

**Cryptotanshinone inhibits lymphatic endothelial cell tube formation by  
suppressing VEGFR-3/ERK and small GTPase pathways**

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## Abstract

Cryptotanshinone (CPT), isolated from the plant *Salvia miltiorrhiza* Bunge, is a potential anticancer agent. However, the underlying mechanism remains to be defined. Here we show that CPT inhibited lymphangiogenesis in an *in vitro* model (tube formation). This effect was partly attributed to inhibiting expression of vascular endothelial growth factor receptor 3 (VEGFR-3) in murine lymphatic endothelial cells (LECs), as overexpression of VEGFR-3 conferred resistance to CPT inhibition of the tube formation, whereas downregulation of VEGFR-3 mimicked the effect of CPT, blocking the tube formation. Furthermore, CPT inhibited phosphorylation of the extracellular signal-related kinase 1/2 (ERK1/2). Overexpression of VEGFR-3 attenuated CPT inhibition of ERK1/2 phosphorylation, whereas downregulation of VEGFR-3 inhibited ERK1/2 phosphorylation in LECs. Expression of constitutively active MKK1 resulted in activation of ERK1/2, and partially prevented CPT inhibition of LEC tube formation. In addition, CPT also inhibited protein expression and activities of Rac1 and Cdc42, but not RhoA. Expression of constitutively active Rac1 and Cdc42 concurrently, but not Rac1 or Cdc42 alone, conferred resistance to CPT inhibition of LEC tube formation. Taken together, the results suggest that CPT inhibits LEC tube formation in part by inhibiting VEGFR-3-mediated ERK1/2 phosphorylation, and in part by inhibiting expression of the small GTPases.

## Introduction

Cryptotanshinone (CPT), a natural compound isolated from *Salvia miltiorrhiza* Bunge (Danshen), has been used in traditional oriental medicine for the treatment of a variety of diseases, such as coronary artery disease (1), hyperlipidemia, acute ischemic stroke (2), chronic renal failure (3), chronic hepatitis (4), and Alzheimer's disease (5). In addition, CPT has been recently shown to possess anticancer activity in a spectrum of human cancer cells (6-10). For instance, CPT inhibits growth of prostate cancer cells (DU145) by inactivating the signal transducer and activator of transcription-3 (Stat3) activity (6); induces apoptosis in DU145 cells by augmenting Fas sensitivity (7); inhibits growth of hepatocarcinoma and gastric cancer cells by arresting cell cycle at S phase (8); and inhibits proliferation of skin cancer cells (B16 and B16BL6) (9). Most recently we have further demonstrated that CPT displayed anticancer activity by inhibiting proliferation of human rhabdomyosarcoma (Rh30), prostate cancer (DU145) and breast cancer cells (MCF-7), by arresting cells in G<sub>1</sub>/G<sub>0</sub> phase of the cell cycle (10). CPT inhibition of cell proliferation was associated with downregulation of cyclin D1 expression and phosphorylation of retinoblastoma (Rb) protein, due to inhibition of the mammalian target of rapamycin (mTOR) signaling pathway (10). These findings suggest that CPT is a potential novel anticancer agent.

Lymphangiogenesis, like angiogenesis, plays an important role in promoting tumor growth and metastasis (11-13). For many types of solid tumors, the lymphatic system acts as the primary conduit for initial metastasis, which is an indication of disease progression and prognosis for reduced survival (14-17). Therefore, inhibition

of lymphangiogenesis is a promising strategy for treatment or prevention of tumor metastasis (18,19). Previous studies have shown that CPT inhibits angiogenesis in bovine aortic endothelial cells (BAECs) (20) and human umbilical vein endothelial cells (HUVEC) (21). However, the effect of CPT on lymphangiogenesis is not known.

Vascular endothelial growth factors (VEGFs) and their receptors are central controllers of vasculogenesis, angiogenesis and lymphangiogenesis (22). Five VEGFs (VEGF or VEGF-A, placenta growth factor, VEGF-B, VEGF-C and VEGF-D) and three VEGF receptors (VEGFR-1, -2 and -3) have been identified in mammals (22). VEGFR-1/2 and VEGFR-3 are primarily expressed on the surface of vascular and lymphatic endothelial cells (LECs), respectively (22). It is known that VEGF-A binds to VEGFR-1/2, regulating vasculogenesis and angiogenesis, while VEGF-C/D binds to VEGFR-3, mediating lymphangiogenesis. In particular, VEGF-C/D binds to and activates VEGFR-3, leading to activation of the downstream signaling molecules, such as phosphatidylinositol 3'-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, which are crucial for LEC survival and lymphangiogenesis (22, 23), as well as metastasis (11-13). Thus, VEGFR-3 pathway has become an attractive target for cancer prevention and treatment.

The small GTPases (Rac1, Cdc42 and RhoA) regulate migration, survival, and vacuole and capillary lumen formation in LECs, which are critical for lymphangiogenesis (24-27). Recent studies have demonstrated a requirement for Rac1 and Cdc42 in capillary lumen formation of LECs, indicating that the small GTPases play key roles in the lymphangiogenic process (28). Therefore, targeting small GTPases is an alternative approach for cancer prevention and treatment.

Using murine LEC tube formation, an *in vitro* lymphangiogenesis model (29), we studied the effect of CPT on lymphangiogenesis. The results indicate that CPT inhibited the LEC tube formation, which was in part by inhibition of VEGFR-3-mediated phosphorylation of extracellular signal-related kinase 1/2 (ERK1/2), and in part by inhibition of expression and activities of Rac1 and Cdc42.

