# Diphenyl Difluoroketone: A Curcumin Derivative with Potent *In vivo* Anticancer Activity

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## Abstract

**Diphenyl difluoroketone (EF24), a molecule having structural similarity to curcumin, was reported to inhibit proliferation of a variety of cancer cells *in vitro*. However, the efficacy and *in vivo* mechanism of action of EF24 in gastrointestinal cancer cells have not been investigated. Here, we assessed the *in vivo* therapeutic effects of EF24 on colon cancer cells. Using hexosaminidase assay, we determined that EF24 inhibits proliferation of HCT-116 and HT-29 colon and AGS gastric adenocarcinoma cells but not of mouse embryo fibroblasts. Furthermore, the cancer cells showed increased levels of activated caspase-3 and increased Bax to Bcl-2 and Bax to Bcl-xL ratios, suggesting that the cells were undergoing apoptosis. At the same time, cell cycle analysis showed that there was an increased number of cells in the $G_2$-M phase. To determine the effects of EF24 *in vivo*, HCT-116 colon cancer xenografts were established in nude mice and EF24 was given i.p. EF24 significantly suppressed the growth of colon cancer tumor xenografts. Immunostaining for CD31 showed that there was a lower number of microvessels in the EF24-treated animals coupled with decreased cyclooxygenase-2, interleukin-8, and vascular endothelial growth factor mRNA and protein expression. Western blot analyses also showed decreased AKT and extracellular signal-regulated kinase activation in the tumors. Taken together, these data suggest that the novel curcumin-related compound EF24 is a potent antitumor agent that induces caspase-mediated apoptosis during mitosis and has significant therapeutic potential for gastrointestinal cancers.** [Cancer Res 2008;68(6):1962–9]

## Introduction

Colorectal carcinoma is the second leading cause of cancer mortality in the United States with ~55,000 deaths in 2006 (1–3). Because conventional therapies, including surgical rejection, chemotherapy, and radiation, are often inadequate at treating the disease, the need for new treatment options has grown more critical. Epidemiologic studies suggest that diet plays a major role in the prevention of many cancers, and curcumin (a common flavoring agent in the spice turmeric) may be a dietary component responsible for lower rates of colorectal cancer in certain part of India (4). Curcumin has also been used in Indian folk medicine to treat several ailments, and recent preclinical and clinical studies show that curcumin has several anticancer and antiangiogenic properties (5, 6). Its antitumor properties include cancer growth inhibition and apoptosis induction in a variety of cultured cancer cell lines *in vitro*. Additionally, curcumin has shown the ability to inhibit tumorigenesis *in vivo* (7–13). The use of curcumin in antiangiogenesis includes the inhibition of vascular endothelial cell proliferation *in vitro* and capillary tube formation and growth *in vivo* (14, 15). The published report and our previous studies showed that curcumin inhibits epidermal growth factor (EGF)-mediated signaling and growth of intestinal adenomas in $APC^{min/+}$ mice (16). However, due to the low cancer-killing potency and poor intestinal absorption characteristics of curcumin (17, 18), bioavailability of the compound has limited its use. Consequently, analogues of curcumin with similar safety profiles but increased anticancer activity and solubility have recently been developed (13, 14, 19). One compound in particular, diphenyl difluoroketone (EF24), is active in anticancer screens and has considerably less toxic effect than the commonly used chemotherapeutic drug cisplatin (17, 20). In this article, we show results of our *in vitro* experiments showing that EF24 inhibits cell proliferation, prevents colony formation, and promotes $G_2$-M arrest of colon and gastric cancer cells. Our *in vivo* studies with HCT-116 colon cancer cell tumor xenografts in nude mice revealed that EF24 suppresses cancer cell proliferation and angiogenesis and induces cell cycle arrest and apoptosis, coupled with the reduction of the expression of colon cancer–promoting genes, including *cyclooxygenase-2* (*COX-2*), *interleukin-8* (*IL-8*), and *vascular endothelial growth factor* (*VEGF*).

## Materials and Methods

**Cells and reagents.** HCT-116, HT-29 colon, and AGS gastric adenocarcinoma cells (all from American Type Culture Collection) were grown in DMEM and RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co.) and 1% antibiotic-antimycotic solution (Mediatech, Inc.) at 37°C in a humidified atmosphere of 5% $CO_2$. Curcumin was purchased from LKT Laboratories. EF24 was synthesized at the Institute of Organic and Medicinal Chemistry, University of Pecs (Pecs, Hungary).

