Methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate, a Derivative of Glycyrrhetinic Acid, Functions as a Potent Angiogenesis Inhibitor

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Abbreviations: VEGF, vascular endothelial growth factor; HUVECs, human umbilical endothelial cells; PPARγ, peroxisome proliferator-activated receptor gamma; CDODA-Me, methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate; mTOR, mammalian target of rapamycin; VEGFR2, vascular endothelial growth factor receptor 2; ERK, extracellular signal-regulated protein kinase; ECGM, endothelial cell growth medium; T2DM, type 2 diabetes mellitus.
Abstract

Methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate (CDODA-Me), a triterpenoid acid derived synthetically from glycyrrhetinic acid, has been characterized as a peroxisome proliferator-activated receptor gamma (PPARγ) agonist with a broad range of receptor-dependent and independent anticancer activities. Although CDODA-Me decreases the expression of some angiogenic genes in cancer cells, the direct effects of this compound on angiogenesis have not been defined. In this study, we have extensively investigated the activities of CDODA-Me in multiple angiogenesis assays. Our results showed that this agent inhibited vascular endothelial growth factor (VEGF)-induced proliferation, migration, invasion, lamellipodium and capillary-like structure formation in human umbilical endothelial cells (HUVECs) in a concentration-dependent manner. Moreover, CDODA-Me abrogated VEGF-induced sprouting of microvessels from rat aortic rings ex vivo and inhibited the generation of new vasculature in the Matrigel plugs in vivo, where CDODA-Me significantly decreased the number of infiltrating von Willebrand Factor-positive endothelial cells.

To understand the molecular basis of this antiangiogenic activity, we examined the signaling pathways in CDODA-Me-treated HUVECs. Our results showed that CDODA-Me significantly suppressed the activation of VEGF receptor 2 (VEGFR2) and interfered with the mammalian target of rapamycin (mTOR) signaling, including mTOR kinase and its downstream ribosomal S6 kinase (S6K), but had little effect on the activities of extracellular signal-regulated protein kinase and AKT. Taken together, CDODA-Me blocks several key steps of angiogenesis by inhibiting VEGF/VEGFR2.
and mTOR/S6K signaling pathways, making the compound a promising agent for the treatment of cancer and angiogenesis-related pathologies.
Introduction

Angiogenesis, defined as a physiological process involving the generation of new vasculature from preexisting vessels, is restricted in adults to some processes related to the reproductive cycle and wound repair, and is carefully regulated by a balance of pro- and anti-angiogenic molecules (Ferrara and Kerbel, 2005). However, many diseases including diabetic retinopathy, age-related macular degeneration, arthritis and psoriasis, are dependent on up-regulated angiogenesis. Moreover, angiogenesis is well-documented as a fundamental process in the transition of tumors from a dormant state to a malignant state and is considered to be one of the hallmarks of cancer (Hanahan and Weinberg, 2000), playing an essential role in tumor growth, invasion, and metastasis (Carmeliet, 2005; Quesada et al., 2006). It is estimated that angiogenesis in tumors contributes to over 90% of all cancer deaths. Stromal-like cells such as fibroblasts, endothelial and inflammatory cells are genetically-stable and less susceptible to drug resistance. Therefore, angiogenesis therapies targeting stroma have become increasingly important for cancer chemotherapy and other diseases (Hafner et al., 2005).

