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

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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 γ 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, and lamellipodium and capillary-like structure formation of 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 proangiogenic and antiangiogenic molecules (Ferrara and Kerbel, 2005). However,

many diseases, including diabetic retinopathy, age-related macular degeneration, arthritis, and psoriasis, depend 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 more than 90% of all cancer deaths. Stromal-like cells such as fibroblasts and 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 the treatment of other diseases (Hafner et al., 2005).

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ABBREVIATIONS: CDODA-Me, methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; HUVEC, human umbilical endothelial cell; PPAR γ , peroxisome proliferator-activated receptor γ ; mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated protein kinase; ECGM, endothelial cell growth medium; FBS, fetal bovine serum; vWF, von Willebrand factor; BrdU, 5'-bromo-2'-deoxyuridine; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; S6K, S6 kinase; Sp, specificity protein; PARP, poly(ADP-ribose) polymerase.

We have previously shown that a variety of known and potential chemopreventive natural compounds target angiogenesis, a concept termed “angioprevention” (Albini et al., 2006; Yi et al., 2008a,b; Pang et al., 2009a,b, 2010.) Our studies have shown that morelloflavone, extracted from *Garcinia dulcis* (Pang et al., 2009a), thymoquinone derived from black seed (*Nigella sativa*) oil (Yi et al., 2008a), gambogic 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., 2010) 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 glycyrrhetic acid, a triterpenoid phytochemical found in licorice extracts. CDODA-Me was initially characterized as a peroxisome proliferator-activated receptor γ (PPAR γ) agonist (Chintharlapalli et al., 2007a) and subsequently shown to inhibit proliferation of colon, pancreatic, and prostate cancer cells (Chintharlapalli et al., 2007a, 2009). CDODA-Me also decreases specificity protein (Sp) transcription factors and Sp-dependent genes, including vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs) (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 PPAR γ -independent, and we hypothesized that, as in other natural compounds, the antiangiogenic activity of CDODA-Me could be a key component of its anticancer actions.

To investigate the effect 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 in vitro and in vivo. Pretreatment of CDODA-Me resulted in the blockade of VEGFR2 activation and the mTOR signaling kinases, but had little effect 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.

Materials and Methods

Reagents, Antibodies, and Cells. CDODA-Me was synthesized as described previously (Chintharlapalli et al., 2007a) and was >98% pure as determined by gas chromatography-mass spectrometry. A 5 mM 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 (Bethesda, MD). Growth factor-reduced Matrigel and a 5'-bromo-2'-deoxyuridine (BrdU) flow kit were purchased from BD Biosciences (San Jose, CA). A CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was purchased from Promega (Madison, WI). Rhodamine-phalloidin was obtained from Invitrogen (Carlsbad, CA). Antibodies against β -actin, caspase 3, and poly(ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology, Inc. (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 CO_2 .

Endothelial Cell Viability and Cell-Cycle Analysis. HUVECs (5×10^3 cells/well) were treated with or without VEGF (50 ng/ml) and various concentrations of CDODA-Me for different intervals (24–72 h). To determine endothelial cell viability, we used a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) inner salt kit from Promega. Cell-cycle analysis (Williams et al., 2006) was carried out in HUVECs synchronized overnight in “starvation medium” consisting of M199 medium (Invitrogen) with 2% FBS (HyClone Laboratories, Logan, UT), 25 $\mu\text{g/ml}$ porcine heparin (Sigma-Aldrich, St. Louis, MO), and 20 mM HEPES buffer (Sigma-Aldrich). Cells were then incubated with 50 ng/ml VEGF for 24 h in starvation medium. Where noted, cells were pulsed with 10 μM BrdU (BD Biosciences) by incubation for 1 h before harvest. Later, cells were detached, fixed, and stained following the protocol of the BrdU flow kit (BD Biosciences). Seven-amino-actinomycin D staining was chosen to determine the total DNA. Data were collected with a fluorescence-activated cell sorting Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

Migration Assay. HUVECs were allowed to grow to full confluence in 6-well plates precoated with 0.1% gelatin (Sigma-Aldrich) 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 to 10 h of incubation at 37°C in a 95:5% (v/v) mixture of air and CO_2 . Three independent experiments were performed.

Transwell Migration Assay. The chemotactic motility of HUVECs was determined by using a Transwell (BD Biosciences) migration assay as described previously (Pang et al., 2009b). In brief, the filter of the Transwell plate was coated with 0.1% gelatin. HUVECs (2×10^4 cells/treatment) were pretreated with different concentrations of CDODA-Me for 1 h and then seeded into the upper chambers of the Transwell plate. The bottom chambers were filled with 600 μl of ECGM supplemented with 20 ng/ml VEGF. After 8 to 10 h of incubation, migrated cells spreading onto the undersurface of the filter were fixed and stained. Images were taken with an inverted microscope (Olympus, Tokyo, Japan; magnification, $\times 100$), 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 previously (Pinkaw 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 . Those cells were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100, rhodamine-phalloidin (Invitrogen) and 4',6-diamidino-2-phenylindole (Biosstatus, Ltd, Leicestershire, UK) solution were applied to the different treatments. Sequentially, stained cells were mounted, and images were taken with a Leica (Solms, Germany) DM 4000B photo microscope (magnification $\times 320$). Lamellipodium formation of cells was indicated.