**Proliferation and apoptosis assays.** To assess proliferation, cells were seeded onto 96-well plates at a density of $1 \times 10^3$ per well and allowed to adhere and grow overnight in 10% heat-inactivated FBS containing DMEM or RPMI 1640. The cells were then treated with increasing doses of EF24 in 10% FBS containing DMEM or RPMI 1640. Analysis of cell proliferation was performed by enzymatic assay as described above (21). For apoptosis,

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-07-6011

caspase-3/7 activity was measured using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega). Both floating and attached cells were included in these analyses.

**Cell cycle analysis.** Cells were plated at a density of $5 \times 10^5$ per well on six-well plates. After treatment with EF24 for the indicated period, both floating and attached cells were collected into flow cytometry tubes and centrifuged at 1,000 rpm for 5 min to obtain cell pellets. The supernatant was discarded, and the cells were washed with PBS and then recentrifuged. The cells were resuspended in 100 μL PBS, 3 mL of −20°C ice-cold 70% ethanol were added, and the cells were then incubated for 1 h at 4°C. The cells were washed twice with PBS and 10 mg/mL RNase A was added. Propidium iodide was added to the tubes at a final concentration of 0.05 mg/mL and incubated at 4°C for 30 min in the dark. Cell cycle analysis was performed with a Becton Dickinson FACScan using an FL2 detector with a bandpass filter at specifications of 585 ± 21 nm. In each analysis, 10,000 events were recorded. Results were analyzed with ModFit LT software (Verity Software House).

**Colony formation assay.** Briefly, six-well dishes were seeded with 500 viable cells in complete medium and allowed to grow for 24 h. The cells were then incubated in the presence or absence of various concentrations of EF24 for up to 48 h. The EF24-containing medium was then removed, and the cells were washed in PBS and incubated for an additional 10 days in complete medium. Each treatment was done in triplicate. The colonies obtained were washed with PBS and fixed in 10% formalin for 10 min at room temperature and then washed with PBS followed by staining with hematoxylin. The colonies were counted and compared with untreated cells.

**HCT-116 cell tumor xenograft in mice.** Five-week-old male athymic nude mice purchased from The Jackson Laboratory were used for *in vivo* experiments; they were maintained with water and standard mouse chow *ad libitum* and used in protocols approved by the University's Animal Studies Committee. Animals were injected with $1 \times 10^6$ HCT-116 cells in the left and right flank and allowed to form xenograft. EF24 (200 μg/kg body weight) in 5% $Na_2HCO_3$ buffer alone was given i.p. daily for 23 days. Tumor size was measured weekly. At the end of treatment, the animals were sacrificed, and the tumors were removed and weighed for use in histology (H&E, COX-2, VEGF, and CD31) and gene expression studies.

**Real-time reverse transcription-PCR analysis.** Total RNA isolated from HCT-116 cells and tumor xenograft using Trizol reagent was reverse transcribed with SuperScript II reverse transcriptase in the presence of random hexanucleotide primers (all from Invitrogen). Complementary DNAs were then used for real-time PCR using JumpStart Taq DNA polymerase (Sigma Chemical) and SYBR Green nucleic acid stain (Molecular Probes). Crossing threshold values for individual genes were normalized to β2 microglobulin. Changes in mRNA expression were expressed as fold change relative to control. Primers used in this study were as follows: β2 microglobulin, 5′-GAGTGCTGTCTCCATGTTTGATG-3′ and 5′-CTCTAAGTTGCCAGCCCTCCT-3′; IL-8, 5′-CTCTTGGCAGCCTTCCTGATT-3′ and 5′-TATGCACTGACATCTAAGTTCCTTTAGCA-3′; COX-2, 5′-GAATCATTCACCAGGCAAATTG-3′ and 5′-TCTGTACTGCGGGTGGAACA-3′; and VEGF, 5′-AGCGCAAGAAATCCCGGTA-3′ and 5′-TGCTTTCTCCGCTCTGAGC-3′.

**Western blot analysis.** Cell lysates were subjected to PAGE and blotted onto Immobilon polyvinylidene difluoride membranes (Millipore). Antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, Inc., and specific proteins were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

**IL-8 ELISA.** The HCT-116 cells were treated with EF24 for 24 h. The cell-free supernatants and tumor xenograft proteins were measured by IL-8 using an enzyme immunoassay kit (Pierce Biotechnology) using the manufacturer's suggested protocol.

