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# The synthetic oleanane triterpenoid, CDDO-methyl ester, is a potent antiangiogenic agent

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## Abstract

**We show that the synthetic oleanane triterpenoid, CDDO-methyl ester (CDDO-Me; methyl 2-cyano-3,12-dioxoolean-1,9-dien-28-oate) is an effective agent for suppressing angiogenesis, both in cell culture and *in vivo*. The potency of CDDO-Me is particularly striking when dosed *in vivo* to inhibit the angiogenic effects of vascular endothelial growth factor and tumor necrosis factor-α in Matrigel sponge assays; activity is seen at i.p. doses of CDDO-Me as low as 0.003 mg/kg of body weight. If the Matrigel sponges are impregnated with CDDO-Me just before implantation in the mice, picomolar doses of CDDO-Me will suppress angiogenesis. CDDO-Me also inhibits growth of endothelial cells in monolayer cultures and suppresses neovascular morphogenesis in three-dimensional cultures, but significantly higher doses (50-200 nmol/L) are required. We also show antiangiogenic effects of CDDO-Me on xenografts of Kaposi's sarcoma cells in immunocompromised mice, using CD31 as a marker. Several known individual molecular targets of CDDO-Me and related triterpenoids that are relevant to all of these findings include nuclear factor-κB signaling, signal transducers and activators of transcription signaling, and transforming growth factor-β signaling, as well as Keap1, the endogenous inhibitor of the transcription factor Nrf2. However, the particularly potent antiangiogenic activity seen *in vivo* in the present experiments suggest that CDDO-Me, as an angioprevention agent, may be interacting with an entire network of molecular and cellular targets, rather than at a single molecular locus or in a single-cell type. [Mol Cancer Ther 2007;6(12):3139–46]**

## Introduction

Overall mortality from cardiovascular diseases, once the most common cause of death in people under 65 years in the United States, has recently dropped below that from cancer. An intensive, proactive approach to prevention, in which pharmacologic interventions are used before clinical disease is manifested, is largely responsible for this success in cardiovascular disease. Chemoprevention of cancer is a rapidly expanding field that has similar promise of significantly reducing mortality (1, 2). To date, most efforts in oncology have focused on new therapies of invasive and metastatic disease. However, it is becoming increasingly clear that early identification of individuals at risk and adoption of adequate preventive measures has the potential to be more effective at saving lives than later intervention and treatment of end-stage disease (1, 2). The concept of angioprevention, which is chemoprevention directed at inhibition of tumor angiogenesis, is now an important component in this overall preventive approach (3).

For effective angioprevention, new molecules are needed to inhibit vascular promotion of tumor growth *in vivo*. This can be assayed either by measuring inhibition of endothelial cell growth and invasion in cell culture or, more meaningfully, *in vivo* as by evaluating suppression of vascularization of Matrigel plugs containing angiogenic growth factors. Many chemopreventive molecules that have powerful anti-inflammatory and antioxidant activity (including sulforaphane, *N*-acetyl cysteine, epigallocatechin gallate, curcumin, sibillin, xanthohumol, and deguelin; refs. 4–7) are also known to inhibit angiogenesis. Many of these agents inhibit the nuclear factor-κB and Akt pathways in endothelial cells (7). These chemopreventive molecules that target the nuclear factor-κB pathway are a large, structurally diverse group, but this common target suggests that it is a critical pathway modulated in endothelial cells during tumor angiogenesis. However, there are undoubtedly other critical molecular targets, as yet unidentified, for angioprevention, and there is a continuing need for new angiopreventive drugs. In this article, we describe the use of the synthetic oleanane triterpenoid, CDDO–methyl ester (CDDO-Me) for this purpose.

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Several hundreds of new synthetic triterpenoids based on oleanolic acid have been synthesized recently (8), and two of them, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me), are now on phase I clinical trials for treatment of leukemia and solid tumors. A third agent, CDDO-imidazolide [1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl)imidazole] is also highly active (9). All of these molecules have potent anti-inflammatory, antioxidative, and antiproliferative activities (8), but their antiangiogenic activity has heretofore not been investigated in depth. However, relevant to the problem of angiogenesis are following known properties of the above triterpenoids: (*a*) they suppress induction of inducible nitric oxide synthase by inflammatory stimuli (10); (*b*) they suppress induction of cyclooxygenase-2 by inflammatory stimuli (10); (*c*) they induce an entire set of antioxidative enzymes (''phase 2 enzymes''; refs. 11–14); (*d*) they inhibit activity of the transcription factor nuclear factor-κB by directly inhibiting its activating kinase, IκB kinase (15, 16); (*e*) they inhibit phosphorylation of signal transducers and activators of transcription (STAT) transcription factors (17, 18), which is required for transcriptional activity of the STATs; and (*f*) they inhibit the ability of tumor necrosis factor-α (TNF-α) to induce expression of vascular endothelial growth factor (VEGF; ref. 19).

