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Reproductive Sciences 2010 17: 931 originally published online 6 August 2010
DOI: 10.1177/1933719110374239

The online version of this article can be found at:
http://rsx.sagepub.com/content/17/10/931
Multiple Anticancer Activities of EF24, a Novel Curcumin Analog, on Human Ovarian Carcinoma Cells

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Abstract
Curcumin, a component of turmeric, has been reported to exhibit potential antitumor activities. This study assessed the effects of a novel synthetic curcumin analog, EF24, on proliferation, apoptosis, and vascular endothelial growth factor (VEGF) regulation in platinum-sensitive (IGROV1) and platinum-resistant (SK-OV-3) human ovarian cancer cells. EF24 time- and dose-dependently suppressed the growth of both cell lines and synergized with cisplatin to induce apoptosis. Although treatment with EF24 had no significant effect on VEGF messenger RNA (mRNA) expression, VEGF protein secretion into conditioned media was dose-dependently reduced with EF24 demonstrating ~8-fold greater potency than curcumin (P < .05). EF24 significantly inhibited hydrogen peroxide (H2O2)-induced VEGF expression, as did the phenolic antioxidant tert-butylhydroquinone (t-BHQ). EF24 upregulated cellular antioxidant responses as observed by the suppression of reactive oxygen species (ROS) generation and activation of antioxidant response element (ARE)-dependent gene transcription. Given its high potency, EF24 is an excellent lead candidate for further development as an adjuvant therapeutic agent in preclinical models of ovarian cancer.

Keywords
curcumin, apoptosis, angiogenesis, VEGF, ovarian cancer, antioxidants

Introduction
Projected to be responsible for more than 15 000 deaths in 2008, ovarian cancer remains the leading cause of death from gynecologic cancer.1 The current standard of clinical care includes primary surgical cytoreduction followed by cytotoxic chemotherapy; however, untoward side effects and recurrence remain significant problems. Cisplatin is an effective and widely used chemotherapeutic agent against various human cancers, including ovarian cancer. Despite its great efficacy, a major limitation of cisplatin treatment is the development of drug resistance. Therefore, the need for effective adjuvant therapeutics well tolerated by patients is paramount.

Curcumin (diferuloylmethane [Cur]), a yellow ingredient isolated from turmeric (Curcuma longa), has been shown to have cytostatic and antiangiogenic effects in preclinical and clinical cancer studies.2,3 Unfortunately, poor intestinal absorption and low bioavailability have limited the use of natural Cur4; however, it remains an excellent compound for the design of more effective analogs. One monoketone analog in particular, EF24 (diphenyl difluoroketone), is efficacious in anticancer screens and considerably less toxic than the commonly used chemotherapeutic drug cisplatin.5,6 EF24 has been shown to induce apoptosis in cancer cells and to inhibit the growth of human breast tumor xenografts in a mouse model with relatively low toxicity and at a dose much lower than that of Cur.5,6 Subramaniam et al7 demonstrated that EF24 significantly reduces both vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8) gene expression in the colon cancer cell line HCT-116, and similar results were observed in EF24-treated HCT-116 xenografts. Furthermore, some studies suggest that the most promising use of EF24 may be as an adjuvant to currently used therapies.8

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Apoptosis is characterized by numerous biochemical and morphological changes in the cell including caspase activation (particularly caspase-3), phosphatidylserine (PS) exposure, cytoplasmic shrinkage, and DNA fragmentation. Anticancer agents that induce apoptosis at doses below those that display cytotoxicity to normal cells could be clinically useful. There is accumulating evidence that the efficiency of chemotherapeutic agents such as cisplatin is related to the intrinsic propensity of the target cells to respond to these agents by apoptosis.

Angiogenesis is pivotal in the development and progression of ovarian cancer and is an ideal target for novel treatment approaches. Early studies suggested that VEGF was a predictive biomarker as well as an important regulator of angiogenesis in epithelial ovarian cancer cells. Vascular endothelial growth factor immunostaining has been demonstrated in ovarian neoplasms and the degree of expression of VEGF and its receptors in ovarian tumors is inversely proportional to prognosis, suggesting that VEGF-induced angiogenesis influences disease progression. However, recent research failed to establish serum VEGF as a clinically relevant independent prognostic indicator for ovarian cancer.