Capillary-Like Tube Formation Assay. Tube formation was assessed as described previously (Pang et al., 2009b). HUVECs ($4-7 \times 10^4$ 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 by using a phase-contrast microscope (Olympus; magnification, $\times 100$) after 5 to 6 h. The tube length of the capillary-

like structure was calculated randomly from five fields, 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 μ l of Matrigel per well. Aortas isolated from 6-week-old male Sprague-Dawley rats were cut into rings of 1 to 1.5 mm in circumference and randomized into wells. VEGF with or without CDODA-Me (20 ng/ml) was added into the wells, and fresh medium with the same formula was replaced every other day. After 6 days, microvessel sprouting was fixed and photographed by using an inverted microscope (Olympus; magnification, $\times 100$). The assay was scored from 0 (least positive) to 5 (most positive) in a double-blind manner. Each data point was assayed three times, and three independent experiments were performed.

In Vivo Matrigel Plug Assay and Immunohistochemistry. Matrigel plug assay was performed as described previously (Pyun et al., 2008). In brief, we subcutaneously injected 0.6 ml of Matrigel containing 3 μ g of CDODA-Me, 100 ng of VEGF, and 20 units of heparin into the ventral area of 6-week-old female C57BL/6 mice (National Rodent Laboratory Animal Resources, Shanghai, China). Six mice were used for each group. After 6 days, the skin of mice was pulled back with scissors to expose intact Matrigel plugs, and plug images were taken with a stereo microscope (Olympus). Immediately, those Matrigel plugs were then fixed with 4% paraformaldehyde and embedded in paraffin. Hematoxylin and eosin staining and von Willebrand factor (vWF) immunohistochemistry (Millipore Bioscience Research Reagents, Temecula, CA) were performed to identify the infiltration of new vasculature on 5- μ m plug sections. Images of newly formed blood vessels were taken with a Leica DM 4000B photo microscope (magnification, $\times 400$).

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, fol-

lowed by stimulation with 50 ng/ml of VEGF. Different stimulation intervals with VEGF led 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 $\text{Na}_4\text{P}_2\text{O}_7$, and 1 mM phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor cocktail (Calbiochem, San Diego, CA). Forty micrograms of cellular protein from each sample was applied to 6 to 12% SDS-polyacrylamide gels and probed with specific antibodies, followed by exposure to a horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody (Cell Signaling Technology). Protein concentration was determined by using the bicinchoninic acid method and equalized before loading.

Statistical Analysis. All experiments were repeated three times independently. Values are given as means and S.D. Data were analyzed by using SPSS 14.0 software (SPSS Inc., Chicago, IL), and statistical results were assessed by Student's *t* test or one-way analysis of variance. *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 by 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 72 h), treatment of 0.5 μ M CDODA-Me significantly reduced VEGF-induced cell viability (Fig. 1A). In contrast, CDODA-Me ($< 5 \mu\text{M}$) did not induce obvious cytotoxicity to HUVECs cultured under normal conditions (20% FBS) (Fig. 1B), suggesting that CDODA-Me at the same

Fig. 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, 48, and 72 h. Cell viability was quantified by MTS assay. Columns show mean from three different experiments with duplicates; bars indicate standard deviation. *, $P < 0.05$; **, $P < 0.01$ versus 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 show mean from three different experiments with duplicates; bars indicate standard deviation. **, $P < 0.01$ versus the untreated cells. C, CDODA-Me blocked the endothelial cell cycle progression by decreasing the S-phase population. Endothelial cells were pulsed with 10 μ M BrdU for 1 h before harvest at 48 h. Cells were consecutively fixed, stained, and analyzed by 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.

concentration exhibited a higher potency to suppress the proliferation of VEGF-activated endothelial cells.

In addition, 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-amino-actinomycin D 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 0.5 μM to 4.74% at 1.25 μM (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 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 that we 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 μM (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 by 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 (Tarabotti 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 postseeding exhibit a clear network formation in the presence of VEGF; however, treatment with 0.5 or 1.25 μM 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 μM , whereas 2.5 μM CDODA-Me greatly decreased the number of microvessel sprouts triggered by VEGF (Fig. 4).

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 1 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 func-

Fig. 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 plates and scratched by pipette, followed by treatments of 20 ng/ml VEGF and indicated concentrations of CDODA-Me. Images were taken after 8 to 10 h of incubation (magnification, $\times 100$), 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 to 10 h of incubation (magnification, $\times 100$). **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 show mean from three different experiments with duplicates; bars indicate standard deviation. **, $P < 0.01$ versus VEGF-positive group.

Fig. 3. CDODA-Me blocks VEGF-triggered formation of lamellipodium and capillary-like 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 4',6-diamidino-2-phenylindole. Images were taken with a Leica DM 4000B photo microscope (magnification, $\times 320$), 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 ($4\text{--}7 \times 10^4$ cells/well). After 6 h, cells were fixed, and tubular structures were photographed by a phase-contrast microscope (magnification, $\times 100$). **C**, the tube length of capillary-like structure of endothelial cells was analyzed statistically. Columns show mean from three different experiments with duplicates; bars indicate standard deviation. *, $P < 0.05$; **, $P < 0.01$ versus VEGF alone.