The role of inflammation in driving tumor angiogenesis and progression is receiving increasing attention (20–22). The presence of inflammatory cues in the tumor microenvironment, including cytokines and chemokines, correlates with and often seems to drive tumor angiogenesis (23). Thus, the induction and inhibition of inflammation and angiogenesis pathways seem to be closely intertwined. The potent and direct action of triterpenoids on the same key pathways involved in angioprevention suggests that these compounds may be potent angioprevention compounds *in vitro* and *in vivo*. In the present article, we report details of the antiangiogenic activity, both *in vitro* and *in vivo*, of CDDO-Me. This triterpenoid has been chosen because of its current evaluation in phase I and phase II clinical trials for treatment of several different carcinomas and lymphomas.

## Materials and Methods

### Reagents and Cells

Sources of reagents were as follows: human and murine recombinant VEGF-A, recombinant murine TNF-α, and recombinant human EGF were from Peprotech, heparin was from Sigma, and Matrigel was made from Engelbreth-Holm-Swarm sarcoma as previously described (24). CDDO-Me was synthesized as described (25) and was dissolved in DMSO before dilution in PBS. Kaposi's sarcoma cells (KS-IMM) are of a spontaneously immortalized cell line from an iatrogenic Kaposi's sarcoma (26).

### Matrigel Sponge Assay (*In vivo* Angiogenesis)

The Matrigel sponge model of angiogenesis was used as described previously (27). VTH mixture (100 ng/mL VEGF-A, 2 ng/mL TNF-α, and heparin), either alone or in combination with CDDO-Me, was added to unpolymerized liquid Matrigel at 4°C, and the mixture was brought to a final volume of 0.6 mL (heparin is always added to Matrigel samples to avoid cytokine/growth factor trapping by proteoglycans). The Matrigel suspension was then slowly injected s.c. into the flanks of C57/BL6 male mice (Charles River, Calco, Italy) with a cold syringe. At body temperature *in vivo*, the Matrigel quickly polymerizes to form a solid gel. In some experiments, the triterpenoid was given by i.p. injection of 150 μL of either 10 or 1 μmol/L CDDO-Me (corresponding to 0.03 and 0.003 mg/kg body weight), whereas controls received the same volume of vehicle (10% DMSO in PBS) alone. Groups of eight mice were used for each treatment. Four days after injection, the gels were recovered, minced, and diluted in water to measure the hemoglobin content with a Drabkin reagent kit (Sigma).

### Monolayer Cell Culture

Human umbilical vascular endothelial cells (HUVEC), obtained from Interlab Cell Line Collection, IST of Genoa, were freshly isolated from umbilical veins and grown on 0.1% gelatin-coated tissue culture plates in M199 endothelial growth medium supplemented with heat-inactivated 10% fetal bovine serum, heparin, and 50 mg/mL endothelial cell growth factor. In all experiments, cells were used between passages 8 and 10 *in vitro*.

### Matrigel Morphogenesis Assay

A 24-microwell plate, prechilled at −20°, was carefully filled with 300 μL per well of liquid Matrigel (10 mg/mL) at 4°C with a prechilled pipette, avoiding bubbles. The Matrigel was then polymerized for 1 h at 37°C, and HUVEC (50,000 cells per well) were suspended in 1 mL of complete medium in the absence or presence of different concentrations of CDDO-Me and carefully layered on the top of the polymerized Matrigel. Effects on the growth and morphogenesis of endothelial cells were recorded after 6 and 24 h with an inverted microscope (Leitz DM-IRB) equipped with CCD optics and a digital analysis system.

### *In vitro* Tube Formation Assay

Angiogenesis *in vitro* was also assessed by measuring the ability of HUVEC, when cocultured with matrix-producing fibroblasts, to form capillary-like structures (AngioKit assay, TCS Biologicals). In selected experiments, monolayers of fibroblasts (obtained from TCS Biologicals) cultured in the absence of HUVEC were used. Briefly, cultures were left untreated or treated with CDDO-Me, in the presence or absence of VEGF-A. Medium (either with or without CDDO-Me) was replaced every 3 days, and the status of cell layers was monitored over time by light microscopy and/or after crystal violet staining. At day 12, the cells were fixed, and HUVECs were stained using an anti-CD31 antibody (TCS Biologicals). Images were captured and analyzed. To measure the formation of capillary networks, the number of connections between three or more capillary-like structures and the total length of tubes were quantified by image analysis at 10× magnification. An ELISA for CD31 (TCS Biologicals) was also used for quantification of the extent of vessel reorganization.