Although it is evident that application of plant-derived drugs can prevent tumor neovascularization, their mechanisms of action remain to be elucidated. The antioxidant property of Cur appears to be an essential component underlying its salutary biological activities. Analysis of its structure reveals a β-diketone moiety with phenolic hydroxyl groups (Figure 1) that are believed to contribute to antioxidation by virtue of Michael reaction acceptor functionalities and electrophilic characteristics. Curcumin also induces the activities of phase II detoxification enzymes, which protect against oxidative stress. The coordinated induction of antioxidant genes by Cur is mediated in part through cis-regulatory DNA sequences located in their promoter or enhancer regions, which are known as antioxidant-responsive elements (AREs). Recent studies indicate that VEGF regulation is influenced by cellular redox processes, linking oxidative stress to angiogenesis and tumor progression.

The goal of this study is to determine whether a novel Cur analog, EF24, which possesses halogenated phenol-like side rings, also might have a role in the prevention and/or treatment of ovarian cancer through antioxidant-mediated mechanisms.

**Materials and Methods**

**Cell Lines**

Two ovarian epithelial adenocarcinoma cell lines: IGROV1 and SK-OV-3, were purchased from ATCC (Manassas, Virginia). IGROV1 was chosen for its high baseline secretion level of VEGF into culture medium (as compared with OVCAR3, OVCAR5, OVCAR8, and SK-OV-3; unpublished data from enzyme-linked immunosorbent assay [ELISA] and protein array screens). SK-OV-3 was chosen from initial cytotoxicity screening and expanded studies because it is platinum-resistant and possesses several key oncogenic characteristics, epidermal growth factor receptor (EGFR) overexpression and p53 mutation. The tumor cell lines were regularly screened for mycoplasma using MycoAlert (Cambrex Bioscience, East Rutherford, New Jersey) as described by the manufacturer. Cells were grown to 80% confluence in complete medium (Gibco, Rockville, Maryland). For all assays, cells were allowed to attach overnight and then treated in medium containing 5% fetal bovine serum (FBS).

**Chemicals**

A 100 mmol/L stock solution of Cur (Sigma, St Louis, Missouri) was prepared in dimethyl sulfoxide (DMSO), stored in aliquots at −20°C and diluted as needed into culture medium. EF24 was synthesized at the Department of Chemistry, Emory University, and freshly dissolved in DMSO immediately before treatment. Tert-butyldihydroquinone (t-BHQ) was obtained from Fisher Scientific (Pittsburgh, Pennsylvania). All compounds were used at final concentrations of 1 to 32 μmol/L with a final DMSO concentration of 0.1% (v/v), using appropriate DMSO vehicle controls. A 100 mg/mL stock solution of N-acetylcysteine (NAC; Sigma) was prepared in double distilled H₂O (ddH₂O) and diluted as needed into culture medium.

**Cell Viability Assay (MTS, SRB)**

Cell viability was determined using CellTiter 96 AQUEous Assay (Promega, Madison, Wisconsin). This colorimetric assay is based on the ability of mitochondria to reduce a substrate (MTS) into a soluble formazan product with an absorbance at 492 nm that is directly proportional to the number of living cells. Briefly, cells (8 × 10⁴) were seeded onto 96-well plates in triplicate and treated with increasing doses of Cur or EF24. Following incubation for 72 hours, MTS solution was added for an additional 3 hours and absorbance was measured at 492 nm wavelength using an Anithos htII ELISA plate reader (Anithos Labtec Instruments, Salzburg, Austria).
Sulforhodamine B (SRB) assay was performed for the toxicity screening of compounds to adherent cells in a 24-well format. After various incubation periods, cell monolayers were fixed with 10% (wt/v) trichloroacetic acid and stained for 30 minutes, after which the excess dye was removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye was dissolved in 10 mmol/L Tris base solution for optical density (OD) determination at 492 nm using the same microplate reader described above.

