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 VEGF receptor 3 (VEGFR-3) in murine lymphatic endothelial cells (LEC), 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. Cancer Prev Res; 4(12): 2083–91. ©2011 AACR.

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 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 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; ref. 9). Most recently, we have further shown 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 G2-M 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 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 (20) and human umbilical vein endothelial cells (21). However, the effect of CPT on lymphangiogenesis is not known. 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 3 VEGF receptors (VEGFR-1, VEGFR-2, and VEGFR-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 (LEC), respectively (22). It is known that VEGF-A binds to VEGFR-1/2, regulating vasculogenesis and angiogenesis, whereas 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 phosphoinositide-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 formation in LECs, which are critical for lymphangiogenesis (24–27). Recent studies have shown 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. 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 VEGF–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 mmol/L), which was aliquoted and stored at −20°C. U0126, a selective inhibitor of MKK1/2, was obtained from LC Laboratories.

**Cell lines and culture**

Murine LECs (30) were grown in antibiotic-free Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 (Mediatech) supplemented with 10% FBS (Hyclone) at 37°C and 5% CO2. Human embryonic kidney 293 (American Type Culture Collection) and 293TD and 293A cells (Invitrogen) were grown in antibiotic-free DMEM (Mediatech) supplemented with 10% heat-inactivated FBS and nonessential amino acids (Mediatech) at 37°C and 5% CO2.

**Plasmids and transfection**

LEC clones stably overexpressing p3xFlag-VEGFR-3-TV1 and p3xFlag-TV1 plasmid (empty vector, as a control) were generated and used as described (29).

**Lentiviral shRNA cloning, production, and infection**

To generate lentiviral short hairpin RNA (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 sites. The oligonucleotides used were as follows: Rac1 sense: 5′-AATTCCTCATCCGAGTCTCATGTCATCATGTCTCCTGATATCCTATCTCTTGCATAGGA-3′, antisense: 5′-GATCCAAAAATACCTGGAGTGCTCAGCTTGCA-GAATTCCCATACCGGAGTGCTCAGCTTGCA-GATC-3′; and Cdc42 sense: 5′-AATTCCTCATGTTCCTGATATCCTATGCAAGAGATA-GGATATCAGGAGACATGTTTTTG-3′, antisense: 5′-GATC-CAAAACATGTCCTCTGATATCCTATCTGTGCATAGGATATCAGGAGACATGTTTTTG-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 hours 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-Cdc4-2L68), 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 conducted 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), phospho-ERK1/2 (Thr202/Tyr204), phospho-Akt (Ser473; Cell Signaling Technology), Rac1 (Cytoskeleton), and β-tubulin (Sigma).

**Cell morphologic analysis**

LEC cells were seeded at a density of 5 × 105 cells per well in 6-well plates. Next day, the cells were treated with CPT (0–10 μmol/L) for 24 hours or with 10 μmol/L CPT for 0 to 24 hours, followed by taking images under an Olympus inverted phase-contrast microscope (Olympus Optical; 200×) equipped with the Quick Imaging system.

**Tube formation assay**

Tube formation assay was conducted as described previously (29).

**Small GTPase activity assay**

The activity of Rac1, Cdc42, or RhoA was determined using Rac/Cdc42 assay kit and Rh assay kit (Millipore), respectively, as described previously (33).
Statistical analysis

Results were expressed as mean values ± SD. The data were analyzed by one-way ANOVA followed by post hoc the Dunnett 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 \textit{in vitro} model for lymphangiogenesis. Treatment with CPT (0–10 μmol/L) for 24 hours did not apparently affect LEC cell viability according to cell morphology (Fig. 1A, bottom). However, pretreatment with CPT (0–10 μmol/L) for 24 hours inhibited LEC tube formation in a concentration-dependent manner (Fig. 1A, top). At 5 and 10 μmol/L, CPT inhibited the tube formation by approximately 65% and 90%, respectively, compared with the control group (Fig. 1C). Furthermore, CPT (10 μmol/L) also inhibited LEC tube formation in a time-dependent manner (Fig. 1B, top), despite no obvious effect on cell viability (Fig. 1B, bottom). After treatment for 4 hours, CPT (10 μmol/L) was able to significantly inhibit the tube formation (by ~20%). When LECs were treated with CPT (10 μmol/L) for 24 hours, the tube formation was suppressed by approximately 90%, compared with the control group (Fig. 1D).

CPT inhibition of LEC tube formation is associated with suppressing VEGFR-3 protein expression

Because 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 μmol/L) for 24 hours, 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 μmol/L, a time-dependent inhibition of VEGFR-3 expression was also observed (Fig. 2B). Treatment with CPT for 8 hours was able to remarkably downregulate VEGFR-3 protein level. Prolonged treatment with CPT resulted in more reduction of VEGFR-3 (Fig. 2B).

