppression of migration was an effect restricted to the HUVEC. We addressed this question by determining the effect of

*z*-guggulsterone treatment on migration by DU145 cells (Fig. 2C). Similar to HUVEC, migration by DU145 cells was inhibited significantly in the presence of *z*-guggulsterone in a concentration- and time-dependent manner (Fig. 2D). These results indicated that *z*-guggulsterone treatment inhibited the migration by HUVEC as well as DU145 cells.

#### ***z*-Guggulsterone Treatment Inhibited Secretion of Proangiogenic Growth Factors in HUVEC and DU145 Cells**

Angiogenic and metastatic potential of cancer cells is regulated by multiple growth factors (VEGF, G-CSF, EGF, FGF), cytokines (IL-12 and IL-17), and MMPs (28–33). We therefore determined the levels of these molecules in medium from HUVEC and DU145 cells cultured for 24 and 48 h in the presence of DMSO (control) or 10 and 20 μmol/L of *z*-guggulsterone. As can be seen in Fig. 3A, the exposure of HUVEC to 10 and 20 μmol/L of *z*-guggulsterone for 24 and/or 48 h resulted in the suppression of secretion into the medium of VEGF (by ~18–32%), G-CSF (up to ~89%), FGF (by ~9–23%), IL-17 (by ~22–33%), and MMP-2 (by ~8–22%; Fig. 3A). A statistically significant decrease in secretion of EGF was observed only after a 48-h treatment of HUVEC with 20 μmol/L of *z*-guggulsterone (~21% decrease compared with DMSO-treated control). Similar to HUVEC, *z*-guggulsterone treatment caused a statistically significant decrease in the secretion of VEGF, G-CSF, IL-17, and MMP-2 in DU145 cells (results not shown). On the other hand, *z*-guggulsterone treatment did not have any appreciable effect on the secretion of MMP-9 and IL-12 in either HUVEC or DU145 cells and FGF in DU145 cells (results not shown).

#### ***z*-Guggulsterone Treatment Decreased the Protein Levels of VEGF-R2 in HUVEC and DU145 Cells**

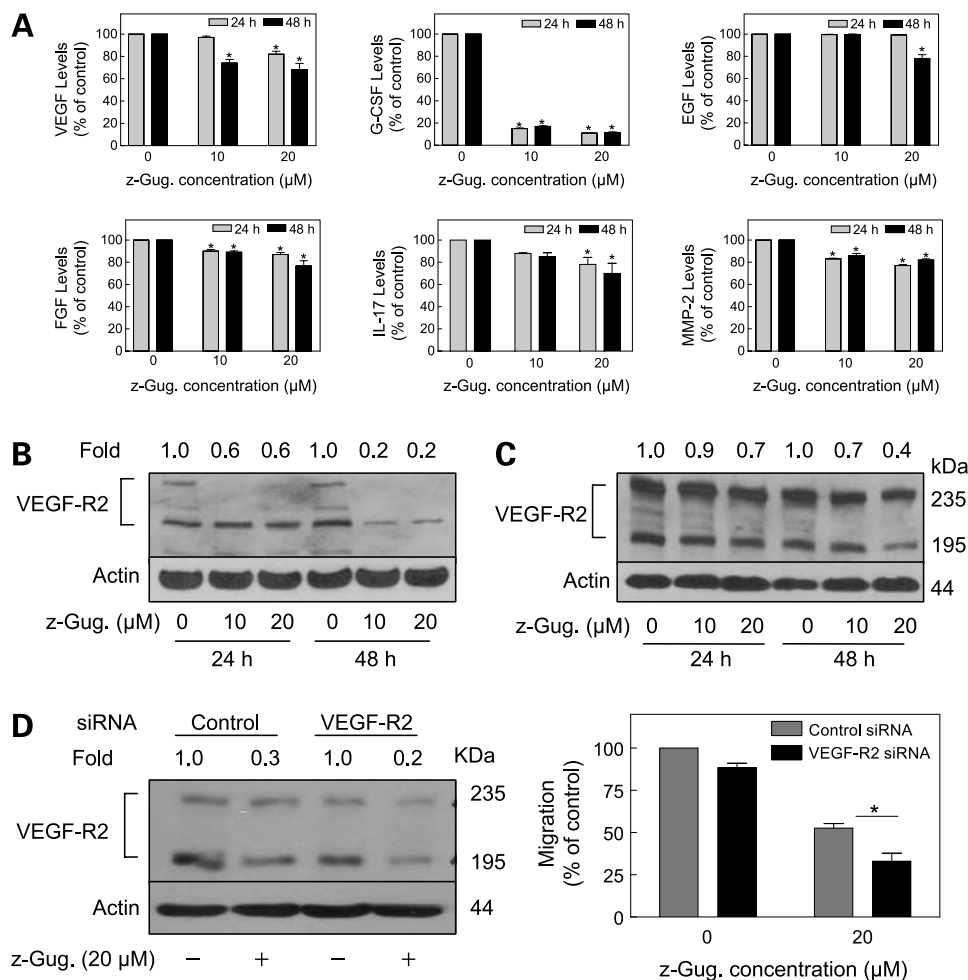
VEGF is one of the most important proangiogenic growth factors, which causes pro-survival signal transduction to the nucleus through interaction with receptors including VEGF-R1, VEGF-R2, and VEGF-R3 (32, 34). Of the three receptors, VEGF-R2 plays an important role in angiogenesis (34). Next, we tested the possibility of whether