## **Materials and Methods**

**Chemicals.** CPT was extracted from the roots of *Salvia miltiorrhiza* Bunge (Danshen), as described previously (10), and dissolved in 100% ethanol to prepare stock solutions (20 mM), which was aliquoted and stored at -20°C. U0126, a selective inhibitor of MKK1/2, was obtained from LC Laboratories (Woburn, MA, USA).

**Cell lines and culture.** Murine LECs (30) were grown in antibiotic-free Dulbecco's modified Eagle medium (DMEM)/F12 (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) at 37°C and 5% CO<sub>2</sub>. Human embryonic kidney 293 (American Type Culture Collection, Manassas, VA, USA), 293TD and 293A cells (Invitrogen, Carlsbad, CA, USA) were grown in antibiotic-free DMEM (Mediatech) supplemented with 10% heat-inactivated FBS and non-essential amino acids (Mediatech) at 37°C and 5% CO<sub>2</sub>.

**Plasmids and transfection.** LEC clones stably overexpressing

p3×Flag-VEGFR-3-TV1 and p3×Flag-TV1 plasmid (empty vector, as a control) were generated and used as described (29).

**Lentiviral shRNA cloning, production and infection.** To generate lentiviral shRNA to Rac1 or Cdc42, oligonucleotides containing the target sequences were synthesized, annealed and inserted into FSIPPW lentiviral vector (31) through the EcoRI/BamHI restriction enzyme site. The oligonucleotides used were: Rac1 sense:

5'-AATTCCCATACCGGAGTGCTCAGCTTGCAAGAGAAGCTGAGCACTCCAG  
GTATTTTTTG -3', antisense: 5'-GATCCAAAAAATACCTGGAGTGCTCAGCTT  
CTCTTGCAAGCTGAGCACTCC AGGTATGGG-3'; Cdc42 sense:

5'-AATTCATGTCTCCTGATATCCTATGCAAGAGATAGGATATCAGGAGA  
CATGTTTTTG-3', antisense: 5'-GATCCAAAACATGTCTCCTGATATCCTA  
TCTCTTGCATAGGATATCAGGAGACATGGGG-3'. Lentiviral shRNAs to Rac1

and Cdc42 were made as described previously (32), and lentiviral shRNAs against VEGFR-3 and green fluorescence protein (GFP, as a control) were shown (29).

Subsequently, LEC cells, when grown to about 70% confluence, were infected with the above lentiviral shRNAs in the presence of 8 µg/ml polybrene and exposed to 2 mg/ml puromycin after 24 h of infection. In 5 days, cells were used for experiments.

**Recombinant adenoviral constructs and infection.** The recombinant adenoviral vectors expressing GFP (Ad-GFP), Flag-tagged constitutively active MKK1 (Ad-MKK1-R4F), RhoA (RhoA-L63) (Ad-RhoA-L63), Cdc42 (Ad-Cdc42-L28),

and Rac1 (Ad-Rac1-L61) were described previously (29,33). All adenoviruses were amplified, titrated and used as described (33).

**Western blot analysis.** Western blot analysis was performed as described previously (32). The primary antibodies used included antibodies to VEGFR-3, Akt, Cdc42, RhoA, ERK2, JNK1, phospho-JNK (Thr183/Tyr185), p38, phospho-p38 (Thr180/Tyr182), MKK1, Flag (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ERK1/2 (Thr202/Tyr204), phospho-Akt (Ser473) (Cell Signaling, Beverly, MA, USA), Rac1 (Cytoskeleton, Denver, CO, USA), and  $\beta$ -tubulin (Sigma, St. Louis, MO, USA).

**Cell morphological analysis.** LECs were seeded at a density of  $5 \times 10^5$  cells/well in 6-well plates. Next day, the cells were treated with CPT (0-10  $\mu$ M) for 24 h, or with 10  $\mu$ M CPT for 0-24 h, followed by taking images under an Olympus inverted phase-contrast microscope (Olympus Optical, Melville, NY, USA) (200 $\times$ ) equipped with the Quick Imaging system.

**Tube formation assay.** Tube formation assay was performed, as described previously (29).

**Small GTPase activity assay.** The activity of Rac1, Cdc42, or RhoA was determined using Rac/Cdc42 assay kit and Rho assay kit (Millipore, Billerica, MA, USA), respectively, as described previously (33).

**Statistical analysis.** Results were expressed as mean values  $\pm$  standard deviation (mean  $\pm$  SD). The data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's *t*-test for multiple comparisons. A level of  $P < 0.05$  was considered to be statistically significant.

## Results

**CPT inhibits LEC tube formation.** Studies have shown that CPT inhibits angiogenesis (20, 21), but the effect of CPT on lymphangiogenesis is not known. To find out whether CPT inhibits lymphangiogenesis, we chose murine LEC tube formation as an *in vitro* model for lymphangiogenesis. Treatment with CPT (0-10  $\mu$ M) for 24 h did not apparently affect LEC cell viability according to cell morphology (Fig.1A, bottom panel). However, pretreatment with CPT (0-10  $\mu$ M) for 24 h inhibited LEC tube formation in a concentration-dependent manner (Fig.1A, upper panel). At 5 and 10  $\mu$ M, CPT inhibited the tube formation by approximately 65% and 90%, respectively, comparing with the control group (Fig.1C). Furthermore, CPT (10  $\mu$ M) also inhibited LEC tube formation in a time-dependent manner (Fig. 1B, upper panel), in spite of no obvious effect on cell viability (Fig.1B, bottom panel). After treatment for 4 h, CPT (10 $\mu$ M) was able to significantly inhibit the tube formation (by ~20%). When LECs were treated with CPT (10 $\mu$ M) for 24 h, the tube formation was suppressed by ~90%, comparing with the control group (Fig.1D).