Figure 1. CPT inhibits LEC tube formation in a concentration- and time-dependent manner. LECs were treated with CPT (0–10 μmol/L) for 24 hours or CPT (10 μmol/L) for 0 to 24 hours, followed by tube formation assay and morphologic analysis, as described in Materials and Methods. Representative images are shown in (A) and (B), respectively. Bar, 100 μm. The length of tube-like formation was evaluated by NIH ImageJ software. Quantitative data are presented as mean ± SD (n = 3) in (C) and (D), respectively. * \( P < 0.05 \), ** \( P < 0.01 \), difference versus control group.
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 p3xFlag-VEGFR-3-TV1 plasmid, as described (29). About 3-fold increase of VEGFR-3 protein expression was detected in LEC/VEGFR-3 cells, compared with the control cells (LEC/V) transfected with the empty vector (Fig. 2C, left). Overexpression of VEGFR-3 did not alter the cell viability and growth rate in LECs. Treatment with CPT (10 μmol/L) for 24 hours inhibited VEGFR-3 protein expression by approximately 80% (Fig. 2C, left) and suppressed the tube formation by approximately 90% in LEC/V cells (Fig. 2C, right). When LEC/VEGFR-3 cells were exposed to CPT (10 μmol/L) for 24 hours, VEGFR-3 protein expression was reduced by approximately 50%, but the VEGFR-3 protein level was still slightly higher than the basal level in the control (LEC/V) cells (Fig. 2C, left). Interestingly, overexpression of VEGFR-3 rendered high resistance to CPT inhibition of the tube formation (Fig. 2C, right), 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 used. Infection with lentiviral shRNA to VEGFR-3 downregulated the protein expression of VEGFR-3 by approximately 90%, in comparison with the controls infected with lentiviral shRNA to GFP (Fig. 2D, left). Silencing VEGFR-3 mimicked the effect of CPT, inhibiting LEC tube formation by approximately 90% (Fig. 2D, right), which supports that VEGFR-3 is essential for LEC tube formation. Addition of CPT (10 μmol/L) 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.

Figure 2. CPT inhibition of LEC tube formation is associated with suppressing VEGFR-3 protein expression. A and B, CPT inhibited protein expression of VEGFR-3 in a concentration- and time-dependent manner. LECs, treated with CPT (0–10 μmol/L) for 24 hours (A) or CPT (10 μmol/L) for 0 to 24 hours (B), were harvested and subjected to Western blot analysis with antibodies to VEGFR-3. β-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 μmol/L) for 24 hours, followed by Western blot analysis with indicated antibodies (left) or tube formation assay (right) as described in Materials and Methods. Quantitative results of tube formation are shown as mean ± SD (n = 3). *, P < 0.05, difference versus control group; #, P < 0.05, difference versus LEC/V group. D, lentiviral shRNA to VEGFR-3 and GFP (control), respectively, were treated with CPT (10 μmol/L) for 24 hours, followed by tube formation assay (right), as described in Materials and Methods. Quantitative results of tube formation are shown as mean ± SD (n = 3). *, P < 0.05, difference versus GFP shRNA control group.
CPT inhibits LEC tube formation by targeting VEGFR-3-mediated ERK1/2 pathway

As PI3K/Akt and MAPK pathways are the 2 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 c-Jun N-terminal kinases (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 μmol/L) for 24 hours, 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 μmol/L) for 24 hours 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 with 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 used to treat the LECs. We observed that 5 μmol/L U0126 inhibited ERK1/2 phosphorylation almost completely and suppressed the tube formation by 90% in LECs. Addition of 10 μmol/L CPT failed to enhance U0126 inhibition of the tube formation (data not shown). However, treatment with either 2.5 μmol/L U0126 or 5 μmol/L CPT alone inhibited ERK1/2 phosphorylation by approximately 50% (Fig. 4C) and inhibited LEC tube formation by approximately 60% and 40%, respectively (Fig. 4D). Combined treatment with 2.5 μmol/L U0126 and 5 μmol/L CPT displayed an additive or synergistic inhibitory effect on ERK1/2 phosphorylation and the tube

Figure 3. CPT inhibits VEGFR-3–mediated ERK1/2 pathway. A and 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 μmol/L) for 24 hours (A) or CPT (10 μmol/L) for 0 to 24 hours (B), were harvested and subjected to Western blot analysis with indicated antibodies. β-Tubulin was used as a loading control. C, overexpression of VEGFR-3 conferred resistance to CPT (10 μmol/L) for 24 hours, 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 μmol/L) for 24 hours, followed by Western blotting with indicated antibodies.
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 μmol/L) for 24 hours or 10 μmol/L CPT for 0 to 24 hours, 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 show 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 expressed 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) and Cdc42 (Fig. 6C, right), respectively. Of interest, downregulation of Rac1 or Cdc42 by approximately 90% alone was able to inhibit LEC tube formation by approximately 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 2 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 antilymphangiogenesis 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.
Selective suppression of ERK1/2 by U0126 (5 μmol/L) also inhibited phosphorylation of ERK1/2 in murine LECs. Here, we found that CPT affected neither cellular protein expression, nor phosphorylation of Akt, JNK, and p38 MAPK pathways (22, 23). Here, we have also observed that expression of constitutively active Rac1 and Cdc42, but not RhoA, in LECs in a concentration- and time-dependent manner. A and B, LECs treated with CPT (0–10 μmol/L) for 24 hours (A) or CPT (10 μmol/L) for 0 to 24 hours (B), were harvested and subjected to Western blotting with indicated antibodies. β-Tubulin was used as a loading control. C and 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 μmol/L) for 24 hours (C) or CPT (10 μmol/L) for 0 to 24 hours (D), were harvested for the small GTPase activity assay as described in Materials and Methods.

VECGR-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 antilymphangiogenic effect of CPT is associated with inhibition of VEGFR-3 protein expression in the LECs. This is supported by the findings that (i) CPT inhibited VEGFR-3 protein expression, (ii) overexpression of VEGFR-3 conferred high resistance to CPT inhibition of LEC tube formation, and (iii) 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. Furthermore, 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 μmol/L) 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 μmol/L; data not shown). However, when LECs were treated with lower concentrations of these 2 compounds, CPT (5 μmol/L) either additively or synergistically enhanced the inhibition of U0126 (2.5 μmol/L) 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. Because 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 showed 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 (24, 25, 28, 45). It is worth 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 (46). 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 used. 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 (Supplementary Fig. 1S). Furthermore, our data indicate that CPT inhibition of LEC tube formation was also, in part, by targeting Rac1 and Cdc42 (Supplementary Fig. 1S). CPT may act as a novel antilymphangiogenic agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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