We have previously shown that a variety of known and potential chemopreventive natural compounds target angiogenesis, a concept termed “angioprevention” (Albini et al., 2006; Pang et al., 2009a; Pang et al.; Pang et al., 2009b; Yi et al., 2008a; Yi et al., 2008b) Recently studies in our laboratory have shown that morelloflavone, extracted from *Garcinia dulcis* (Pang et al., 2009a) , thymoquinone derived from
black seed (*Nigella sativa*) oil (Yi et al., 2008a), gambogenic acid from *Gamboge hanburyi* (Yi et al., 2008b), boswellic acid from gum resin of *Boswellia serrata* and *Boswellia carterii Birdw* (Pang et al., 2009b) and celastrol derived from *Trypterygium wilfordii* Hook F. (“Thunder of God Vine”) (Pang et al.) are functional angiogenesis inhibitors, acting on one or several biological functions of activated endothelial cells, including proliferation, adhesion, migration, invasion, capillary-structure formation and angiogenic signaling pathways. Methyl 2-cyano-3, 11-dioxo-18-olean-1, 12-dien-30-oate (CDODA-Me) is a synthetic derivative of glycyrrhetinic acid, a triterpenoid phytochemical found in licorice extracts. CDODA-Me was initially characterized as a peroxisome proliferator-activated receptor gamma (PPARγ) agonist (Chintharlapalli et al., 2007a), and subsequently shown to inhibit proliferation of colon, pancreatic and prostate cancer cells (Chintharlapalli et al., 2009; Chintharlapalli et al., 2007a). CDODA-Me also decreases specificity protein (Sp) transcription factors and Sp-dependent genes, including vascular endothelial growth factor (VEGF) and VEGF receptors (Chintharlapalli et al., 2009), suggesting great potential for this compound as an inhibitor of angiogenesis. The anticancer activities of CDODA-Me in colon and prostate cancer cells were primarily PPARγ-independent and we hypothesized that, like other natural compounds, the antiangiogenic activity of CDODA-Me could be a key component of its anticancer actions.

To investigate the effects of CDODA-Me on angiogenesis, we examined how this
compound specifically regulates endothelial cells and the underlying mechanism. Our results showed that CDODA-Me interfered with various key steps of angiogenesis \textit{in vitro} and \textit{in vivo}. Pretreatment of CDODA-Me resulted in the blockade of VEGFR2 activation and the mTOR signaling kinases, but had little effects on the phosphorylation of AKT and extracellular signal-regulated protein kinase (ERK), suggesting that CDODA-Me could be used as an antiangiogenic agent for tumor angiogenesis and angiogenesis-related diseases.
Methods

**Reagents, antibodies and cells.** CDODA-Me was synthesized as previously described (Chintharlapalli et al., 2007a) and >98% pure as determined by GC-MS. A 5 mmol/L stock solution of CDODA-Me was prepared and then stored at –20°C as small aliquots until needed. Bacteria-derived recombinant human VEGF (VEGF-A) was from the Experimental Branch of the National Institutes of Health (NIH; Bethesda, MD). Growth factor–reduced Matrigel and BrdU Flow kit were purchased from BD Biosciences (San Jose, CA). CellTiter 96 AQueous One Solution Cell Proliferation Assay kit was purchased from Promega (Madison, WI). Rhodamine-phalloidin was obtained from Invitrogen Molecular Probes (Eugene, OR). Antibodies against β-actin, caspase 3 and poly (ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against AKT, ERK1/2, mTOR, p70S6K, and phospho-specific anti-AKT (Ser473), anti-ERK1/2 (Thr202/Tyr204), anti-mTOR (Ser2448), anti-mTOR (Ser2481), anti-p70S6K (Thr389), anti-p70S6K (Thr421/Ser424) and anti-VEGFR2 (Tyr1175) were purchased from Cell Signaling Technology (Danvers, MA). Primary human umbilical vascular endothelial cells (HUVECs) were cultured in endothelial cell growth medium (ECGM) as described previously (Pang et al., 2009a). HUVECs were cultured at 37°C under a humidified 95%:5% (v/v) mixture of air and CO2.

**Endothelia cell viability and cell-cycle analysis.** HUVECs (5×10³ cells/well) were treated with or without VEGF (50 ng/mL) and various concentrations of CDODA-Me
for different intervals (24-72h). To determine endothelial cell viability, we used MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] kit from Promega (Madison, WI). Cell-cycle analysis (Williams et al., 2006) was carried out in HUVECs synchronized overnight in “starvation medium” consisting M199 (Gibco-Invitrogen) with 2% FBS (Hyclone Laboratories), 25 μg/mL porcine heparin (Sigma Chemical Corporation) and 20 mmol/L HEPES buffer (Sigma Chemical Corporation) for overnight. Cells were then incubated with 50 ng/mL VEGF for 24 h in starvation medium. Where noted, cells were pulsed with 10 μM 5′-bromo-2′-deoxyuridine (BrdU; BD Biosciences; San Jose, CA) by incubation for 1 h prior to harvest. Later, cells were detached, fixed and stained following the protocol of the BrdU Flow kit (BD Biosciences; San Jose, CA). Seven-amino-actinomycin D (7-AAD) staining was chosen to determine the total DNA. Data was collected using a fluorescence activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson) and analyzed using CELLQuest software (Becton Dickinson).