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### Tumor Growth *In vivo* and Histochemistry

Kaposi's sarcoma tumors were obtained by s.c. injection of $5 \times 10^6$ KS-IMM cells mixed with liquid Matrigel (final volume, 250 μL) in the flanks of 7-week-old nude nu/nu (CD-1)BR mice (Charles River) as previously described (28). Treated animals were given 150 μL of either 10 or 1 μmol/L CDDO-Me (corresponding to 0.03 and 0.003 mg/kg body weight) by i.p. injection thrice a week starting at day 7, at which time the established tumors were already palpable. Controls received the same volume of vehicle (10% DMSO in PBS) alone. The animals were weighed, and tumor growth was monitored at regular intervals by measuring two tumor diameters with calipers and calculating the tumor volumes with the formula length × width$^2$ / 2. On day 25, the animals were sacrificed and the tumors were removed, weighed, fixed in formalin, embedded in paraffin, and sectioned. Sections (3 μm) were stained with H&E for histologic examination. The percentage of necrotic area was evaluated by morphologic criteria in different fields for each histologic section.

To evaluate formation of new blood vessels, paraffin sections of KS-IMM tumor xenografts were immunostained with a monoclonal antibody to CD31 (Hycult Biotechnology) after antigen unmasking with proteinase K. The Dako REAL EnVision system was used to visualize staining. CD31-positive vessels were counted in three different areas of each histologic section.

## Results

### CDDO-Me Inhibits Angiogenesis in Matrigel Sponge Assays *In vivo*

Striking inhibition of angiogenesis by CDDO-Me was seen in Matrigel sponge assays *in vivo*. In these assays, angiogenesis is induced by incorporation of angiogenic factors, such as VEGF-A and TNF-α, into unpolymerized liquid Matrigel before this material is injected into the flank of a mouse. As the Matrigel warms to the body temperature in the mouse, it polymerizes to form a solid gel, which then becomes vascularized within 4 days, in response to the VEGF and TNF-α. The extent of vascularization can be quantitated by measuring the hemoglobin content of the recovered sponges.

Inhibitory effects of CDDO-Me were evaluated in two ways, either (*a*) by adding the drug directly to the liquid Matrigel before s.c. implantation into a mouse or (*b*) not by adding the drug directly to the liquid Matrigel but by treating the mouse *in vivo* by i.p. injection of CDDO-Me. Both assays showed (Fig. 1A and B) that CDDO-Me is highly effective in suppressing angiogenesis *in vivo*. Figure 1A shows that direct addition of CDDO-Me, even at a 100 pmol/L ($10^{-10}$ mol/L) concentration, markedly inhibited angiogenesis ($P = 0.0036$). Even more importantly, as shown in Fig. 1B, extremely small doses of the drug can be given systemically to a mouse and still suppress the combined angiogenic effects of VEGF and TNF-α in a sponge at a distant site. These striking results, obtained from three different dose schedulings at two different i.p. doses, are shown in Fig. 1B. CDDO-Me, whether given to mice at either 0.003 or 0.03 mg/kg body weight, at either 1 day before gel implantation or both on day 0 and day 2 of implantation, or on all 3 days (−1, 0, +2), markedly suppressed angiogenesis. Representative photographs of individual Matrigel sponges recovered at autopsy are shown in Fig. 1C. In the assays shown in Fig. 1B and C, CDDO-Me was well tolerated. There were no evident effects of the drug on the general health of the animals as determined by behavioral observation and gross examination upon necropsy.

### CDDO-Me Inhibits Endothelial Cell Growth *In vitro*

The *in vitro* effects of CDDO-Me on freshly isolated human umbilical vein endothelial cell growth in monolayer cell culture were directly assessed using crystal violet staining and counting of the cells after exposure to a wide range of concentrations of the drug from 1 to 1,000 nmol/L. Figure 2 shows that although CDDO-Me did not inhibit cell growth significantly at the lowest concentrations tested, but at higher concentrations (250 nmol/L and above), significant inhibition was seen. However, as noted above, in the more physiologic *in vivo* angioprevention assays shown in Fig. 1, CDDO-Me was much more potent.