### **CPT inhibition of LEC tube formation is associated with suppressing VEGFR-3**

**protein expression.** Since VEGFR-3 is primarily expressed in LECs (34), and essential for lymphangiogenesis (11-13,22), we studied whether CPT inhibits LEC tube formation by targeting VEGFR-3. When LECs were treated with CPT (0-10  $\mu$ M) for 24 h, a concentration-dependent reduction of VEGFR-3 protein expression was detected by Western blotting (Fig.2A). When the cells were exposed to CPT at 10  $\mu$ M, a time-dependent inhibition of VEGFR-3 expression was also observed (Fig.2B). Treatment with CPT for 8 h was able to remarkably downregulate VEGFR-3 protein level. Prolonged treatment with CPT resulted in more reduction of VEGFR-3 (Fig.2B).

To define the role of VEGFR-3 in CPT inhibition of LEC tube formation, LEC cells (LEC/VEGFR-3) stably overexpressing VEGFR-3 were generated by transfection with p3 $\times$ Flag-VEGFR-3-TV1 plasmid, as described (29). About 3-fold increase of VEGFR-3 protein expression was detected in LEC/VEGFR-3 cells, comparing with the control cells (LEC/V) transfected with the empty vector (Fig.2C, left panel). Overexpression of VEGFR-3 did not alter the cell viability and growth rate in LECs. Treatment with CPT (10  $\mu$ M) for 24 h inhibited VEGFR-3 protein expression by ~80% (Fig. 2C, left panel), and suppressed the tube formation by ~90% in LEC/V cells (Fig.2C, right panel). When LEC/VEGFR-3 cells were exposed to CPT (10  $\mu$ M) for 24 h, VEGFR-3 protein expression was reduced by ~50%, but the VEGFR-3 protein level was still slightly higher than the basal level in the control (LEC/V) cells (Fig.2C, left panel). Interestingly, overexpression of VEGFR-3 rendered high resistance to CPT inhibition of the tube formation (Fig.2C, right panel),

suggesting that CPT suppresses LEC tube formation in part by reducing VEGFR-3 protein expression.

To further substantiate the role of VEGFR-3 in CPT inhibition of LEC tube formation, RNA interference was utilized. Infection with lentiviral shRNA to VEGFR-3 downregulated the protein expression of VEGFR-3 by ~90%, in comparison with the controls infected with lentiviral shRNA to GFP (Fig.2D, left panel). Silencing VEGFR-3 mimicked the effect of CPT, inhibiting LEC tube formation by ~90% (Fig.2D, right panel), which supports that VEGFR-3 is essential for LEC tube formation. Addition of CPT (10  $\mu$ M) did not further enhance VEGFR-3 shRNA inhibition of LEC tube formation, suggesting that downregulation of VEGFR-3 by 90% might have maximally inhibited the tube formation.

### **CPT inhibits LEC tube formation by targeting VEGFR-3-mediated ERK1/2**

**pathway.** As PI3K/Akt and MAPK pathways are the two major downstreams of VEGFR-3 (22), we next wondered whether CPT inhibits LEC tube formation through targeting these pathways. Treatment with CPT failed to alter protein expression or phosphorylation of Akt (Fig.3A and B), and JNK and p38 MAPK pathways obviously (data not shown), but resulted in a concentration- and time-dependent inhibition of phosphorylation of ERK1/2, despite no effect on total protein level of ERK2 (Fig.3A and B), suggesting a selective inhibition of the ERK pathway in the LECs.

To determine whether CPT inhibition of phosphorylation of ERK1/2 is through regulation of VEGFR-3 pathway, LEC/VEGFR-3 and LEC/V cells were treated with CPT (10  $\mu$ M) for 24 h, respectively. As expected, overexpression of VEGFR-3

enhanced ERK1/2 phosphorylation and rendered high resistance to CPT inhibition of ERK1/2 phosphorylation (Fig.3C). In contrast, silencing VEGFR-3 by lentiviral shRNA mimicked the effect of CPT, decreasing ERK1/2 phosphorylation (Fig.3D). The results reveal that CPT inhibition of ERK1/2 phosphorylation is a consequence of downregulation of VEGFR-3 protein expression in the LECs.

To further verify whether CPT inhibition of LEC tube formation is truly attributed to inhibition of ERK1/2 pathway, we generated recombinant adenoviral vector (Ad-MKK1-R4F) expressing Flag-tagged constitutively active MKK1, which activates ERK1/2 (35). As shown in Fig.4A, Flag-MKK1 was expressing in the LECs infected with Ad-MKK1-R4F, but not Ad-GFP (control). Expression of constitutively active MKK1 elevated phosphorylation of ERK1/2 in LECs. Treatment with CPT (10  $\mu$ M) for 24 h suppressed phosphorylation of ERK1/2 in both Ad-GFP-infected (control) and Ad-MKK1-R4F-infected cells. However, the ERK1/2 phosphorylation level in Ad-MKK1-R4F-infected cells exposed to CPT was comparable to the basal level in the control cells (Fig.4A). Of notice, expression of constitutively active MKK1, but not GFP, conferred high resistance to CPT inhibition of LEC tube formation (Fig.4B). As a control, U0126 (a selective inhibitor of MKK1/2, upstream of ERK1/2) was employed to treat the LECs. We observed that 5  $\mu$ M U0126 inhibited ERK1/2 phosphorylation almost completely and suppressed the tube formation by 90% in LECs. Addition of 10  $\mu$ M CPT failed to enhance U0126 inhibition of the tube formation (data not shown). However, treatment with either 2.5  $\mu$ M U0126 or 5  $\mu$ M CPT alone inhibited ERK1/2 phosphorylation by ~50% (Fig.4C), and inhibited LEC tube formation by approximately 60% and 40%, respectively (Fig.4D). Combined

treatment with 2.5  $\mu$ M U0126 and 5  $\mu$ M CPT displayed an additive or synergistic inhibitory effect on ERK1/2 phosphorylation and the tube formation (Fig.4C and D). The results suggest that CPT inhibits LEC tube formation partly through targeting VEGFR-3-mediated ERK pathway.