**Migration assay.** HUVECs were allowed to grow to full confluence in 6-well plates precoated with 0.1% gelatin (Sigma; St. Louis, MO) and wounded by pipette tips. Indicated concentrations of CDODA-Me were added into the wells with or without 20 ng/mL VEGF in starvation medium (2% FBS). Images of the cells were taken after 8–10 h of incubation at 37°C in a 95%:5% (v/v) mixture of air and CO₂. Three independent experiments were performed.
Transwell migration assay. The chemotactic motility of HUVECs was determined using a Transwell (BD Biosciences; San Jose, CA) migration assay as described previously (Pang et al., 2009b). Briefly, the filter of the Transwell plate was coated with 0.1% gelatin. HUVECs (2×10^4 cells/treatment) was first pretreated with different concentrations of CDODA-Me for 1 h and then seeded into the upper chambers of Transwell. The bottom chambers were filled with 600 μL ECGM supplemented with 20 ng/mL VEGF. After 8-10 h incubation, migrated cells spreading onto the undersurface of the filter were fixed and stained. Images were taken using an inverted microscope (Olympus; magnification, ×100), and invasive cells were quantified by manual counting. The percentage of migrated cells inhibited by CDODA-Me was expressed on the basis of untreated control.

Lamellipodium formation assay. To examine the dynamic cytoskeleton change in cell movement after treatment, the lamellipodium formation assay was performed as described (Pinkaew et al., 2009). In brief, HUVECs were starved for 4 h in serum-free medium and then pretreated with various concentrations of CDODA-Me for 2 h, followed by stimulation with 20 ng/mL VEGF for 30 min at 37°C. After treated cells being washed, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, rhodamine-phalloidin (Invitrogen-Molecular Probes) and 4',6-diamidino-2-phenylindole (DAPI; Biostatus, Ltd, Leicestershire, UK) solution were applied to the different treatments respectively. Sequentially, stained cells were mounted and images were taken by a Leica DM 4000B photo microscope (Solms, Germany; magnification
×320). Lamellidodium formation of cells was indicated with arrows.

**Capillary-like tube formation assay.** Tube formation was assessed as described previously (Pang et al., 2009b). HUVECs (4-7×10⁴ cells per treatment) were pretreated with various dilutions of CDODA-Me for 1 h in serum-free medium and then seeded onto the Matrigel layer in 24-well plates. Subsequently, 20 ng/mL VEGF was supplemented into the wells. Tubular structure of endothelial cells was photographed using a phase-contrast microscopy (Olympus; magnification, ×100) after 5-6 h. The tube length of capillary-like structure was calculated from 5 fields randomly and three independent experiments were performed.

**Rat aortic ring assay.** Rat aortic ring assay was carried out as described previously (Pang et al., 2009b). In brief, 48-well plates were first coated with 120 μL of Matrigel per well. Aortas isolated from 6-week-old male Sprague-Dawley rats were cut into rings of 1-1.5 mm in circumference and randomized into wells. Twenty ng/mL of VEGF with or without CDODA-Me was added into the wells and the fresh medium with the same formula was replaced for every other day. After 6 d, microvessel sprouting was fixed and photographed using an inverted microscope (Olympus, Center Valley, PA; magnification, ×100). The assay was scored from 0 (least positive) to 5 (most positive) in a double-blind manner. Each data point was assayed 3 times and three independent experiments were performed.
**In vivo Matrigel plug assay and immunohistochemistry.** Matrigel plug assay was performed as described (Pyun et al., 2008). Briefly, we subcutaneously injected 0.6 mL of Matrigel containing 3 μg of CDODA-Me, 100 ng of VEGF, and 20 units of heparin into the ventral area of six-week-old female C57BL/6 mice (National Rodent Laboratory Animal Resources, Shanghai, China). Six mice were used for each group. After 6 d, the skin of mice was pulled back with scissors to expose intact Matrigel plugs and plug images were taken by stereo microscope (Olympus, Center Valley, PA). Immediately, those Matrigel plugs were then fixed with 4% paraformaldehyde and embedded in paraffin. Hematoxylin and eosin (H&E) staining and a von Willebrand Factor immunohistochemistry (vWF; Chemicon, Temecula, CA) were performed to identify the infiltration of new vasculature on 5-μm plug sections. Images of new-formed blood vessels were taken using a Leica DM 4000B photo microscope (Solms, Germany; magnification, ×400).