DNA Fragmentation (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) Assay

Immunocytochemical terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) staining was performed for specific labeling of nuclear DNA fragmentation, an important biochemical hallmark of apoptosis, with the in situ cell death detection kit (Roche, Indianapolis, Indiana). The fluorescent label was converted into a colorimetric signal using the TUNEL peroxidase as a secondary detection system. The stained mounted cells were examined microscopically at 40× magnification and DNA fragmentation was visualized as brown staining of nuclei. Cells incubated with TUNEL only solution instead of the TdT reaction mix served as negative control whereas those incubated with DNase to induce DNA strand breaks served as positive control.

Western Blots for Caspase-3 Cleavage and Bcl-2 Expression

Cellular proteins were prepared and separated by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis. Immunoblotting was performed using rabbit monoclonal antibodies against cleaved caspase-3 or Bcl-2 (1:1000 dilution, Cell Signaling Technology, Danvers, Massachusetts). Immunodetection was obtained using a goat antirabbit secondary antibody (1:20 000 dilution, Pierce, Rockford, Illinois). Antibody binding was visualized using the enhanced chemiluminescence (ECL) system (Amersham International, Buckinghamshire, UK) in accordance with the manufacturer’s protocol.

Vascular Endothelial Growth Factor ELISA

Each well of 96-well flat-bottomed microtiter plates (Maxisorp, Nunc, Denmark) was coated with 100 μL phosphate buffered saline (PBS) containing antihuman VEGF (specific for VEGF165 and VEGF121, the major soluble isoforms of VEGF) protein (0.5 μg/mL) by overnight incubation at room temperature. The plates were then washed with PBS (pH 7.2) containing 0.05% (v/v) Tween-20 (phosphate buffered saline Tween-20 [PBST]) and blocked with ELISA-coating stabilizer (Antigenix America Inc, New York) for 1 hour at room temperature. The plates were washed with PBST and allowed to air-dry for 4 to 6 hours before storing it with desiccant at 4°C. VEGF levels in cell-free supernatants were determined under standard procedure and the plate was read at 450 nm wavelength using an Anthos htII ELISA plate reader (Anthos Labtec Instruments) with the correction wavelength set at 570 nm. Experiments were repeated at least 4 times and results were normalized to a standard gradient of reference VEGF protein included in each plate.

Reverse Transcription Real-time Quantitative Polymerase Chain Reaction

Total RNA isolated from IGROV1 cells using TRIzol Reagent (Life Technologies, Inc, Rockville, Maryland) was reverse transcribed with SuperScript II reverse transcriptase in the presence of random hexanucleotide primers (all from Invitrogen, Carlsbad, California). Complementary DNAs were then used for quantitative polymerase chain reaction (qPCR) using iQ SYBR Green Supermix (Bio-Rad, Hercules, California). Crossing threshold values for individual genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Changes in messenger RNA (mRNA) expression were shown as fold change relative to control. Primers (Eurofins MWG Operon, Huntsville, Alabama) used in this study were as follows: 72 bp VEGF, sense (5’-GCCACCATGGGCAAGGA-3’), antisense (5’-GCTGCGCTGATAGACATC-3’); 185 bp GAPDH, sense (5’-CCATGGAGAGGCTGGGG-3’), antisense (5’-CAAAGTTGTACATGCCAACC-3’).

Detection of Intracellular Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) were quantified using a fluorescent probe 2’7’-dichlorofluoresceindiacetate (DCFH-DA; Invitrogen). 2’7’-Dichlorofluoresceindiacetate diffused through the cell membrane readily and is enzymatically hydrolyzed by intracellular esterases to generate nonfluorescent DCFH, which is rapidly oxidized to highly fluorescent DCF in the presence of intracellular ROS. Cells (5 × 10^6) seeded in 24-well plates were incubated with 10 μmol/L DCFH-DA at 37°C for 30 minutes. Media containing experimental conditions were added and fluorescence of the wells measured kinetically using a Synergy 2 Microplate Reader (BioTek Instruments, Winooski, Vermont) maintained at 37°C, with excitation and emission wavelengths of 460 and 530 nm, respectively.