### Inhibition of Endothelial Morphogenesis *In vitro*

This interesting contrast between the potent antiangiogenic activity *in vivo* and the lesser antiproliferative activity *in vitro* of CDDO-Me led to examination of the mechanisms by which this triterpenoid inhibits angiogenesis. HUVEC tend to organize into ''capillary-like'' structures when cultured in a three-dimensional Matrigel layer, mimicking *in vitro* the events that occur *in vivo* during angiogenesis. Figure 3A shows that CDDO-Me partially inhibited the ability of endothelial cells, when grown in such Matrigel layers, to form capillary-like networks, as evaluated at both 6 and 24 h after treatment. To examine this process further, we used the TCS CellWorks AngioKit, in which human endothelial cells are cocultured with fibroblasts.

With the use of this kit, endothelial cells initially form small islands within the culture matrix; subsequently, they begin to proliferate and then enter into a migratory phase to form threadlike tubule structures. Network formation was followed daily for 11 days, and quantification of the endothelial tubule differentiation was done using the AngioKit ELISA for CD31. Figure 3B shows that treatment of the endothelial cells with CDDO-Me inhibited endothelial tubule formation, starting at concentrations of 50 nmol/L; inhibition was essentially complete at 250 nmol/L, regardless of the presence or absence of VEGF. Figure 3C shows that CDDO-Me dose-dependently interfered with endothelial cell morphogenesis. As quantified by CD31 ELISA analysis, we found that CDDO-Me was significantly more potent than known angiogenesis inhibitors, such as suramin (included in the AngioKit as a positive control) and fenretinide (4HPR; ref. 29); thus, at 250 nmol/L, CDDO-Me is more effective than fenretinide (5 μmol/L) or suramin (14 μmol/L). The results in this assay are similar to those observed for CDDO-Me on endothelial cell proliferation as

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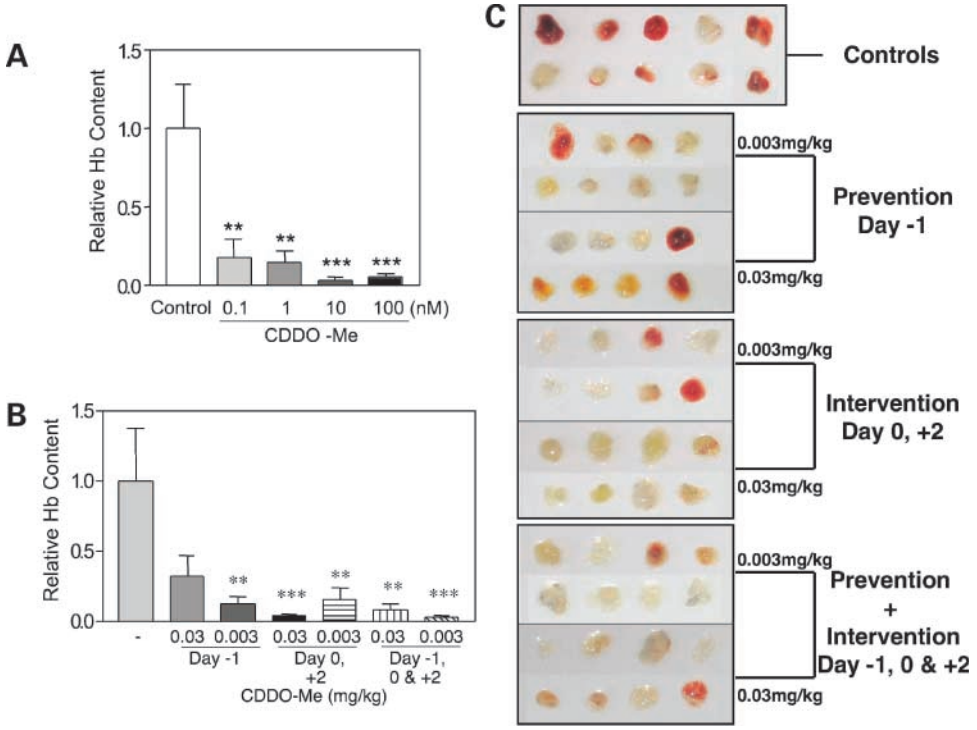