**Rac1 and Cdc42, but not RhoA, are involved in CPT inhibition of LEC tube formation.** As the small GTPases play critical roles in lymphangiogenesis (24-28), we were also curious to ask whether CPT inhibits LEC tube formation by targeting the small GTPases. When LECs were treated with CPT (0-10  $\mu$ M) for 24 h or 10 $\mu$ M CPT for 0-24 h, a concentration- and time-dependent inhibition of protein expression of Rac1 and Cdc42, but not RhoA, was observed (Fig.5A and B). Similarly, CPT treatment also markedly decreased the active (GTP-bound) protein levels of Rac1 and Cdc42, but not RhoA (Fig.5C and D), indicating inhibition of the activities of Rac1 and Cdc42.

To demonstrate the roles of the small GTPases in the CPT inhibition of LEC tube formation, we constructed recombinant adenoviruses encoding constitutively active Rac1 and Cdc42. LECs infected with these adenoviruses were treated with or without CPT, followed by tube formation assay. As shown in Fig.6A, considerable levels of constitutively active Rac1 (Rac1-L61) and Cdc42 (Cdc42- L28) were expressing in LECs, respectively, as detected by Western blotting using antibodies to Flag and individual small GTPases. Expression of Rac1-L61 or Cdc42-L28 alone neither significantly altered the basal tube formation, nor rendered apparent resistance to CPT inhibition of LEC tube formation (Fig.6B). However, interestingly, concurrent

expression of Rac1-L61 and Cdc42-L28 conferred high resistance to CPT inhibition of LEC tube formation (Fig.6B).

To further corroborate the roles of the small GTPases in CPT inhibition of LEC tube formation, we generated lentiviral shRNAs to silence Rac1 (Fig.6C, left panel) and Cdc42 (Fig.6C, right panel), respectively. Of interest, downregulation of Rac1 or Cdc42 by ~90% alone was able to inhibit LEC tube formation by ~70% and ~80%, respectively (Fig.6D). The data suggest that both Rac1 and Cdc42 are essential for LEC tube formation, and CPT inhibits LEC tube formation partly by suppressing expression/activities of the two small GTPases.

## Discussion

Recent studies have shown that CPT exhibits anticancer activity by inhibiting proliferation and inducing apoptosis in cancer cells, as well as inhibiting angiogenesis (6-10, 20-21). Here, for the first time, we present evidence that CPT inhibited LEC tube formation, an *in vitro* lymphangiogenesis model, suggesting that CPT inhibits lymphangiogenesis. It is well accepted that lymphangiogenesis, like angiogenesis, is pivotal for tumor growth and metastasis, and anti-lymphangiogenesis has become a new avenue for prevention and treatment of cancer (11-13). Therefore, our findings strongly support the notion that CPT is a potential anticancer agent.

VEGFR-3 has been characterized as a key player for lymphangiogenesis, and disruption of this pathway has been explored for the development of anticancer drugs (36-41). In the present study, we found that the anti-lymphangiogenic effect of CPT is

associated with inhibition of VEGFR-3 protein expression in the LECs. This is supported by the findings that (1) CPT inhibited VEGFR-3 protein expression; (2) overexpression of VEGFR-3 conferred high resistance to CPT inhibition of LEC tube formation; and (3) downregulation of VEGFR-3 mimicked the effect of CPT, blocking LEC tube formation. The data are consistent with previous reports that blockade of the VEGFR-3 pathway by soluble receptor form (38, 40), small molecule inhibitors (23, 39, 42), or specific antibodies (43) effectively inhibited lymphangiogenesis.

It has been described that activation of VEGFR-3 pathway promotes LEC proliferation, migration and survival through PI3K/AKT and MAPK pathways (22, 23). Here we found that CPT affected neither cellular protein expression nor phosphorylation of Akt, JNK and p38 MAPK pathways, but inhibited phosphorylation of ERK1/2 in murine LECs. Further, we identified that CPT inhibition of ERK1/2 pathway was a consequence of downregulation of VEGFR-3 protein expression, as overexpression of VEGFR-3 attenuated CPT inhibition of ERK1/2 phosphorylation, whereas downregulation of VEGFR-3 mimicked the effect of CPT, reducing ERK1/2 phosphorylation in the LECs. In addition, we also observed that expression of constitutively active MKK1 increased ERK1/2 phosphorylation and rendered high resistance to CPT inhibition of LEC tube formation. Selective suppression of ERK1/2 by U0126 (5  $\mu$ M) directly blocked LEC tube formation by 90%, whereas no additive or synergistic inhibitory effect on the tube formation was observed by treatment with CPT (10  $\mu$ M) (data not shown). However, when LECs were treated with lower concentrations of these two compounds, CPT (5  $\mu$ M) either additively or synergistically enhance the inhibition of U0126 (2.5  $\mu$ M) on the tube formation,

implying that ERK pathway is a critical controller for the LEC tube formation, and CPT inhibited LEC tube formation, at least partly through inhibition of VEGFR-3-mediated ERK1/2 pathway.