Transient Transfection and Reporter Gene Activity Assay

The Dual-luciferase Reporter Assay System (Promega) was used to examine reporter gene activity in transiently transfected cells. Briefly, cells were seeded at a density of 5 × 10^4 per well in 24-well plates and transfected with an antioxidant response element (ARE)-firefly luciferase reporter construct (4 × ARE luc; kindly provided by Dr Mark Hannink, Department of Biochemistry, University of Missouri) using FuGENE 6 (Roche Applied Science), according to the manufacturer’s instructions. A control plasmid encoding Renilla luciferase under regulation of the thymidine kinase promoter (pRL-TK), was used to normalize transfection efficiency. Twenty-four hours following transfection, media were aspirated and cells were treated with
25 µmol/L t-BHQ or other compounds at desired concentrations overnight. Firefly and Renilla luciferase activities in cell lysates were measured sequentially using a luminometer (Turner Designs Model TD-20/20, Promega) following the addition of luciferase assay reagent II and stop and glo reagent (Promega), respectively. The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of Renilla luciferase.

Statistical Analysis

All experiments were done in triplicate and independently repeated at least 3 times. Statistical analysis was done using Statistical Package for the Social Sciences 12.0 for windows (SPSS, Inc). All values are expressed as the mean ± standard deviation (SD). Student t test or repeated measures analysis of variance (ANOVA) were used to compare the effects of multiple treatments. A 2-tailed P = .05 threshold was set for significant differences.

Results

Effects of EF24 on Inhibition of Cancer Cell Proliferation

EF24 significantly suppressed proliferation of both cell lines within 24 hours and continued to inhibit cell growth for up to 72 hours (Figure 2). The compound demonstrated approximately 50% inhibition at 1.6 µmol/L (IGROV1) and 2.4 µmol/L (SK-OV-3) after a 72-hour treatment, with the former cell line appearing to be more sensitive to EF24. The apparent potency of EF24 was >8-fold that of the parent agent, Cur. More importantly, the effects were observed at an EF24 concentration ≤2 µmol/L, a dose at which Cur had no significant effect on cell proliferation. Similar differences in the effects of EF24 were observed on VEGF secretion and ARE activation, which will be discussed later, and indicate the enhanced potency of EF24.

Effects of EF24 on Induction of Apoptosis

At a sufficiently high dose, Cur is known to induce apoptosis of cancer cells. Caspase-3 is a key effector molecule in the apoptosis pathway involved in amplifying the signal from initiator caspases, such as caspase-8.20 Treatment of both cell lines with Cur and EF24 induced apoptosis as manifested by the presence of TUNEL-positive cells (Figure 3A). These effects were confirmed by caspase-3 activation and downregulation of the antiapoptotic protein Bcl-2 (Figure 3B). Moreover, it was noted that in platinum-resistant SK-OV-3 cells, the cleavage of caspase-3 induced by 20 µg/mL cisplatin was significantly potentiated by pretreatment with Cur or EF24, which alone
did not induce much caspase-3 cleavage (Figure 3C). These data suggest that curcuminoids can sensitize cancer cells to cisplatin.

**Effects of EF24 on VEGF Secretion**

Having determined the IC_{50}s of the 2 curcuminoids on proliferation, we carefully selected optimal doses of Cur (16 μmol/L) and EF24 (2 μmol/L) for the following experiments to determine their effects on VEGF protein secretion. Under these conditions, Cur had no effect on cell viability and EF24 only showed a minor inhibition of <20%. Human VEGF was measured in ovarian cancer cells supernatants to determine the effect of Cur and EF24 on the release of this angiogenic protein into conditioned media. We observed that these compounds dose-dependently inhibited IGROV1 cell VEGF secretion after 24-hour treatment (Figure 4A). These data suggest that EF24 has more potent and profound suppression of angiogenesis than Cur. Interestingly, we also found that t-BHQ (25 μmol/L), a phenolic antioxidant, also suppressed VEGF secretion by 30% (P < .01) in this model but had no effect on IGROV1 proliferation (data not shown).

**Effects of EF24 on VEGF mRNA Levels**

To determine whether curcuminoids downregulate VEGF mRNA expression, RT-qPCR was performed using GAPDH as internal constitutive control. Neither Cur nor EF24 treatments had significant effect on VEGF mRNA expression after 6, 12, or 24 hours (Figure 4B; data for 6 and 12 hours not shown).
Thus, although VEGF protein secretion was significantly reduced, VEGF mRNA levels were not affected by Cur or EF24 treatment, suggesting posttranscriptional mechanisms of VEGF inhibition by curcuminoids. Actinomycin D (1 μg/mL) was used as positive control and, as expected, significantly reduced VEGF mRNA accumulation by >80%.