z-guggulsterone-mediated inhibition of *in vitro* angiogenesis was associated with a change in protein levels of VEGF-R2. The z-guggulsterone treatment caused a decrease in the level of VEGF-R2 protein in HUVEC (Fig. 3B) and DU145 cells (Fig. 3C). As can be seen in Fig. 3D (left), the level of VEGF-R2 protein was decreased by ~60% in DU145 cells transiently transfected with a VEGF-R2-targeted siRNA compared with DU145 cells transfected with a control nonspecific siRNA. Knockdown of VEGF-R2 protein level only slightly inhibited DU145 migration (compare the percentage of migration in the control nonspecific siRNA-transfected and VEGF-R2-targeted siRNA-transfected DU145 cells in the absence of z-guggulsterone treatment;

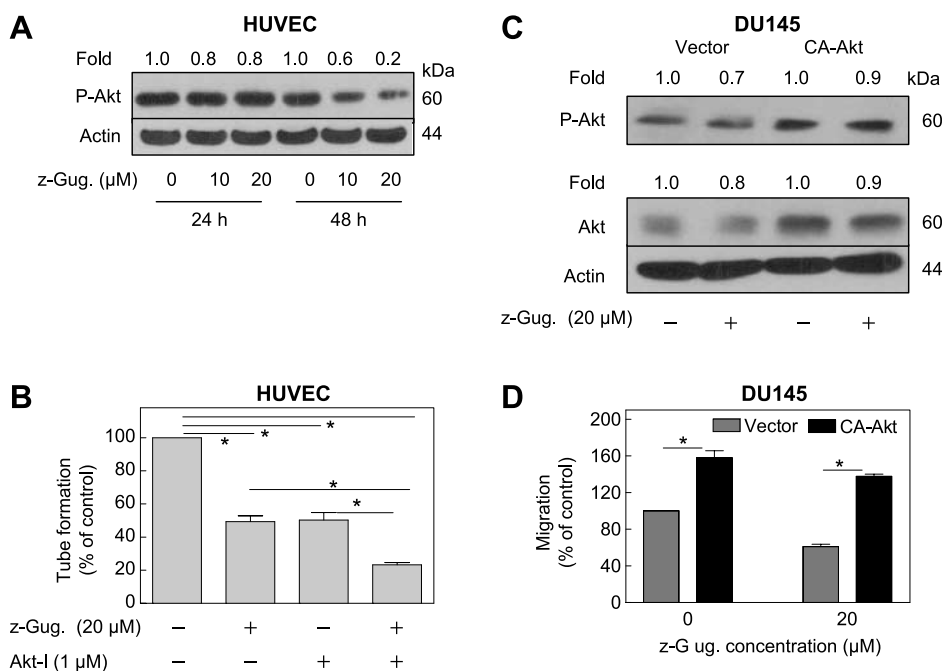
Fig. 3D, right). On the other hand, inhibition of DU145 cell migration resulting from a 24-h exposure to 20  $\mu\text{mol/L}$  of z-guggulsterone was relatively more pronounced in DU145 cells with knockdown of VEGF-R2 protein levels compared with the cells transfected with the control nonspecific siRNA (Fig. 3D, right). Collectively, these results indicated that the z-guggulsterone-mediated inhibition of angiogenesis was probably accomplished, at least in part, by down-regulation of VEGF-R2 protein level.