Fig. 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 days. **A**, representative photographs of endothelial cell sprouts from the margins of aortic rings (magnification, $\times 100$). **B**, sprouts were scored from 0 (least positive) to 5 (most positive) in a double-blinded manner. Columns show mean from three different experiments with duplicates; bars indicate standard deviation. **, $P < 0.01$ versus VEGF alone.

tional new vasculature generation, with the Matrigel plugs being pale and weak in color (Fig. 5A).

We next used hematoxylin and eosin 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 lineage 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 with 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 down-regulation of both VEGFR1 and VEGFR2 mRNA levels in endothelial cells (data not shown). Because 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 μM 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 effect on the phosphorylation of ERK and AKT (Fig. 6B), but significantly inhibited the phosphorylation of mTOR kinase (at both Ser2448 and Ser248) and its downstream ribosomal S6 kinase (at Thr389 and Thr421/Ser424) at concentrations of 0.5 and 1.25 μM (Fig. 6C), suggesting that CDODA-Me-mediated suppression of angiogenesis is partially caused by the inhibition of mTOR signaling pathways.

Discussion

PPARs belong to a large superfamily of nuclear hormone receptors, which play an important role in the regulation of lipid homeostasis and glucose metabolism (Stienstra et al., 2007; Christodoulides and Vidal-Puig, 2010). So far, three

Fig. 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 μ g of CDODA-Me, 100 ng of VEGF, and 20 units of heparin into the ventral area ($n = 6$). After 6 days, 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 immunohistochemistry were applied to 5- μ M plug sections. Images were taken with a Leica DM 4000B photo microscope (magnification, $\times 400$). Infiltrating endothelial cells and vWF-positive cells are indicated by arrowheads.

different isoforms have been identified: PPAR α , PPAR β/δ , and PPAR $\gamma 1/2$. Apart from the endogenous natural ligands, a multitude of synthetic PPAR γ agonists have been developed to treat type 2 diabetes mellitus. Unexpectedly, those PPAR γ agonists exhibit antineoplastic effects beyond their original “metabolic” indications (Giaginis et al., 2008; Hafner et al., 2005; Simpson-Haidaris et al., 2010). For example, CDODA-Me (Fig. 7), a synthetic PPAR γ agonist and a derivative of 18 β -glycyrrhetic acid, inhibits growth of colon (Chintharlapalli et al., 2007a, 2009), prostate (Papineni et al., 2008), pancreatic (Chintharlapalli et al., 2007b), and bladder cancer cells primarily through PPAR γ -independent signaling pathways. However, most studies on the anticancer activities of PPAR γ agonists suggest that these effects are primarily receptor-independent in many cancer cell and tumor models (Chintharlapalli et al., 2007b, 2009; 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 (Figs. 1–4). These phenomena were consistent with previous reports that PPAR γ ligands, pioglitazone and rosiglitazone, possess inhibitory properties on vascular endothelial growth factor- and basic fibroblast growth factor-induced angiogenesis and endothelial cell migration (Aljada et al., 2008). Furthermore, the antiangiogenic

Fig. 6. CDODA-Me targets VEGFR2 and mTOR signaling pathways in endothelial cells. A, CDODA-Me blocked VEGFR2 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 kinase. 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), and anti-p70S6K (Thr421/Ser424) antibodies.

Methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate
(CDODA-Me)
 $C_{32}H_{43}NO_4$ FW. 505.69

Fig. 7. The chemical structure of CDODA-Me.

activities of CDODA-Me were 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 of these observations, we deem that CDODA-Me is a potential angiopreventive agent. In contrast, evidence has been raised that some PPAR γ agonists represent proangiogenic actions (Chintalgattu et al., 2007; Biscetti et al., 2008; Huang et al., 2008), which might be caused by 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 VEGFR2 and blocked the mTOR/S6K signaling pathway. In the early stages of angiogenesis, VEGF-A and VEGFR2 play a crucial role in vessel sprouting and new vessel initiation through

induction of proliferation, migration, and survival of endothelial cells (Helmlinger et al., 2000; Ferrara and Kerbel, 2005). 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, 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. mTOR is a serine/threonine kinase that 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. 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 effect 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- β -boswellic acid showed that this compound also inhibits the mTOR kinase pathway (10). However, unlike CDODA-Me, the effects of acetyl-11-keto- β -boswellic acid were accompanied by inhibition of the mitogen-activated protein kinase and phosphatidylinositol 3-kinase 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 microRNA-27a and induction of ZBTB10 (Chinthalapalli 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. 1, A and B), 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/day CDODA-Me did not cause any obvious toxic side effects but exhibited significant tumor inhibition (Chinthalapalli 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. That is currently being investigated. Our studies demonstrate that in addition to the effects of CDODA-Me on the down-regulation of Sp transcription factors in a cancer cell context, this compound inhibits 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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