**Effects of EF24 on Inducible VEGF Secretion**

Studies from our own laboratory and others indicate that VEGF can be posttranscriptionally regulated through redox changes. This fact, combined with the evidence that Cur can act as an antioxidant under certain circumstances, suggests that such activity may contribute to the inhibitory effect of curcuminoids on VEGF secretion. This contention was supported by our observation that the antioxidant t-BHQ suppressed VEGF secretion in IGROV1 cells. IGROV1 cells were exposed to H2O2 and the supernatant was analyzed for VEGF protein levels. H2O2 dose-dependently stimulated release of VEGF protein (Figure 4A). These responses were redox-specific, as shown by their complete reversal after application of 1 mmol/L NAC, an ROS scavenger. Pretreatment with 2 μmol/L EF24 and 25 μmol/L t-BHQ also significantly reversed the H2O2-induced effect on VEGF secretion, whereas Cur at a concentration of 16 μmol/L only had a weak effect on H2O2-induced VEGF secretion (Figure 5B). None of the treatments caused a loss of cell viability, as can be seen by constant SRB detection.

**Effects of EF24 on ROS Generation**

We investigated the general antioxidant effects of EF24 for its potential to inhibit total ROS, as represented by DCF fluorescence. Immediately after the application of different treatments, we measured DCF intensity kinetically. H2O2, 100 μmol/L, induced intracellular oxidation that reached a plateau after 2 hours (Figure 6A) and demonstrated an overall 6-fold increase in ROS generation (Figure 6B). EF24 (2 μmol/L) did not generate any intracellular ROS compared to untreated group and negative control (NAC-treated cells), with its generation curve staying at the basal line during the observed period. The 4-hour pretreatment of EF24 diminished DCF-sensitive ROS production following H2O2 exposure in IGROV1 cells; whereas NAC showed a complete ROS-scavenging effect.

**Effects of EF24 on Induction of ARE**

Cur has been reported as a bifunctional antioxidant, scavenging ROS directly and inducing certain endogenous enzymes that function against oxidative stress. EF24, likewise a strong Michael acceptor, may act in the same way. Indirect effects of the curcuminoid compounds as antioxidants were tested using ARE reporter assay. EF24 was screened in its effective dosage range. We selected t-BHQ as positive control to challenge IGROV1 cells because it was used in the original experiments to define antioxidants and their effects on the ARE consensus sequence. ARE luciferase activity in IGROV1 cells was strongly induced by t-BHQ and in a dose-dependent manner by the curcuminoids (Figure 7). The potency of EF24 (2 μmol/L) was ~16-fold greater than natural Cur on ARE activation.

**Discussion**

The high mortality of ovarian cancer, in addition to the significant morbidity of adjuvant chemotherapy, has led searches for more effective and less toxic alternative treatments. A variety of plant-derived compounds that are present naturally in our daily foods and used as traditional herbal medications, have been identified to elicit chemopreventive and therapeutic effects. Our results indicate that EF24, a novel Cur analog, possesses great promise as an antiovarian cancer therapeutic. The key findings of the current study are that EF24 has efficacy in ovarian carcinoma models and EF24-induced inhibition of VEGF secretion may be attributed to its antioxidant
activity. As shown in our studies, EF24 inhibits proliferation and induces caspase-mediated apoptosis of both platinum-sensitive and platinum-resistant ovarian cancer cells; whereas Cur only has similar effects at relatively high doses. EF24 also potentiated the proapoptotic effect of cisplatin via caspase-3 activation. Furthermore, treatment with EF24 suppressed constitutive and H$_2$O$_2$-induced VEGF expression in ovarian cancer cells, which appears to occur at a posttranscriptional level and is possibly mediated through antioxidant pathways, including ARE-dependent gene expression.