**Western blotting analysis.** To determine the signaling mechanism of CDODA-Me involved in angiogenesis, HUVECs were first starved in serum-free ECGM for 4 h and pretreated sequentially with or without various concentrations of CDODA-Me for 2 h, followed by stimulation with 50 ng/mL of VEGF. Different stimulation intervals with VEGF lead to the activation of different signaling substrates. The whole-cell extracts of various treatments were prepared in RIPA buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM Na4P2O7, and 1 mM PMSF) supplemented with proteinase inhibitor cocktail (Calbiochem, San
Diego, CA). Forty micrograms of cellular protein from each sample was applied to 6%–12% SDS-polyacrylamide gels and probed with specific antibodies, followed by exposure to a horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody (Cell Signaling Technology, Danvers, MA). Protein concentration was determined using bicinchoninic acid (BCA) method and equalized before loading.

**Statistical analysis**

All experiments were repeated three times with independence. Values are given as means and standard deviations (SD). Data was analyzed using SPSS 14.0 software and statistical was assessed by Student's *t*-test or one-way ANOVA. *P* values ≤ 0.05 were considered statistically significant.
Results

**CDODA-Me blocked HUVEC proliferation through a caspase-dependent pathway**

To determine the effect of CDODA-Me on VEGF-induced HUVEC proliferation, we first examined its cytotoxicity using the MTS assay. The endothelial cells were synchronized by starvation and then stimulated with 50 ng/mL of VEGF. At subsequent time points (24, 48 and 72h), treatment of 0.5 μM CDODA-Me significantly reduced VEGF-induced cell viability (Fig. 1A). In contrast, CDODA-Me (< 5 μmol/L) did not induce obvious cytotoxicity to HUVECs cultured under normal condition (20% FBS) (Fig. 1B), suggesting that CDODA-Me at the same concentration exhibited a higher potency to suppress the proliferation of VEGF-activated endothelial cells.

Additionally, the antiproliferative action of CDODA-Me was confirmed by measuring the fraction of cells in the S phase of the cell cycle based on BrdU and 7-AAD incorporation during VEGF stimulation. The results showed that the proportion of BrdU-positive cells in the S phase (in the presence of VEGF) was 19.06% in VEGF group. However, BrdU-positive cells decreased rapidly with increasing concentrations of CDODA-Me from 10.06% at 0.5μmol/L to 4.74% at 1.25μmol/L (Fig. 1C), indicating that treatment with CDODA-Me inhibited endothelial cell-cycle progression and arrested cells in G1 phase.
We next examined whether the antiproliferative effects of CDODA-Me on HUVECs were accompanied by induction of apoptosis using by western blot analysis. Our results showed that CDODA-Me dose-dependently induced endothelial cells to undergo apoptosis, with a clear accumulation of cleaved PARP and cleaved caspase-3 (Fig. 1D), suggesting that CDODA-Me inhibited HUVEC proliferation by a caspase-dependent pathway.

**CDODA-Me inhibits VEGF-induced chemotactic motility and lamellipodium formation**

Migration is a key process required for angiogenesis and VEGF-A is a potent and specific mitogen for endothelial cells and was used as a chemoattractant in migration assays. We observed that CDODA-Me inhibited VEGF-induced HUVEC migration (Fig. 2A) and invasion (Fig. 2B) in a dose-dependent manner, with significant inhibition at 1.25 μmol/L (Fig. 2C). To further assess the cytoskeleton changes involved in CDODA-Me-dependent inhibition of cell migration and invasion, we investigated lamellipodium formation of HUVECs triggered by VEGF using rhodamine-phalloidin staining (Fig. 3A). Our results showed that CDODA-Me disrupted lamellipodium formation and F-actin activation at the exterior edge of endothelial cells in a dose-dependent manner.