In our study, we also noticed that overexpression of VEGFR-3 (Fig.2C) or constitutively active MKK1 (Fig.4B) failed to fully rescue the tube formation inhibited by CPT, implying that there may be other signaling molecules involved in LEC tube formation. Since the small GTPases play important roles in cytoskeletal reorganization, cell motility and protrusion formation, which are essential for tube formation (44), we then investigated the effect of CPT on the small GTPases. Our results demonstrated that CPT inhibited protein expression and activities of Rac1 and Cdc42, but not RhoA. Furthermore, only concurrent expression of constitutively active Rac1 and Cdc42 conferred significant resistance to CPT inhibition of LEC tube formation. This is in agreement with other findings that both Rac1 and Cdc42 are necessary for the tube formation (25, 28, 45, 46). It is worthy mentioning that in the studies, we observed that silencing RhoA by shRNA alone also inhibited LEC tube formation (data not shown), suggesting that RhoA is essential for LEC tube formation as well, although RhoA is not a target of CPT. However, expression of constitutively active RhoA (RhoA-L63) alone, or even concurrent expression of RhoA-L63 + Cdc42-L28 or RhoA-L63 + Rac1-L61, failed to prevent CPT inhibition of LEC tube formation (data not shown).

Studies have shown that Rac1 is regulated by VEGFR-3 pathway in normal mouse endothelial cells (47). Following observation that CPT inhibits VEGFR-3 and Rac1/Cdc42, we originally hypothesized that CPT inhibition of Rac1 and Cdc42 is

probably by inhibiting VEGFR-3. However, to our surprise, neither overexpression nor downregulation of VEGFR-3 had any influence on activities/expression of Rac1 and Cdc42, regardless of presence or absence of CPT (data not shown), suggesting that Rac1 and Cdc42 are not regulated by VEGFR-3 pathway in our murine LECs. This is probably due to the different cell lines or experimental conditions employed. Further studies are needed to address whether CPT inhibits lymphangiogenesis *in vivo* and uncover the underlying molecular mechanisms. Also, more studies should be helpful to unveil how CPT inhibits expression of VEGFR-3, Rac1 and Cdc42.

In summary, we have shown that CPT inhibited LEC tube formation in a concentration- and time-dependent manner. CPT inhibition of the LEC tube formation was related to suppression of VEGFR-3-mediated ERK pathway (Fig.1S). Furthermore, our data indicate that CPT inhibition of LEC tube formation was also in part by targeting Rac1 and Cdc42 (Fig.1S). CPT may act as a novel anti-lymphangiogenic agent.

#### **Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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## Figure legends

**Figure 1.** CPT inhibits LEC tube formation in a concentration- and time-dependent manner. LECs were treated with CPT (0-10  $\mu$ M) for 24 h, or CPT (10  $\mu$ M) for 0-24 h, followed by tube formation assay and morphological analysis, as described in “Materials and Methods”. Representative images are shown in (A) and (B), respectively. Bar = 100  $\mu$ m. The length of tube-like formation was evaluated by NIH Image J software. Quantitative data are presented as mean  $\pm$  SD ( $n = 3$ ) in (C) and (D), respectively. \* $P < 0.05$ , \*\* $P < 0.01$ , difference vs. control group.

**Figure 2.** CPT inhibition of LEC tube formation is associated with suppressing VEGFR-3 protein expression. *A, B*, CPT inhibited protein expression of VEGFR-3 in a concentration- and time-dependent manner. LECs, treated with CPT (0-10  $\mu$ M) for 24 h (*A*) or CPT (10  $\mu$ M) for 0-24 h (*B*), were harvested and subjected to Western blot analysis with antibodies to VEGFR-3.  $\beta$ -tubulin was used as a loading control. *C*, Overexpression of VEGFR-3 partially prevented CPT inhibition of LEC tube formation. LEC/V (control) and LEC/VEGFR-3 cells were treated with CPT (10  $\mu$ M) for 24 h, followed by Western blot analysis with indicated antibodies (Left panel), or tube formation assay (Right panel), as described in “Materials and Methods”. Quantitative results of tube formation are shown as mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , difference vs. control group; # $P < 0.05$ , difference vs. LEC/V group. *D*, Lentiviral shRNA to VEGFR-3, but not GFP, downregulated VEGFR-3 protein expression in LECs, as detected by Western blotting (Left panel). LECs, infected with lentiviral

shRNAs to VEGFR-3 and GFP (control), respectively, were treated with CPT (10  $\mu$ M) for 24 h, followed by tube formation assay (Right panel), as described in “Materials and Methods”. Quantitative results of tube formation are shown as mean  $\pm$  SD ( $n = 3$ ).

\*  $P < 0.05$ , difference vs. GFP shRNA control group.

**Figure 3.** CPT inhibits VEGFR-3-mediated ERK1/2 pathway. *A, B*, CPT inhibited phosphorylation of ERK1/2, but not AKT, in LECs in a concentration- and time-dependent manner. LECs treated with CPT (0-10 mM) for 24 h (*A*) or CPT (10  $\mu$ M) for 0-24 h (*B*) were harvested and subjected to Western blot analysis with indicated antibodies. b-tubulin was used as a loading control. *C*, Overexpression of VEGFR-3 conferred resistance to CPT (10  $\mu$ M) for 24 h, followed by Western blotting with indicated antibodies. *D*, Downregulation of VEGFR-3 mimicked the effect of CPT, inhibiting phosphorylation of ERK1/2 in LECs. LECs, infected with lentiviral shRNAs to VEGFR-3 and GFP (control), respectively, were treated with CPT (10  $\mu$ M) for 24 h, followed by Western blotting with indicated antibodies.