**Figure 1.** Matrigel sponge assays. Matrigel sponges containing angiogenic factors (VEGF and TNF-α) become rapidly vascularized when implanted s.c., as assayed by measurement of hemoglobin content on the fourth day after implantation. **A,** CDDO-Me inhibits angiogenesis *in vivo* when added directly to Matrigel before implantation in mice. Even the lowest dose of CDDO-Me used significantly inhibited vascularization of the gels. *Columns,* mean; *bars,* SE (**, $P < 0.01$; ***, $P < 0.001$, Mann-Whitney). Concentrations of CDDO-Me shown are final concentrations in the liquid gel before implantation. Relative hemoglobin (*Hb*) content is the amount of hemoglobin in a sponge, relative to the total weight of the sponge. Control values have been normalized to 1.0. **B,** CDDO-Me inhibits angiogenesis *in vivo* when given i.p. to mice. Mice were divided in four groups: controls received vehicle (10% DMSO in PBS) alone on the day before the implantation of the Matrigel sponge (day −1), on the day of Matrigel injection (day 0), and 2 days after Matrigel injection (day +2). Mice were treated with either a low or high dose of CDDO-Me on three schedules: on day −1 alone; on days 0 and +2; or on days -1, 0, and +2. In all groups, at both doses, CDDO-Me significantly inhibited angiogenesis. *Columns,* mean; *bars,* SE (**, $P < 0.01$; ***, $P < 0.001$, Mann-Whitney). **C,** representative photographs of Matrigel sponges from the experiment shown in **B**.

shown in Fig. 2; again, the doses required to inhibit morphogenesis in Fig. 3C were significantly higher that those causing inhibition of angiogenesis *in vivo*, as shown in Fig. 1.

### CDDO-Me Inhibits Growth of Kaposi's Sarcoma Cells in Nude Mice

KS-IMM Kaposi's sarcoma cells form highly vascularized, rapidly growing tumors when injected in immunocompromised mice. To test the ability of CDDO-Me to suppress the growth of KS-IMM cells *in vivo*, nude mice were s.c. injected $5 \times 10^6$ KS-IMM cells in Matrigel, and then these cells were allowed to become established palpable tumors. After 7 days, the mice were randomized into three groups, of which two were treated with i.p. injections of CDDO-Me thrice weekly at two different doses (0.03 and 0.003 mg/kg body weight); the third group (control) was treated with vehicle on the same schedule. Figure 4 shows that, upon autopsy at day 25, tumor size in the animals receiving CDDO-Me was significantly smaller than in the controls. Interestingly, we could detect no gross difference between the two treatment groups.

Histologic analysis of the tumors (Fig. 5A–C) showed extensive areas of necrosis in the mice treated with CDDO-Me that was substantially less than that seen in the controls. At autopsy, many mice treated with CDDO-Me showed areas of intact Matrigel vehicle, suggesting an almost

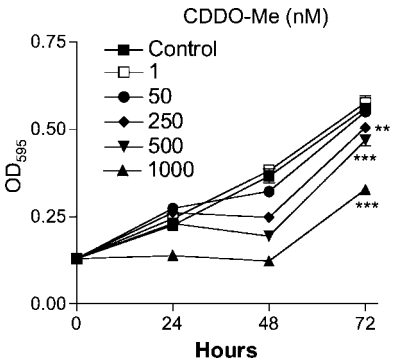


**Figure 2.** Effects of CDDO-Me on *in vitro* growth of endothelial (HUVE) cells as quantified by crystal violet assay (**, $P < 0.01$; ***, $P < 0.001$, two-way ANOVA).

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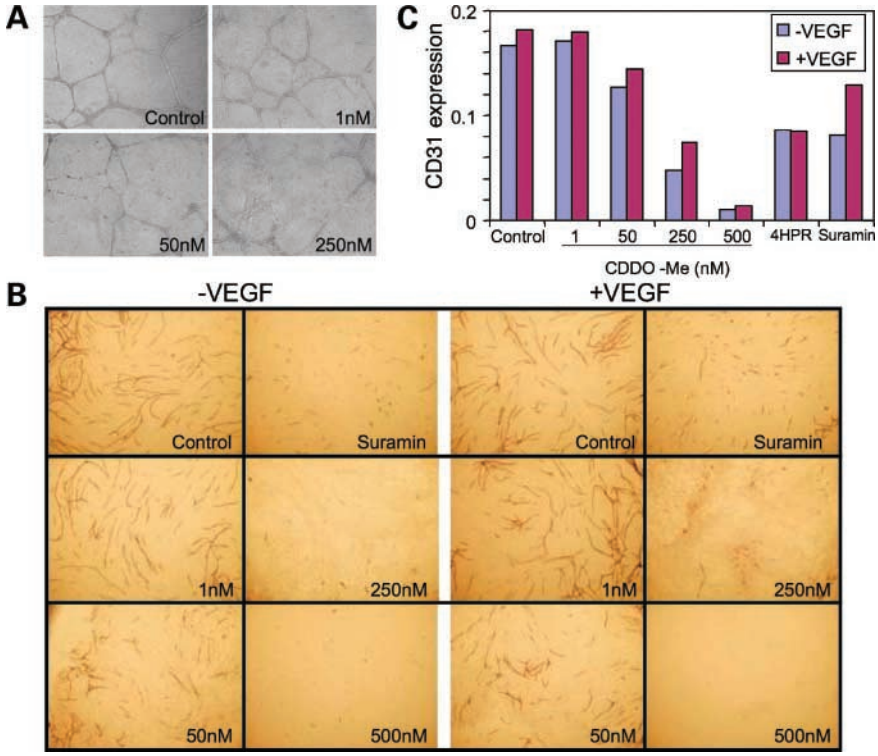