### z-Guggulsterone Treatment Caused Inactivation of Akt in HUVEC and DU145 Cells

Akt is implicated in the promotion of endothelial cell survival and VEGF-stimulated endothelial cell migration



**Figure 3.** **A**, secretion of VEGF, G-CSF, EGF, FGF, IL-17, and MMP-2 into the media by the HUVEC cultured for 24 or 48 h in the presence of DMSO (control) or the indicated concentrations of z-guggulsterone. Columns, mean; bars, SE; \*,  $P < 0.05$ , significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test ( $n = 3$ ). Immunoblotting for VEGF-R2 using lysates from HUVEC (**B**) and DU145 (**C**) cells treated with DMSO (control) or 10 and 20  $\mu\text{mol/L}$  of z-guggulsterone for 24 or 48 h. The blots were stripped and reprobed with antiactin antibody to ensure equal protein loading. Immunoblotting was done twice using independently prepared lysates and the results were comparable. Changes in protein levels relative to DMSO-treated control, after correction for actin loading control, are shown on top of the immunoreactive bands. **D**, immunoblotting for VEGF-R2 using lysates from DU145 cells transiently transfected with a control nonspecific siRNA or VEGF-R2 targeted siRNA and treated for 24 h with either DMSO (control) or 20  $\mu\text{mol/L}$  of z-guggulsterone (left). The blots were stripped and reprobed with antiactin antibody to ensure equal protein loading. Right, migration by DU145 cells transiently transfected with a control nonspecific siRNA or VEGF-R2-targeted siRNA and treated for 24 h with either DMSO (control) or 20  $\mu\text{mol/L}$  of z-guggulsterone. Columns, mean; bars, SE; \*,  $P < 0.05$ , significantly different between the indicated groups by  $t$  test ( $n = 3$ ). Similar results were observed in two experiments.



**Figure 4.** **A**, immunoblotting for phosphorylated (Ser<sup>473</sup>)-Akt using lysates from HUVEC treated with DMSO (*control*) or 10 and 20  $\mu$ mol/L of *z*-guggulsterone for 24 or 48 h. The blots were stripped and reprobed with antiactin antibody to ensure equal protein loading. Immunoblotting was done twice using independently prepared lysates and the results were comparable. **B**, quantitation of HUVEC tube formation following 24 h of treatment with DMSO (*control*) or 20  $\mu$ mol/L of *z*-guggulsterone in the absence or presence of 1  $\mu$ mol/L of Akt-1/2 inhibitor. *Columns*, mean; *bars*, SE; \*,  $P < 0.05$ , significantly different between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test ( $n = 3$ ). Similar results were obtained in two independent experiments. **C**, immunoblotting for phosphorylated (Ser<sup>473</sup>)-Akt and total Akt using lysates from DU145 cells transiently transfected with empty vector or expression vector encoding constitutively active Akt and treated for 24 h with DMSO (*control*) or 20  $\mu$ mol/L of *z*-guggulsterone. The blots were stripped and reprobed with antiactin antibody to ensure equal protein loading. **D**, quantitation of DU145 cell migration in cells transiently transfected with the empty vector or constitutively active Akt and treated for 24 h with DMSO (*control*) or 20  $\mu$ mol/L of *z*-guggulsterone. Results are expressed as percentage of DMSO-treated control in empty vector-transfected DU145 cells. *Columns*, mean; *bars*, SE; \*,  $P < 0.05$ , significantly different between the indicated groups by *t* test ( $n = 3$ ). Similar results were observed in two independent experiments.