**CDODA-Me inhibits VEGF-induced capillary-structure formation**

Although angiogenesis is a very complex process involving several steps, endothelial
cell differentiation is the crucial checkpoint (Patan, 2004). HUVECs when seeded on Matrigel become elongated and form capillary-like structures mimicking the *in vivo* neoangiogenesis process (Taraboletti and Giavazzi, 2004). We used this assay to examine the potential effects of CDODA-Me on the tubular structure formation of endothelial cells. As shown in Fig. 3B, HUVECs at 6 h post-seeding exhibit a clear network formation in the presence of VEGF; however, treatment with 0.5 μmol/L or 1.25 μmol/L of CDODA-Me dramatically blocked VEGF-induced capillary-like structure formation (Fig. 3C). This phenomenon was observed within a relatively short interval and provides evidence that CDODA-Me-mediated suppression of HUVEC network formation was possibly through targeting certain signaling pathways rather than nonspecific cytotoxicity.

**CDODA-Me inhibits VEGF-induced microvessel sprouting *ex vivo***

To further explore whether CDODA-Me inhibited VEGF-induced angiogenesis *ex vivo*, we examined the sprouting of microvessels from aortic rings in the absence or presence of CDODA-Me. As shown in Fig 4A, VEGF (20 ng/mL) alone significantly triggered microvessel sprouting, leading to the formation of a complex network of microvessels around the aortic rings, whereas treatment with CDODA-Me antagonized the VEGF-induced sprouting (Fig. 4A). It is noteworthy that the minimal effective concentration of CDODA-Me in inhibiting microvessel sprouting was 1.25 μmol/L while 2.5 μmol/L CDODA-Me greatly decreased the number of microvessel sprouts triggered by VEGF (Fig. 4A and 4B).
CDODA-Me inhibits angiogenesis in vivo

To validate CDODA-Me-mediated inhibitory functions on angiogenesis in a whole animal, we used an established in vivo angiogenesis model, namely the mouse Matrigel plug assay. After being embedded subcutaneously into mice for a week, Matrigel plugs containing VEGF alone appeared dark red (Fig. 5A), indicating that infiltrating vasculatures had formed inside the Matrigel via angiogenesis. In contrast, addition of CDODA-Me in the Matrigel plugs at 3 μg per plug dramatically inhibited functional new vasculature generation, with the Matrigel plugs being pale and weak in color (Fig. 5A).

We next used hematoxylin and eosin (H&E) staining and immunohistochemistry with vWF antibody to identify the infiltrating endothelial cells and vasculature content in the Matrigel plugs. Our data indicated that infiltrating endothelial cells in VEGF positive plugs polarized and formed linage around the vasculature, whereas the endothelial cells in CDODA-Me treated plugs were still scattered (Fig. 5B). Moreover, there were fewer vWF-positive cells in the CDODA-Me-treated group compared to the control group, suggesting that CDODA-Me strongly suppress angiogenesis in vivo.

VEGF/VEGFR2 and mTOR signaling are molecular targets of CDODA-Me in angiogenesis
In previous studies, we showed that CDODA-Me decreased expression of Sp1, Sp3, Sp4 and Sp-dependent genes, including VEGF and VEGF receptors in colon cancer cells after treatment for 24, 48 or 72 h (Chintharlapalli et al., 2009). In this study we found that CDODA-Me treatment of HUVECs for 24 h also resulted in the downregulation of both VEGFR1 and VEGFR2 mRNA levels in endothelial cells (data not shown). Since VEGFR2 is the primary receptor that mediates VEGF-induced angiogenesis signaling pathways, we examined the function of CDODA-Me in blockage of VEGFR2 activation after a relatively short treatment period (pretreatment for 2 h with CDODA-Me). Our results showed that 0.5 μmol/L of CDODA-Me significantly suppressed VEGFR2 phosphorylation without affecting VEGFR2 protein levels (Fig. 6A). To further investigate the intracellular kinases affected by CDODA-Me, we screened some key kinases involved in angiogenesis signaling. Our data showed that CDODA-Me had little effects on the phosphorylation of ERK and AKT (Fig. 6B), but significantly inhibited the phosphorylation of mTOR kinase (both at Ser 2448 and Ser 2481 site) and its downstream ribosomal S6 kinase (at Thr 389 and Thr 421/Ser 424 site) at concentrations of 0.5 and 1.25 μmol/L (Fig. 6C), suggesting that CDODA-Me-mediated suppression of angiogenesis is partially due to the inhibition of mTOR signaling pathways.
Discussion