**Figure 3.** Inhibition of endothelial cell morphogenesis by CDDO-Me. **A**, CDDO-Me inhibits formation of networks of HUVEC cultured in a three-dimensional Matrigel assay. The figure shows dose and time dependency of this action. **B**, CDDO-Me inhibits endothelial tubule formation in TCS AngioKit assay, as described in text. Inhibitory activity was found either in the absence or presence of VEGF; VEGF concentration was 2 ng/mL. Suramin (14 μmol/L) is used as a positive control. **C**, quantitation of results shown in **B**. 4HPR concentration was 5 μmol/L.

complete arrest of growth of the Kaposi's cells; this was never observed in specimens taken from control animals. The extent of necrosis in the tumors in the three groups was quantitated by histopathologic analysis of sections stained with H&E, as shown in Fig. 5A–D. Figure 5D shows that the higher dose of CDDO-Me greatly enhanced the area of necrosis in the tumors compared with the control group. These data indicate that although the tumors in the two treated groups were of similar size, those in the group treated with the higher dose of CDDO-Me contained significantly fewer viable cells. Again, these data further underscore the potency of CDDO-Me to suppress tumor growth *in vivo* and correlate with the inhibition of angiogenesis *in vivo*, as shown in Fig. 1. Staining of the vessels in cryostat sections with CD31 clearly showed significantly fewer vessels in the treated groups, as shown in Fig. 6. Taken together, these data indicate that inhibition of angiogenesis is a key contributing mechanism for inhibition of these tumor cells, which are highly vascularized *in vivo* (26), although a direct effect on the Kaposi's cells themselves cannot be excluded.

## Discussion

We have found that the synthetic triterpenoid CDDO-Me is a highly potent antiangiogenic agent when tested in *in vivo* assays that evaluate neoangiogenesis in a physiologic context. The Matrigel sponge assay measures the formation of new blood vessels in a three-dimensional matrix *in vivo*, and in this assay, CDDO-Me is exceptionally potent, showing strong inhibitory activity at picomolar concentrations of drug if it is added directly to sponges before implantation and at nanomolar amounts of administrated drug if the recipient mice are treated with the drug by i.p. injection. These potent effects *in vivo* are in sharp contrast to the lesser antiangiogenic potency of CDDO-Me that we have seen in simpler, but less physiologic, assays in monolayer culture and emphasize the nature of angiogenesis as a complex process involving many components of the microenvironment (1, 30).

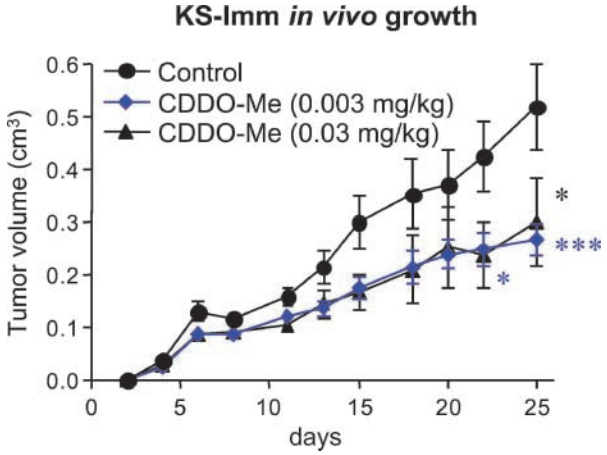


**Figure 4.** CDDO-Me, given i.p., inhibits tumor growth by KS-IMM Kaposi's cells when injected in nude mice. Details of assay are given in text. Both concentrations of drug were effective. *Points,* mean; *bars,* SE (*, $P < 0.05$; ***, $P < 0.001$, two-way ANOVA).