and differentiation (35–37). Activation of Akt is mediated by receptor tyrosine kinase-mediated phosphorylation (38). The Ser<sup>473</sup> phosphorylation of Akt occurs in response to growth factor stimulus and is necessary for its activation (38). As can be seen in Fig. 4A, *z*-guggulsterone treatment caused a decrease in the phosphorylation of Akt in HUVEC in a dose-dependent manner especially at the 48-h time point. The *z*-guggulsterone-mediated suppression of Akt phosphorylation was not due to a reduction in protein levels of total Akt (results not shown). To further examine the relationship between *z*-guggulsterone-mediated suppression of angiogenesis and Akt inactivation, we did tube formation assays using HUVEC exposed to 20  $\mu$ mol/L of *z*-guggulsterone in the absence or presence of a selective inhibitor of Akt-1/2 (39, 40). The Akt-1/2 inhibitor used in the present study has been shown to be cell-active and blocks phosphorylation (activation) of Akt in human prostate, cervical carcinoma, breast carcinoma, and colon cancer cells (39, 40). As can be seen in Fig. 4B, the HUVEC tube formation was inhibited by ~50% after 24 h of treatment with *z*-guggulsterone and Akt-1/2 inhibitor alone. Combined treatment of HUVEC with *z*-guggulsterone and Akt-1/2 inhibitor resulted in a nearly 77% inhibition of capillary-like tube formation (Fig. 4B). We also determined the effect of Akt-1/2

inhibitor on *z*-guggulsterone-mediated inhibition of DU145 cell migration. Combined treatment of DU145 cells with *z*-guggulsterone (20  $\mu$ mol/L, 24 h) and Akt-1/2 inhibitor (1  $\mu$ mol/L, 24 h) resulted in a much greater inhibition of cell migration (~88% inhibition compared with DMSO-treated control) when compared with single agents (~45% and 37% inhibition of cell migration, respectively, in the presence of *z*-guggulsterone and Akt-1/2 inhibitor alone; data not shown). These results indicated that *z*-guggulsterone-mediated inhibition of HUVEC tube formation and DU145 cell migration correlated with Akt inactivation.

#### Ectopic Expression of Constitutively Active Akt Conferred Protection against *z*-Guggulsterone-Mediated Suppression of DU145 Cell Migration

Next, we determined the effect of overexpression of constitutively active Akt on *z*-guggulsterone-mediated inhibition of DU145 cell migration. Cells transfected with the empty vector were used as controls. As can be seen in Fig. 4C, the levels of total Akt and phosphorylated (Ser<sup>473</sup>)-Akt were ~2-fold higher in DU145 cells transfected with constitutively active Akt compared with the empty vector-transfected control cells. As expected, similar to untransfected DU145 cells (Fig. 2C), the *z*-guggulsterone treatment inhibited migration in empty vector-transfected control

DU145 cells (Fig. 4D). The z-guggulsterone-mediated inhibition of DU145 cell migration was attenuated by ectopic expression of constitutively active Akt (Fig. 4D). These results confirmed that the z-guggulsterone-mediated inhibition of DU145 cell migration was partly dependent on Akt inactivation.

#### z-Guggulsterone Treatment Inhibited *In vivo* Angiogenesis in Matrigel Plug Assay

To assess the *in vivo* significance of these cellular findings, we proceeded to determine the effect of z-guggulsterone administration on *in vivo* angiogenesis using DU145-Matrigel plug assay in male nude mice. The dose of guggulsterone used in the present study was (1 mg/mouse corresponding to ~40 mg/kg body weight) selected from pharmacokinetic data in rats (41). Oral gavage of z-guggulsterone (5 times/week) resulted in a statistically significant decrease in tumor volume (Fig. 5A) and wet tumor weight (Fig. 5B) compared with vehicle-treated control mice. The average body weights of the control and z-guggulsterone-treated mice did not differ throughout the experimental period (Fig. 5C). The z-guggulsterone-treated mice otherwise seemed healthy and did not exhibit any signs of distress including impaired movement or posture, indigestion, and areas of redness or swelling.