Peroxisome proliferator-activated receptors (PPARs) belong to a large superfamily of nuclear hormone receptors, which play an important role in the regulation of lipid homoeostasis and glucose metabolism (Christodoulides and Vidal-Puig, 2009; Stienstra et al., 2007). So far, three different isoforms are identified, including PPARα, PPARβ/δ and PPARγ1/2. Apart from the endogenous natural ligands, a multitude of synthetic PPARγ agonists have been developed to treat type 2 diabetes mellitus (T2DM). Unexpectedly, those PPARγ agonists exhibit anti-neoplastic effects beyond their original “metabolic” indications (Giaginis et al., 2008; Hafner et al., 2005; Simpson-Haidaris et al.). For example, methyl 2-cyano-3, 11-dioxo-18-olean-1,12-dien-30-oate (CDODA-Me), a synthetic PPARγ agonist and a derivative of 18beta-glycyrrhetinic acid, inhibits growth of colon (Chintharlapalli et al., 2009; Chintharlapalli et al., 2007a), prostate (Papineni et al., 2008), pancreatic (Chintharlapalli et al., 2007b), and bladder cancer cells primarily through PPARγ-independent signaling pathways. However, most studies on the anticancer activities of PPARγ agonists suggest that these effects are primarily receptor-independent in many cancer cell and tumor models (Chintharlapalli et al., 2009; Chintharlapalli et al., 2007b; Papineni et al., 2008). In the present study, we observed that CDODA-Me was a potent inhibitor of angiogenesis in vitro and ex vivo, in endothelial cell proliferation, apoptosis, migration, invasion and tubulogenesis assays (Fig 1-4). These phenomena was consistent with previous reports that PPAR gamma ligands, pioglitazone and rosiglitazone, possess inhibitory properties on
vascular endothelial growth factor- and basic fibroblast growth factor-induced angiogenesis and on endothelial cell migration (Aljada et al., 2008). Furthermore, the antiangiogenic activities of CDODA-Me are validated in a whole animal model using an established in vivo Matrigel plug assay, demonstrating CDODA-Me significantly decreased the number of infiltrating vWF-positive endothelial cells and vasculature density (Fig. 5). Based on all these observations, we deem that CDODA-Me is a potential angiopreventive agent. Oppositely, evidences has been raised that some PPARγ agonists represent pro-angiogenic actions (Biscetti et al., 2008; Chintalgattu et al., 2007; Huang et al., 2008), which might be due to diverse mechanisms in adipogenesis and inflammation with different mediators and different pathological processes.

We also investigated the molecular events associated with the antiangiogenic activity of CDODA-Me in HUVECs. CDODA-Me significantly inhibited activation of VEGF receptor 2 (VEGFR2) and blocked the mTOR/S6K signaling pathway. In the early stages of angiogenesis, VEGF-A and VEGFR-2 play a crucial role in vessel sprouting and new vessel initiation through induction of proliferation, migration and survival of endothelial cells (Ferrara and Kerbel, 2005; Helmlinger et al., 2000). Thus, at an earlier time point when the angiogenic controller is switched on, CDODA-Me can rapidly exert an antiangiogenic effect by inhibiting VEGF/VEGFR2 pathway activation and suppressing the induction of proangiogenic chemokines in endothelial cells. However, recent preclinical and clinical trials have shown that the blockage of
VEGF-A signaling results in activation of alternative VEGF-A-independent proangiogenic signaling pathways (Casanovas et al., 2005; Roodhart et al., 2008). Investigation of alternative signaling pathways involved in angiogenesis such as the AKT/mTOR/S6K pathway has been initiated recently. mTOR is a serine/threonine kinase which stands at the crossroads of various signaling pathways leading to mRNA, ribosome, protein synthesis and translation of significant biomolecules (Strimpakos et al., 2009), making it a crucial drug target in tumor prevention and therapy. Currently, rapamycin and rapamycin analogs are mTOR inhibitors in clinical trials (Dancey et al., 2009). Our present data showed that CDODA-Me significantly interfered with the mTOR pathway, including mTOR kinase and its downstream ribosomal S6 kinase in a concentration-dependent manner (Fig. 6C), but had little effects on ERK and AKT kinase activities, suggesting CDODA-Me probably acts at the junction of mTOR upstream kinases and mTOR kinase. Our recent studies on the triterpenoid, acetyl-11-keto-beta-boswellic acid (AKBA) showed that this compound also inhibit the mTOR kinase pathway (10). However, unlike CDODA-Me, the effects of AKBA were accompanied by inhibition of the MAPK and PI3K pathways (Pang et al., 2009b). Structure-dependent antiangiogenic activities of triterpenoids are currently being investigated. Based on our results, the dual inhibition of the VEGF-A/VEGFR2 and mTOR signal pathways of CDODA-Me would lead cooperative antiangiogenic effects through inhibition of initiation and further critical stages of blood vessel formation. In addition, previous studies show that CDODA-Me decreased expression of Sp transcription factors and Sp-dependent proangiogenic genes (Papineni et al.,
2008) by repression of miR-27a and induction of ZBTB10 (Chintharlapalli et al., 2009). Through targeting different pathways in both endothelial cells and cancer cells, CDODA-Me blocks interactions of tumors with surrounding stromal cells, thus inhibiting both tumor angiogenesis and tumor growth.