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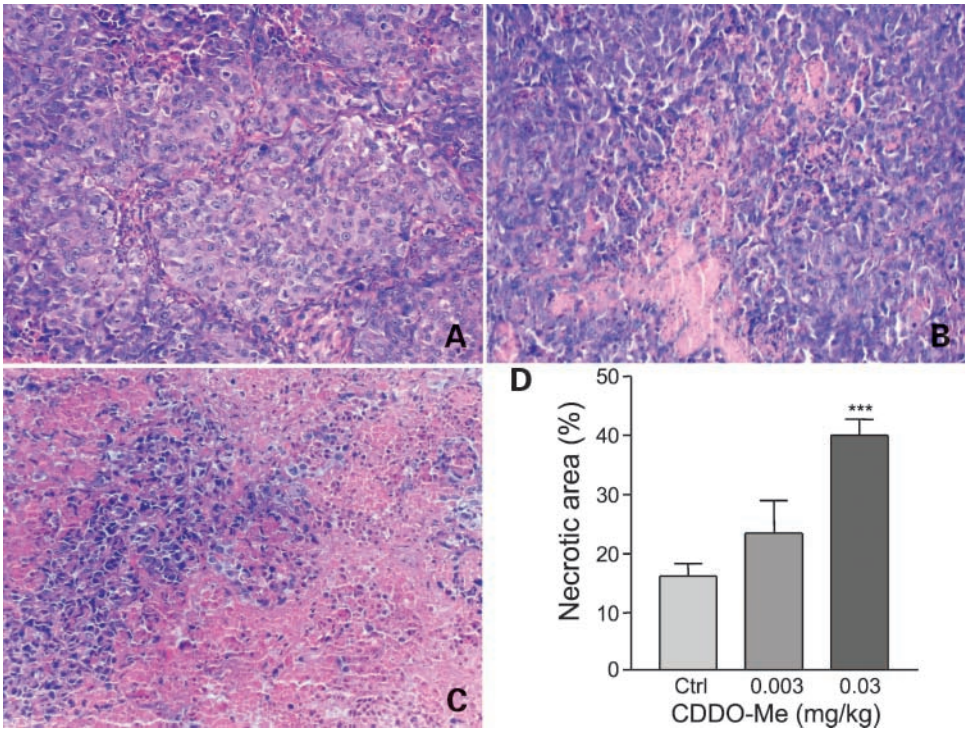


**Figure 5.** Histologic analysis of Kaposi's tumor xenografts. **A,** representative control tumor. **B** and **C,** representative tumors from mice treated with either low (**B**) or high (**C**) doses of CDDO-Me, showing extensive necrotic areas at autopsy. **D,** the percentage of the microscopic field that was necrotic was significantly higher in the group treated with the higher dose of CDDO-Me. *Columns,* mean; *bars,* SE (**, $P < 0.01$; ***, $P < 0.001$, Mann-Whitney).

Our results clearly show that CDDO-Me is a model angioprevention drug, in that it is not only effective at blocking angiogenesis *in vivo* at very low doses, but it also does this without exerting apparent toxicity toward normal endothelium, as no depolarization of mitochondria, an indicator of apoptosis as observed using the JC1 dye, was observed in normal endothelial cells treated even at concentrations of CDDO-Me as high as 500 nmol/L (data not shown). Ideally, cancer chemopreventive agents should inhibit tumor growth without any side effects that would interfere with long-term administration to healthy individuals. Inhibition of angiogenesis, and more precisely the