#### z-Guggulsterone Administration Decreased Microvessel Area and Expression of VEGF-R2 Protein in DU145-Matrigel Plugs

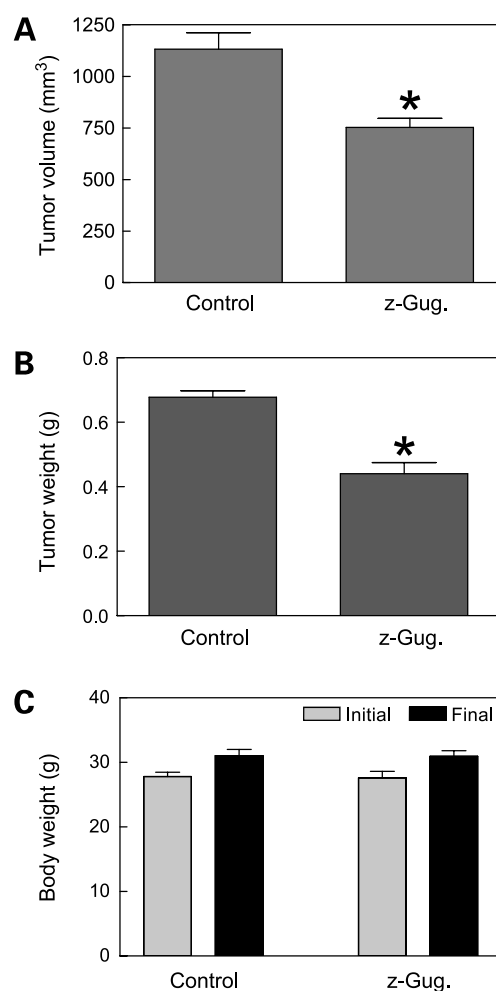
We did immunohistochemistry for angiogenic markers (factor VIII and CD31) to determine the effect of z-guggulsterone administration on *in vivo* angiogenesis. Immunohistochemistry for factor VIII and CD31 in sections from DU145-Matrigel plugs removed from control mice exhibited a large number of endothelial cells organized into capillaries or large vascular cavities (Fig. 6A). The microvessel area, based on immunostaining for factor VIII and CD31, was significantly lower in the plugs removed from the z-guggulsterone-treated mice compared with controls (Fig. 6B). Consistent with cellular data, the z-guggulsterone administration also caused a statistically significant decrease in protein levels of VEGF-R2 compared with control mice (Fig. 6). These results provided evidence to indicate the antiangiogenic effect of z-guggulsterone *in vivo*.

## Discussion

The present study reveals that the z-isomer of guggulsterone is a potent suppressor of *in vitro* angiogenesis as evidenced by the inhibition of HUVEC tube formation and inhibition of HUVEC and DU145 cell migration. In addition, oral gavage of z-guggulsterone to male nude mice implanted with DU145-Matrigel plugs significantly inhibits *in vivo* angiogenesis as judged by immunohistochemical analyses for angiogenic markers (factor VIII and CD31). To the best of our knowledge, the present study is the first published report to document the antiangiogenic activity of z-guggulsterone. Statistically significant inhibition of *in vitro* tube formation and migration is evident at 5 to 20  $\mu\text{mol/L}$  concentrations of the