**Figure 4.** CPT inhibition of LEC tube formation is through targeting VEGFR-3-mediated ERK1/2 pathway. *A, B*, Expression of constitutively active MKK1 attenuated CPT inhibition of ERK1/2 phosphorylation and the tube formation in LECs. LECs, infected with Ad-MKK1-R4F and Ad-GFP (control), respectively, were treated with or without CPT (10  $\mu$ M) for 24 h, followed by Western blotting with indicated antibodies (*A*), or by tube formation assay (*B*) as described in “Materials and Methods”. Quantitative results are shown as mean  $\pm$  SD ( $n = 3$ ). \* $P <$

0.05, difference vs. control group; <sup>#</sup>*P* < 0.05, difference vs. Ad-GFP group. *C, D*, LECs were treated with U0126 (2.5 μM) or CPT (5 μM) alone, or both for 24 h, followed by Western blotting using the indicated antibodies (*C*), or by tube formation assay (*D*) as described in “Materials and Methods”. Quantitative results of tube formation are shown as mean ± SD (*n* = 3). \**P* < 0.05, difference vs. control group. <sup>#</sup>*P* < 0.05, difference vs. U0126 or CPT treatment group.

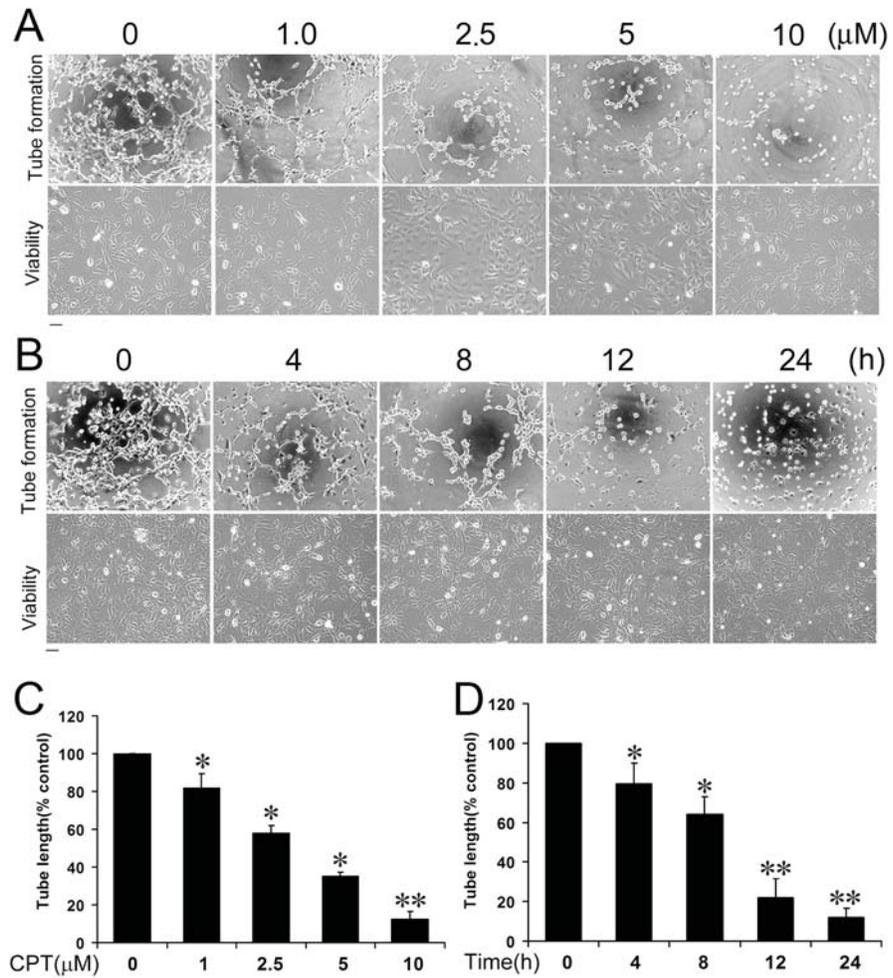
**Figure 5.** CPT inhibits protein expression and activities of Rac1 and Cdc42, but not RhoA, in a concentration- and time-dependent manner. *A, B*, LECs, treated with CPT (0-10 μM) for 24 h (*A*) or CPT (10 μM) for 0-24 h (*B*), were harvested and subjected to Western blotting with indicated antibodies. b-tubulin was used as a loading control. *C, D*, CPT inhibited activity of Rac1 and Cdc42, but not RhoA, in LECs in a concentration- and time-dependent manner. LECs treated with CPT (0-10 μM) for 24 h (*C*) or CPT (10 μM) for 0-24 h (*D*), were harvested for the small GTPase activity assay as described in “Materials and Methods”.

**Figure 6.** Rac1 and Cdc42 pathways are involved in CPT inhibition of LEC tube formation. *A, B*, Expression of constitutively active Rac1 and Cdc42 concurrently, but not Rac1 or Cdc42 alone, rendered resistance to CPT inhibition of LEC tube formation. LECs, infected with Ad-Cdc42-L28, Ad-Rac1-L61, Ad-Cdc42-L28+Ad-Rac1-L61, or Ad-GFP (control), respectively, were treated with or without CPT (10 μM) for 24 h, followed by Western blotting using the indicated antibodies (*A*), or by tube formation assay (*B*) as described in “Materials and