It is noteworthy that CDODA-Me at the same concentration exhibits a higher potency to suppress proliferation of activated endothelial cells (at angiogenic state) compared with that of normal cultured endothelial cells (Fig. 1A and Fig. 1B), suggesting CDODA-Me is a relatively affordable drug that specifically targets activated endothelial cells and tumors. Previously, we have reported that oral gavage with 15 mg/kg/d CDODA-Me did not cause any obvious toxic side-effects but exhibited significant tumor inhibition (Chintharlapalli et al., 2009). Although this study did not completely address clinical utility of this compound in vivo, we anticipate that a long-term use of CDODA-Me at low doses will be well tolerated and these studies are currently being investigated. Our studies demonstrate that in addition to the effects of CDODA-Me on the downregulation of Sp transcription factors in a cancer cell context, this compound also inhibit angiogenic pathways in HUVECs, suppressing critical VEGF-mediated kinase pathways that play a key role in cell survival, growth and angiogenesis. The multiple activities of CDODA-Me suggest a potential clinical role for this compound alone as an anticancer drug or in combination with other compounds such as cytotoxic drugs or other VEGF inhibitors (bevacizumab) (Laquente et al., 2007) in cancer chemotherapy.
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Footnotes

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Legends for Figures

Figure 1. CDODA-Me blocked HUVECs proliferation and produced cytotoxicity by a caspase-dependent pathway.

A, CDODA-Me inhibited VEGF-induced cell viability in a dose-dependent manner. HUVECs (5×10^3 cells/well) were starved and then treated with VEGF (50 ng/mL) and various dilutions of CDODA-Me for 24 h, 48 h and 72 h. Cell viability was quantified by MTS assay. Columns, mean from three different experiments with duplicates; bars, standard deviation; *, P < 0.05; **, P < 0.01 vs. VEGF positive group. B, CDODA-Me did not exhibit obvious cytotoxicity to normal cultured HUVECs. HUVECs were directly treated with CDODA-Me for 48 h, and cell viability was analyzed by MTS assay. Columns, mean from three different experiments with duplicates; bars, standard deviation; **, P < 0.01 vs. the untreated cells. C, CDODA-Me blocked the endothelial cell-cycle progression by decreasing S-phase population. Endothelial cells were pulsed with 10 μM BrdU for 1 h prior to harvest at 48 h. Cells were consecutively fixed, stained and analyzed by a fluorescence activated cell sorting. D, HUVECs underwent apoptosis after CDODA-Me treatment. The whole-cell sample from endothelial cells treated by CDODA-Me was applied to western blotting and probed with apoptosis indicators, cleaved caspase 3 and cleaved PARP.