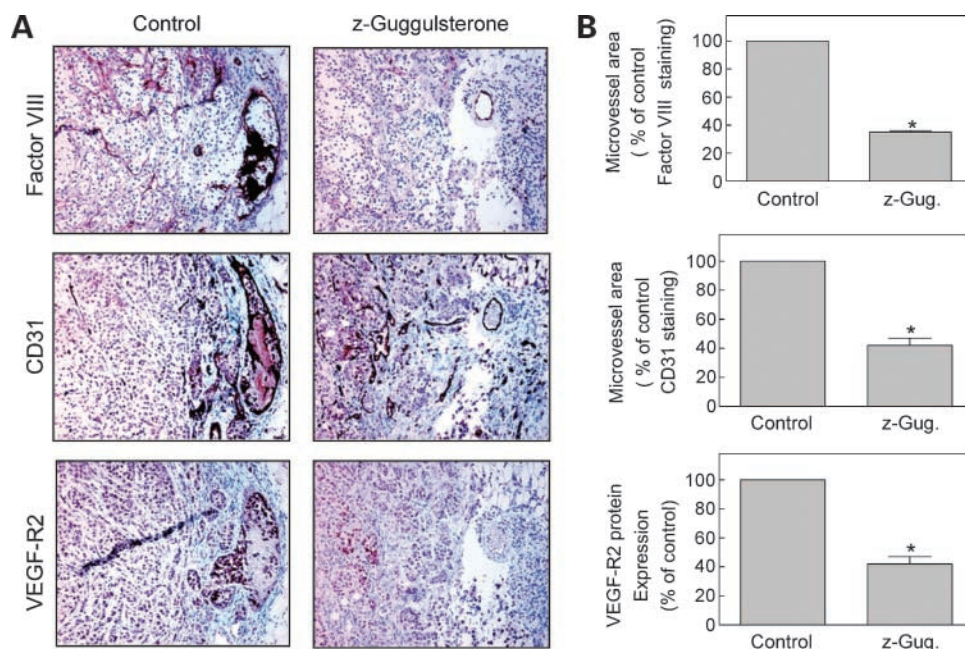
z-guggulsterone, especially at the 48-h time point. Even though pharmacokinetic variables for z-guggulsterone have not been determined in humans, the maximal plasma concentration of guggulsterone was shown to be ~3.3  $\mu\text{mol/L}$  in rats orally gavaged with 50 mg of guggulsterone/kg body weight (41). The rat pharmacokinetic study also documented a reasonably good oral bioavailability of z-guggulsterone (~43%; ref. 41). Thus, it is possible that the concentrations of z-guggulsterone needed to inhibit tumor angiogenesis may be achievable in humans.

VEGF is most closely associated with aggressive human cancers (22, 32, 33). VEGF provides prosurvival signals to



**Figure 5.** Effect of oral administration of 1 mg of z-guggulsterone (Monday through Friday) to male nude mice s.c. implanted with DU145 cell-containing Matrigel plugs on tumor volume (A), wet tumor weight (B), and body weight (C). Mice were pretreated for 2 weeks with vehicle (PBS) or z-guggulsterone prior to DU145-Matrigel plug implantation and treatment was continued for 2 more weeks. Columns, mean; bars, SE; \*,  $P < 0.05$ , significantly different compared with vehicle-treated control by *t* test ( $n = 5$ ). The body weights of the vehicle-treated control and guggulsterone-treated mice did not differ significantly throughout the experimental protocol.





**Figure 6.** **A**, representative immunostaining for factor VIII, CD31, and VEGF-R2 in sections of DU145-Matrigel plugs removed from the vehicle-treated control mouse and z-guggulsterone-treated mouse. Plugs from five mice from each group were analyzed. **B**, quantitation of microvessel area representing factor VIII and CD31-positive vasculature and VEGF-R2 protein expression using Image Analysis Program. *Columns*, mean; *bars*, SE; \*,  $P < 0.05$ , significantly different compared with vehicle-treated control by paired *t* test ( $n = 5$ ).

normal and tumor-derived endothelial cells and this signal transduction to the nucleus is mediated by VEGF receptors including VEGF-R1, VEGF-R2, and VEGF-R3 (22, 32–34). Although VEGF-R2 is believed to play an important role in angiogenesis, VEGF-R3 is responsible for lymphangiogenesis. VEGF-R2 is a receptor tyrosine kinase which, upon activation by ligand binding, phosphorylates and activates secondary messengers including Akt to regulate endothelial cell proliferation and migration (35). The present study reveals that exposure of HUVEC and DU145 cells to z-guggulsterone results in the suppression of VEGF secretion into the medium as well as down-regulation of VEGF-R2 protein expression. Moreover, the z-guggulsterone-mediated suppression of DU145 cell migration is increased upon the knockdown of VEGF-R2 protein levels. Even though elucidation of the mechanism by which z-guggulsterone causes suppression of VEGF secretion and VEGF-R2 expression awaits further investigation, various possibilities exist to explain these effects. For instance, a number of transcription factors including hypoxia-inducible factor-1 $\alpha$ , NF- $\kappa$ B, activator protein 1, and Sp-1 bind to the VEGF promoter to initiate and activate its transcription (42). Likewise, hypoxia-inducible factor-2 $\alpha$  is highly expressed by vascular endothelial cells and activates the transcription of endothelial cell-specific receptor tyrosine kinases and VEGF-R2 (42). It is possible that z-guggulsterone inhibits some of these transcription factor(s) to suppress VEGF and VEGF-R2 expression. Suppression of constitutive (U266 multiple myeloma cells and MDA 1986 head and neck cancer cells) and tumor necrosis factor-induced activation of NF- $\kappa$ B (A549 lung cancer cells and leukemia cells) and inhibition of NF- $\kappa$ B-regulated gene expression including VEGF by z-guggulsterone treatment has been documented previously (17). Besides VEGF, other growth factors and cytokines, includ-