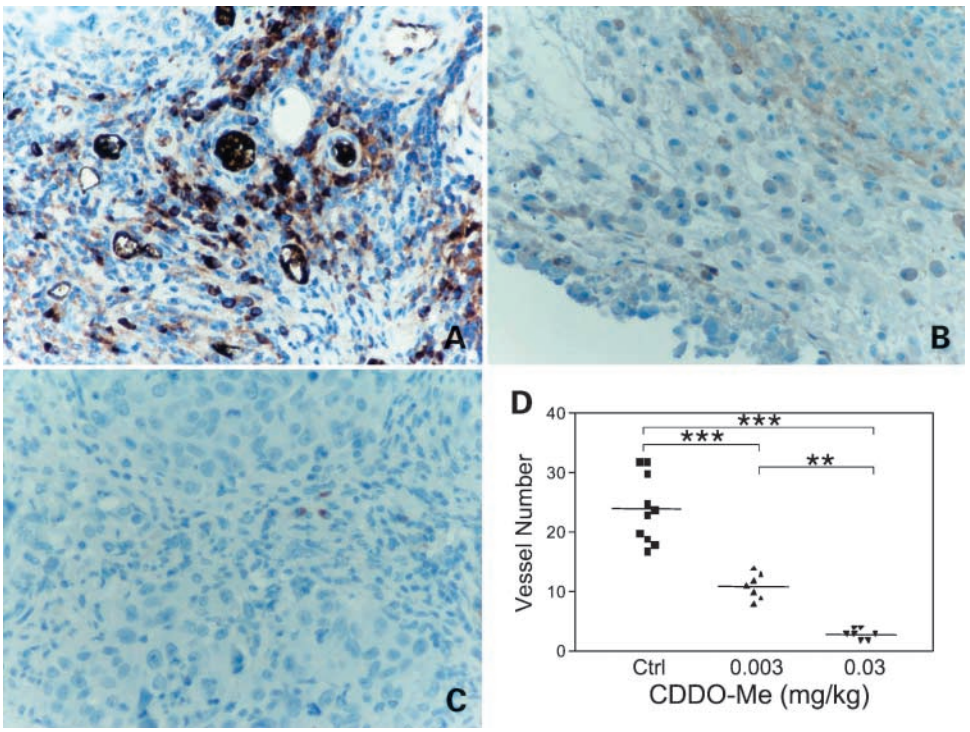


**Figure 6.** CD31 staining for vessels in the Kaposi's tumor xenografts. **A,** representative control tumor. **B** and **C,** representative tumors from mice treated with either low (**B**) or high (**C**) doses of CDDO-Me. **D,** the number of vessels per high power field was significantly higher in the control group compared with both treated groups, and the number of vessels in the group treated with the high dose of CDDO-Me was significantly lower than in the low-dose group. *Points,* mean; *bars,* SE (**, $P < 0.01$; ***, $P < 0.001$, one-way ANOVA).

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angiogenic switch that is critical for progression toward malignancy (31), is an ideal approach to this goal. Physiologic angiogenesis in adults only occurs during wound healing and in the female estrous cycle (32), indicating that blocking angiogenesis should have limited adverse systemic effects. During the progression phase of carcinogenesis *in vivo*, foci of transformed cells, in the absence of the ability to induce angiogenesis, are generally limited to small foci a few millimeters in diameter (32). Hyperplastic foci without apparent angiogenic potential are often found at autopsy in many tissues, such as the prostate (33).

Specific molecular targets of CDDO-Me that may be responsible for its antiangiogenic activity in the present experiment have been elucidated recently. Thus, there are at least three known molecular targets for CDDO-Me and its close relative, CDDO-imidazolide, that are highly relevant to the regulation of angiogenesis. The first is suppression of nuclear factor-κB signaling (15, 16), which is linked to suppression of angiogenesis (7). Both CDDO-Me and CDDO-imidazolide have been shown to be potent direct inhibitors of IκB kinase, which activates nuclear factor-κB; a specific thiol group on cysteine-179 of inhibitors of IκB kinase has been implicated as a specific target of these two triterpenoids (15, 16). The second target is suppression of STAT signaling, as mediated by the ability of both CDDO-Me and CDDO-imidazolide to suppress the phosphorylation of both STAT3 and STAT5, which is required for the activity of these proteins as transcription factors (17, 18). STATs are known to play an important role in mediating both wound healing and angiogenesis (34). A third important pathway regulated by synthetic oleanane triterpenoids is transforming growth factor-β/BMP/Smad signaling (35, 36), and in this case, the triterpenoids enhance the transcriptional activity of the respective Smads that are involved in regulating gene activity. Again, there is abundant evidence for the role of transforming growth factor-β/Smad signaling as a critical regulator of both wound healing and angiogenesis (37). A fourth molecular target of CDDO-Me is Keap1, the endogenous inhibitor of the transcription factor Nrf2, although the effects of genes regulated by Nrf2 on the process of angiogenesis is still unclear. Interestingly, sulforaphane, which is the prototypical agent for activating Nrf2 by binding to Keap1 (38), has been found to be a potent inhibitor of angiogenesis (4–6).

In summary, the ability of synthetic oleanane triterpenoids to control the expression and activity of several transcriptional regulators would seem to be intimately involved in the antiangiogenic activity we have reported here. At the molecular level, it is clear that CDDO-Me and related synthetic triterpenoids are chemically reactive with activated thiol groups on proteins, leading to the formation of Michael adducts, which may be reversible (8). Moreover, the chemistry of this interaction suggests that it is possible that there are yet other protein targets, still unidentified, that may contribute to the antiangiogenic effect of CDDO-Me.

We would also suggest that the particularly strong antiangiogenic activity we have observed for CDDO-Me in the present *in vivo* experiments, as contrasted to its somewhat less potent effects in cell cultures of endothelial cells, may additionally be the result of interactions of CDDO-Me with the numerous cells in the microenvironment, beyond any direct effects on endothelial cells (1, 30). Thus, in each of the stromal cells of the microenvironment, there is potential for CDDO-Me to signal through multiple molecular targets by virtue of formation of Michael adducts, which may even be reversible under some conditions. It is possible that there may be an entire signaling network, both molecular and cellular, that is regulated by CDDO-Me, resulting in an effective and coordinated suppression of angiogenesis *in vivo*.

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