Figure 2. CDODA-Me inhibits VEGF-induced chemotactic motility of endothelial cells. Activated endothelial cell motility was evaluated by migration
assay and Transwell invasion assay. A, CDODA-Me inhibited the migration of endothelial cells. HUVECs were grown into full confluence in 6-well plate and scratched by pipette, followed by treatments of 20 ng/mL VEGF and indicated concentrations of CDODA-Me. Images were taken after 8–10 h of incubation (magnification, ×100) and three independent experiments were performed. B, CDODA-Me inhibited endothelial cell invasion. HUVECs were seeded in the upper chamber of a gelatin-coated Transwell and treated with different concentrations of CDODA-Me. The bottom chamber was filled with ECGM supplemented with 20 ng/mL VEGF. Images were taken after 8–10 h of incubation (magnification, ×100). C, Those invasive cells (spreading onto the undersurface of the filter) were quantified by manual counting. Five fields were randomly selected and three independent experiments were performed. Columns, mean from three different experiments with duplicates; bars, standard deviation; **, \( P < 0.01 \) vs. VEGF positive group.

**Figure 3. CDODA-Me blocks VEGF-triggered formation of lamellipodium and capillary- structure of endothelial cells.**

A, CDODA-Me inhibited the lamellipodium formation of endothelial cells. Lamellipodium formation was detected by rhodamine-phalloidin staining. Endothelial cells were starved, pretreated with different dilutions of CDODA-Me and then stimulated with 50 ng/mL VEGF for 30 min. Next, cells were stained with rhodamine-phalloidin and DAPI. Images were taken by a Leica DM 4000B photo microscope (magnification ×320), and three independent experiments were performed.
B, CDODA-Me inhibited the VEGF-induced tubular formation of HUVECs. Treated endothelial cells were seeded into 24-well plates coated with Matrigel (4-7×10^4 cells/well). After 6 h, cells were fixed, and tubular structures were photographed by a phase-contrast microscopy (magnification, ×100). C, The tube length of capillary-like structure of endothelial cells was analyzed statistically. Columns, mean from three different experiments with duplicates; bars, standard deviation; *, P < 0.05; **, P < 0.01 vs. VEGF alone.

Figure 4. CDODA-Me inhibits VEGF-induced microvessel sprouting ex vivo. Aortic segments isolated from Sprague-Dawley rats were placed into the Matrigel-covered wells and treated with VEGF (20 ng/mL) in the presence or absence of CDODA-Me for 6 d. A, Representative photographs of endothelial cell sprouts from the margins of aortic rings (magnification, ×100). B, Sprouts were scored from 0 (least positive) to 5 (most positive) in a double-blinded manner. Columns, mean from three different experiments with duplicates; Bars, standard deviation; **, P < 0.01 vs. VEGF alone.

Figure 5. CDODA-Me inhibits VEGF-induced angiogenesis in vivo. Six-week-old female C57/BL/6 mice were injected with 0.6 mL of Matrigel containing 3 μg of CDODA-Me, 100 ng of VEGF, and 20 units of heparin into the ventral area (n=6). After 6 d, the skin of mice was pulled back to expose the intact Matrigel plugs. A, CDODA-Me inhibits VEGF-induced angiogenesis in Matrigel plug assays.
Representative Matrigel plugs with control, VEGF, and VEGF plus CDODA-Me were photographed. *B*, CDODA-Me inhibited blood vasculature formation. The Matrigel plugs were fixed, embedded in paraffin and sectioned. Hematoxylin and eosin staining and vWF immunohischemistry were applied to 5-μM plug sections. Images were taken using a Leica DM 4000B photo microscope (magnification, ×400) and infiltrating endothelial cells and vWF-positive cells were marked with arrows.

**Figure 6. CDODA-Me targets VEGFR2 and mTOR signaling pathways in endothelial cells.**  
*A*, CDODA-Me blocked VEGF receptor 2 activation. Endothelial cells were starved, pretreated with CDODA-Me for 2 h and then stimulated with 50 ng/mL VEGF for 2 min. Proteins harvested from different treatments were subjected to Western blotting and probed with phospho-specific anti-VEGFR2 (Tyr1175) antibody.  
*B*, CDODA-Me had little effect on the phosphorylation of ERK and AKT kinases.  
*C*, CDODA-Me inhibited the mTOR signaling pathway in endothelial cells. Proteins from different treatments were probed with specific mTOR signaling kinase antibodies, including phospho-specific anti-mTOR (Ser2448), anti-mTOR (Ser2481), anti-p70S6K (Thr389) anti-p70S6K (Thr421/Ser424) antibodies.

**Figure 7. The chemical structure of CDODA-Me.**
Methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate
(CDODA-Me)

C_{32}H_{43}NO_{4}  FW. 505.69