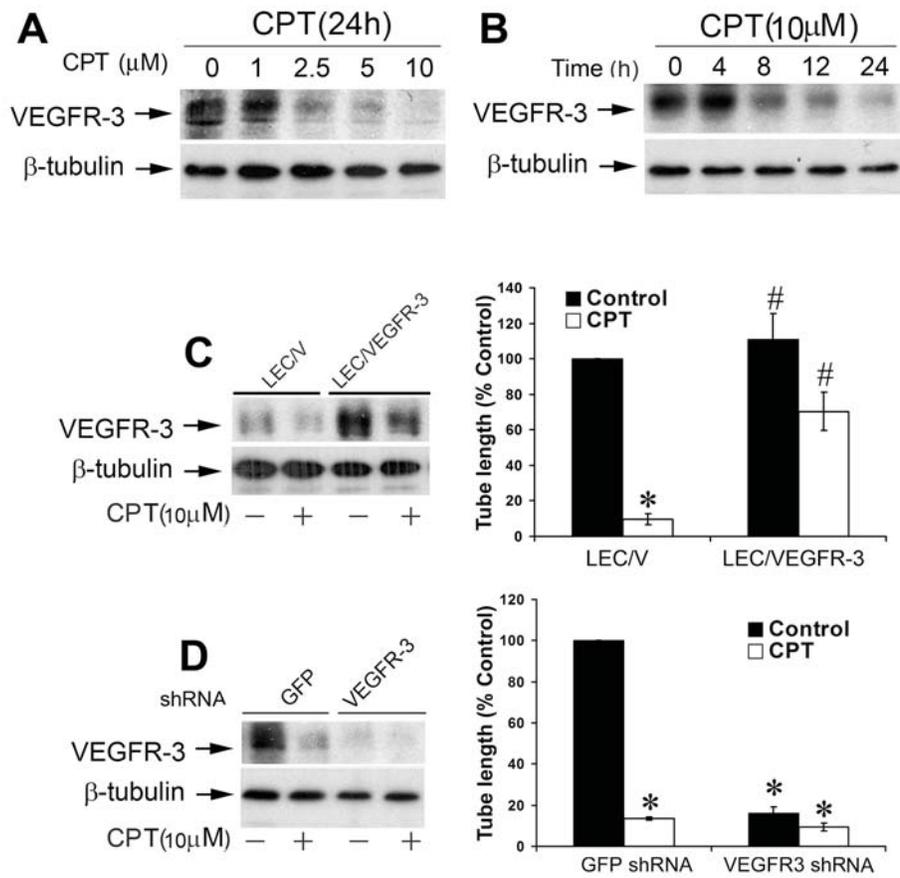
Methods". Quantitative results of tube formation are shown as mean  $\pm$  SD ( $n = 3$ ).  $*P < 0.05$ , difference vs. Ad-GFP control group;  $\#P < 0.05$ , difference vs. Ad-GFP/CPT, Ad-Cdc42-L28/CPT, or Ad-Rac1-L61/CPT treatment group. *C*, Lentiviral shRNA to Rac1 or Cdc42, but not GFP, downregulated Rac1 or Cdc42 in LECs, respectively, as detected by Western blotting. *D*, Downregulation of Rac1 or Cdc42 inhibited tube formation in LECs. Quantitative results are shown with mean  $\pm$  SD ( $n = 3$ ).  $*P < 0.05$ , difference vs. GFP shRNA control group.

### Supplementary data

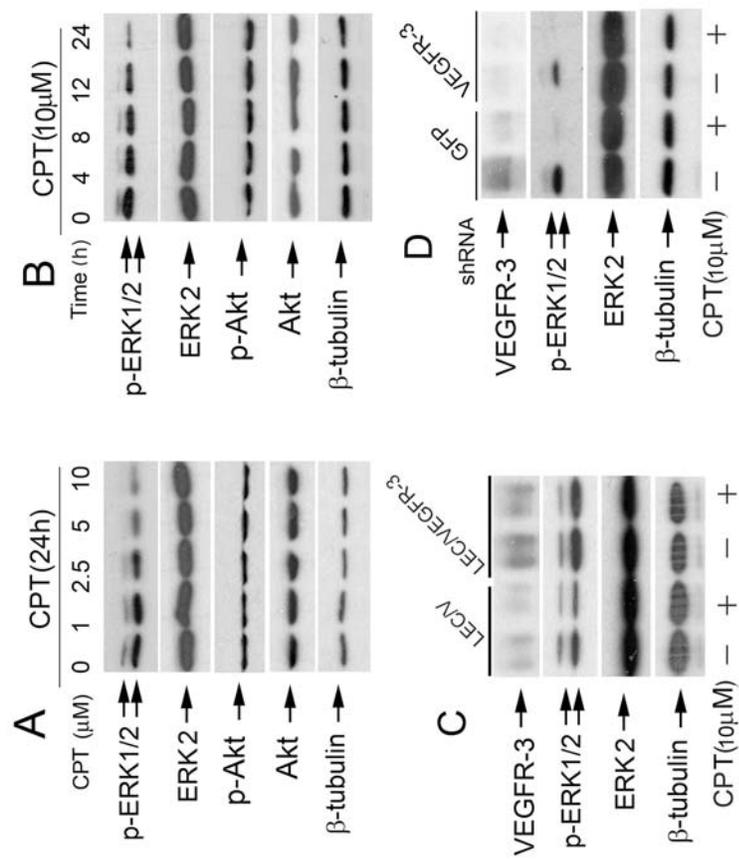
**Figure 1S.** A model showing that CPT inhibits LEC tube formation in part by inhibiting VEGFR-3-mediated ERK1/2 phosphorylation, and in part by inhibiting expression of Rac1/Cdc42. Arrows represent activation, whereas bars represent inhibition.