ing EGF, G-CSF, FGF, IL-12, and IL-17 also play an important role in angiogenesis (28–31). For instance, IL-17 increases angiogenic activity and *in vivo* growth of human non-small lung cancer cells in severe combined immunodeficiency mice (31). Similarly, G-CSF promotes tumor angiogenesis by increasing circulating endothelial progenitor cells (43). Tumor invasion and metastasis is facilitated by MMPs, which are zinc-dependent proteolytic enzymes (44, 45). The expression of MMPs is associated with grade, stage, and survival of solid tumors (44, 46). MMP-2 in particular is believed to have an important role in the initial steps of tumor invasion (46). The present study reveals that z-guggulsterone treatment significantly inhibits the secretion of G-CSF, MMP-2, and IL-17 in both HUVEC and DU145 cells. Thus, it is reasonable to conclude that the antiangiogenic effect of z-guggulsterone is mediated by the suppression of G-CSF, IL-17, and MMP-2 secretion.

VEGF and VEGF-R2 regulate endothelial cell survival and angiogenesis through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway (37, 38). Jiang et al. reported that overexpression of constitutively active Akt and its upstream kinase phosphatidylinositol 3'-kinase induced angiogenesis in chicken embryo (47). The present study reveals that the antiangiogenic effect of z-guggulsterone is associated with the inactivation of Akt. The z-guggulsterone treatment decreases activating phosphorylation of Akt in HUVEC and DU145 cells and the z-guggulsterone-mediated inhibition of HUVEC tube formation and DU145 cell migration (data not shown) are intensified in the presence of cell-permeable and selective inhibitor of Akt-1/2.

Tumor angiogenesis (neovascularization) is a highly complex process that is regulated by multiple proangiogenic



growth factors and their corresponding receptors (28–33). Based on the results of the present study, it seems reasonable to conclude that inhibition of the VEGF–VEGF-R2–Akt signaling axis may be an important mechanism in the antiangiogenic effects of z-guggulsterone. This conclusion is supported by the following observations: (a) z-guggulsterone–mediated inhibition of tube formation and migration correlates with the suppression of secretion of VEGF, which provides prosurvival signals to normal and tumor-derived endothelial cells mediated by receptor tyrosine kinase VEGF-R2 (22, 32–34); (b) z-guggulsterone treatment down-regulates the protein levels of VEGF-R2; (c) z-guggulsterone–mediated suppression of DU145 cell migration is intensified by the knockdown of VEGF-R2 protein levels; (d) z-guggulsterone inhibits Akt in HUVEC and DU145 cells and inhibition of HUVEC tube formation by this agent is intensified by pharmacologic inhibition of Akt. However, the precise mechanism by which z-guggulsterone reduces the secretion of VEGF or down-regulates VEGF-R2 protein level is not clear and requires further investigation.

In conclusion, the present study reveals that z-guggulsterone inhibits angiogenesis *in vitro* and *in vivo*. The z-guggulsterone–mediated inhibition of angiogenesis is associated with the inactivation of Akt, suppression of growth factor (VEGF and G-CSF), IL-17 and MMP-2 secretion, and down-regulation of VEGF-R2 protein expression.

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