Currently, there is growing interest in the development of adjuvant therapies offering additive or synergistic effects to traditional antineoplastic drugs for ovarian cancer. Phytochemicals, like Cur and its analogs, could be such agents. By acting as chemosensitizers, they increase drug efficacy by either modulating the disease process itself or affecting specific
cellular pathways known to cause resistance to standard cytotoxics. The potential therapeutic role of EF24 in conjunction with other therapeutic agent has been explored in an in vitro setting. Simultaneous treatment of cells with EF24 and platinum-based therapy resulted in a striking increase in tumor cell apoptosis. It might be expected that in vivo such adjuvants could reduce the cytotoxic effects of alkylating chemotherapeutics on normal cells by simply reducing the dose of drug needed for effect. Interestingly, the magnitude of proapoptotic effect from combination therapy was above that elicited by cisplatin therapy alone, thus suggesting that combining these 2 agents may actually be a superior therapeutic choice. Moreover, even in a chemorefractory model, we showed proapoptotic activity by administering EF24 alone and in combination with cisplatin.

Neovascularization is a general characteristic of tumor growth and dissemination and VEGF is a critical angiogenic factor.29 In ovarian cancer, VEGF protein expression and

Figure 6. Effects of EF24 on H$_2$O$_2$-induced ROS generation. Here we show ROS production (DCF fluorescence) measured in real time (A) and total end point fluorescence 2 hours after the initiation of treatment (B). IGROV1 cells were treated with 100 µmol/L H$_2$O$_2$ followed by the addition of 2 µmol/L for 4 hours. H$_2$O$_2$ demonstrates a marked increase in fluorescence whereas EF24 treatment alone did not show any ROS production when compared to the unstimulated control. H$_2$O$_2$-induced ROS generation was suppressed >30% by EF24 pretreatment and completely reversed by NAC (1 mmol/L). EF24 indicates diphenyl difluoroketone; Cur, curcumin; DCF, dichlorofluorescein; ROS, reactive oxygen species; NAC, N-acetylcysteine.
elevated VEGF levels in ascites have been correlated to advanced stage disease and poor prognosis. Thus, antiangiogenic therapy targeting VEGF in ovarian cancer models is very appealing, and innovative drug delivery systems have been developed. In our study, EF24 suppresses in vitro VEGF secretion in a concentration-dependent manner. Curcumin has potentially therapeutic effects at relatively high doses; whereas EF24 interferes with the proliferation of cancer cells and downregulates VEGF secretion with up to 8-fold more efficacy.

Under the circumstance of oxidative stress produced by H2O2, ovarian cancer cells secreted more VEGF into the supernatant. However, when pretreated with NAC or t-BHQ, we noticed inhibition of H2O2-induced VEGF secretion. These findings suggest that the angiogenic properties of tumor cells are likely influenced by the redox state of their environment. Indeed, VEGF is sensitive to alterations in oxygen tension, redox state and intracellular levels of ROS. EF24 also exhibited a significant reversal of H2O2-induced VEGF secretion.

Oxidative stress is potentially harmful to cells and ROS are known to be generated in the initiation and progression of cancer. The antioxidant function of Cur is predicted by its phenolic structure, which can disrupt the oxidative chain reaction by trapping free radicals. The findings that EF24 reduced ROS generation and scavenged ROS during H2O2 stress also indicate its antioxidant potency. In addition to its inherent ability to attenuate oxygen free radicals, Cur has been shown to enhance the activities of detoxifying enzymes such as glutathione-S-transferase (GST). In this study, we were able to mimic the effect of EF24 with t-BHQ, a potent activator of the ARE pathway. Whether the induction of distinct antioxidant genes contributes to pharmacological actions mediated by curcuminoids has yet to be examined, but this hypothesis is substantially induced by EF24. Correlation of the apparent abilities of EF24 and t-BHQ to activate ARE-driven gene expression and suppress H2O2-induced VEGF secretion suggests that antioxidants contribute to inhibition of VEGF expression.

Collectively, the results of this study emphasize the notion that the pharmacological activity attributed to plant-derived drugs relies on their innate ability to activate endogenous intracellular antioxidant systems. Our in vitro data reveal that EF24 demonstrates higher antiproliferative potency and downregulates VEGF secretion more effectively than natural Cur and hence represents a model compound for further development of natural product-derived antiovary cancer agents to be tested in vivo ovarian cancer models.

Declaration of Conflicting Interests
The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research and/or authorship of this article: supported by grants from the Caldwell Family Foundation and the Vesa W. and William J. Hardman, Jr. Charitable Foundation Inc.

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