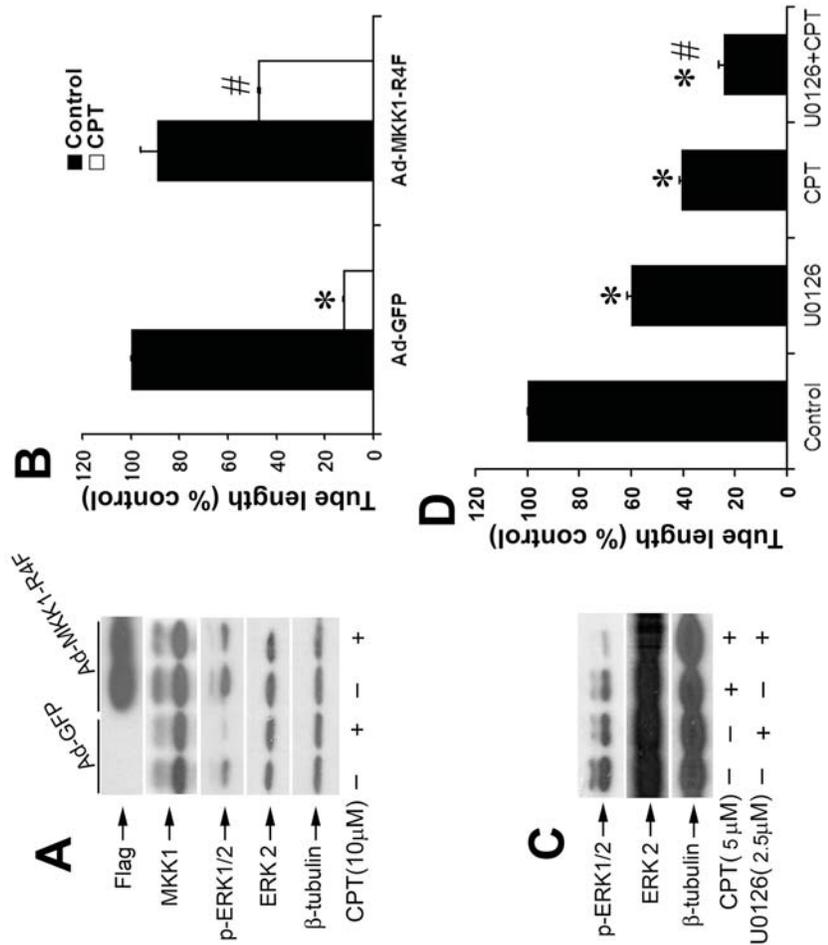
Luo et al., Fig.1



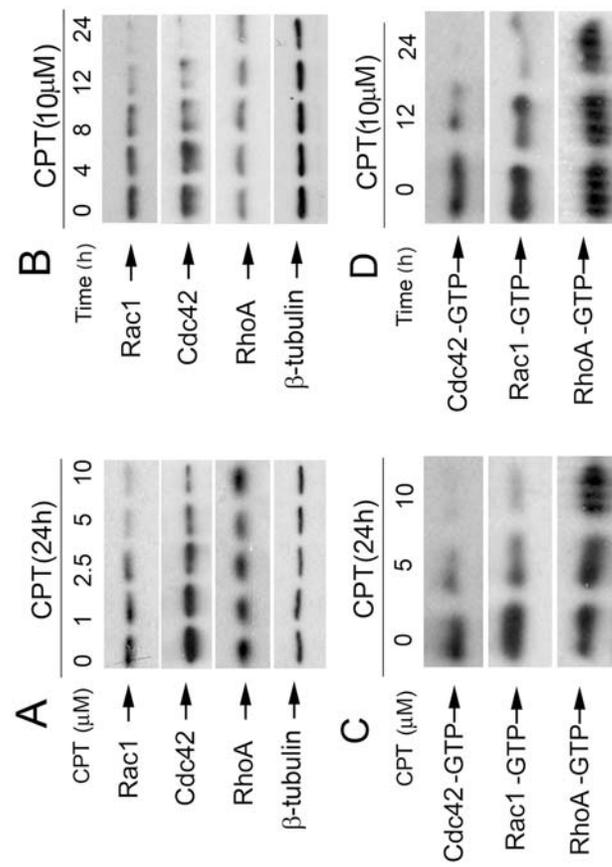
Luo et al., Fig.2



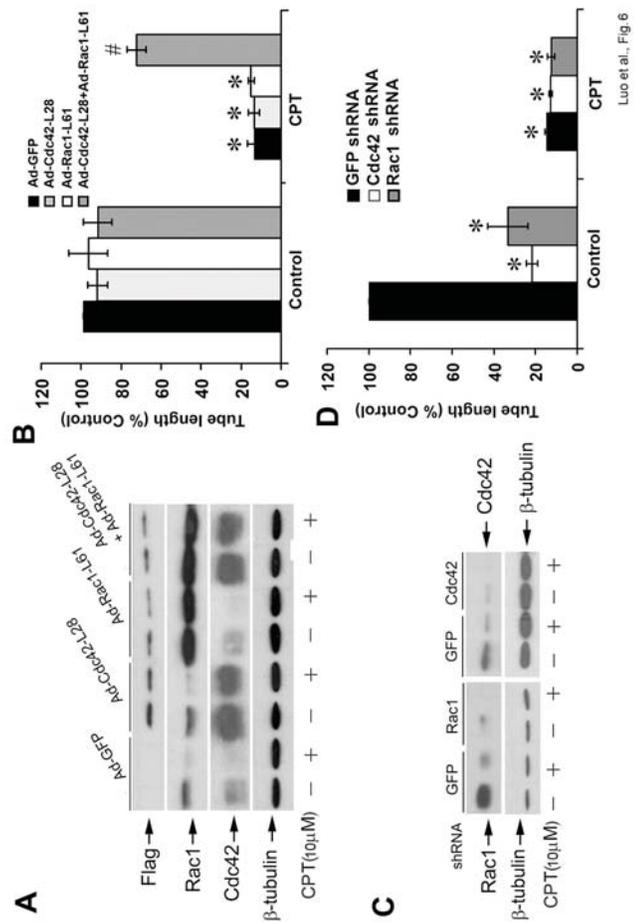
Luo et al., Fig.3



Luo et al., Fig. 4



Luo et al., Fig.5



Luo et al., Fig. 6

# Cancer Prevention Research

## Cryptotanshinone inhibits lymphatic endothelial cell tube formation by suppressing VEGFR-3/ERK and small GTPase pathways

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