**Immunohistochemistry.** Tissues embedded in paraffin were cut to a section of 4 μm, deparaffinized, and treated with citrate buffer. Then, they were blocked with avidin/biotin for 20 min. The slides were incubated with anti–COX-2 or VEGF or CD31 for overnight at 4°C. Subsequently, the slides were treated with secondary antibody with horseradish peroxidase goat anti-rabbit for COX-2 or VEGF and goat anti-rat for CD31 staining for 1 h and developed with 3,3′-diaminobenzidine (Sigma-Aldrich). Finally, the slides were counterstained with hematoxylin.

**Statistical analysis.** All values are expressed as the mean ± SE. Data were analyzed using a paired two-tailed $t$ test. A $P$ value of <0.05 was considered statistically significant.

## Results

**EF24 inhibits intestinal cancer cell proliferation.** We first determined the effect of EF24 and curcumin on cell proliferation in a variety of cultured cancer cell lines (Fig. 1*A*). EF24 significantly suppresses proliferation of colon cancer cell lines HCT-116 and HT-29 and a gastric cancer cell line (AGS) within a 24-h period, which continues to 72 h (Fig. 1*B*). More importantly, the effects were observed at a dose of 1 μmol/L, a dose at which curcumin had no significant effect on HCT-116 cell proliferation. Similar results were observed with esophageal, lung, breast, pancreatic, and cervical cancer cells (data not shown). In contrast, EF24 did not affect the proliferation of normal mouse embryonic fibroblasts cells treated with 5 μmol/L concentration, whereas hydrogen peroxide significantly affected it (Fig. 1*C*), suggesting that EF24 is not toxic to normal cells. To determine the long-term effect of EF24 treatment, cells were treated with 1 μmol/L EF24 for 24 h, following which the cells were allowed to grow in normal medium. EF24 treatment suppresses colony formation in a dose-dependent manner in all three cell lines (Fig. 1*D*), suggesting that EF24 effects on the cells are irreversible. Again, at this 1 μmol/L dose, curcumin did not affect cell growth and colony formation (Fig. 1*D*).

**EF24 induces cell cycle arrest and apoptosis.** At a sufficiently high dose, curcumin is known to induce apoptosis of cancer cells. Cell cycle analysis was performed to determine the mechanism by which EF24 affects cancer cells. EF24 (1 μmol/L) induced growth arrest of the HT-29 and AGS cells within 24 h at the $G_2$-M stage and subsequently at the $G_0$ hypodiploid/fragmented DNA stage at 48 hours (Fig. 2*A*; Supplementary Fig. S1). Similar results were observed in HCT-116 cells (data not shown). These data suggest that the cells were undergoing apoptosis. Caspase-3 is a key effector molecule in the apoptosis pathway involved in amplifying the signal from initiator caspases, such as caspase-8 (22, 23). Increased activation of caspase-3 and caspase-7 was observed within 24 h in HCT-116 and AGS cells treated with either 1 or 5 μmol/L of EF24 (Fig. 2*B*). However, whereas 1 μmol/L EF24 did not induced caspase-3 and caspase-7 activity in HT-29 cells at 24 hours, 5 μmol/L EF24 did (Fig. 2*B*). These data further show that EF24 was inducing apoptosis (Fig. 2*B*). Western blot analyses of HCT-116 cell lysates showed a significant increase in the activated caspase-3 in cells treated with 5 μmol/L EF24 (Fig. 2*C*). In addition, 5 μmol/L EF24 inhibited the expression of antiapoptotic genes *Bcl-2* and *Bcl-xL* protein but it had no detectable effect on the expression levels of apoptosis-promoting total Bax protein (Fig. 2*D*). This resulted in decreased Bcl-2 to Bax and Bcl-xL to Bax protein ratios (Supplementary Fig. S2). These data suggest that EF24 is a potent inducer of apoptosis even at low doses where no such effect is observed with curcumin.

**EF24 inhibits tumor growth.** To evaluate the role of EF24 in tumor proliferation *in vivo*, we examined the ability of EF24 to suppress the growth of human cancer cell xenografts in nude mice. Colon cancer cell–induced xenograft tumors were allowed to develop and grow to a size of 500 $mm^3$, following which EF24 was given i.p. for 3 weeks daily. EF24 significantly inhibits the growth of the tumor xenografts (Fig. 3*A*). The excised tumors from the EF24-treated animals ranged from 200 to 300 mg, whereas those from the control group weighed <120 mg (Fig. 3*B* and *C*). In addition, tumor volumes were significantly decreased (Fig. 3*D*). Whereas the

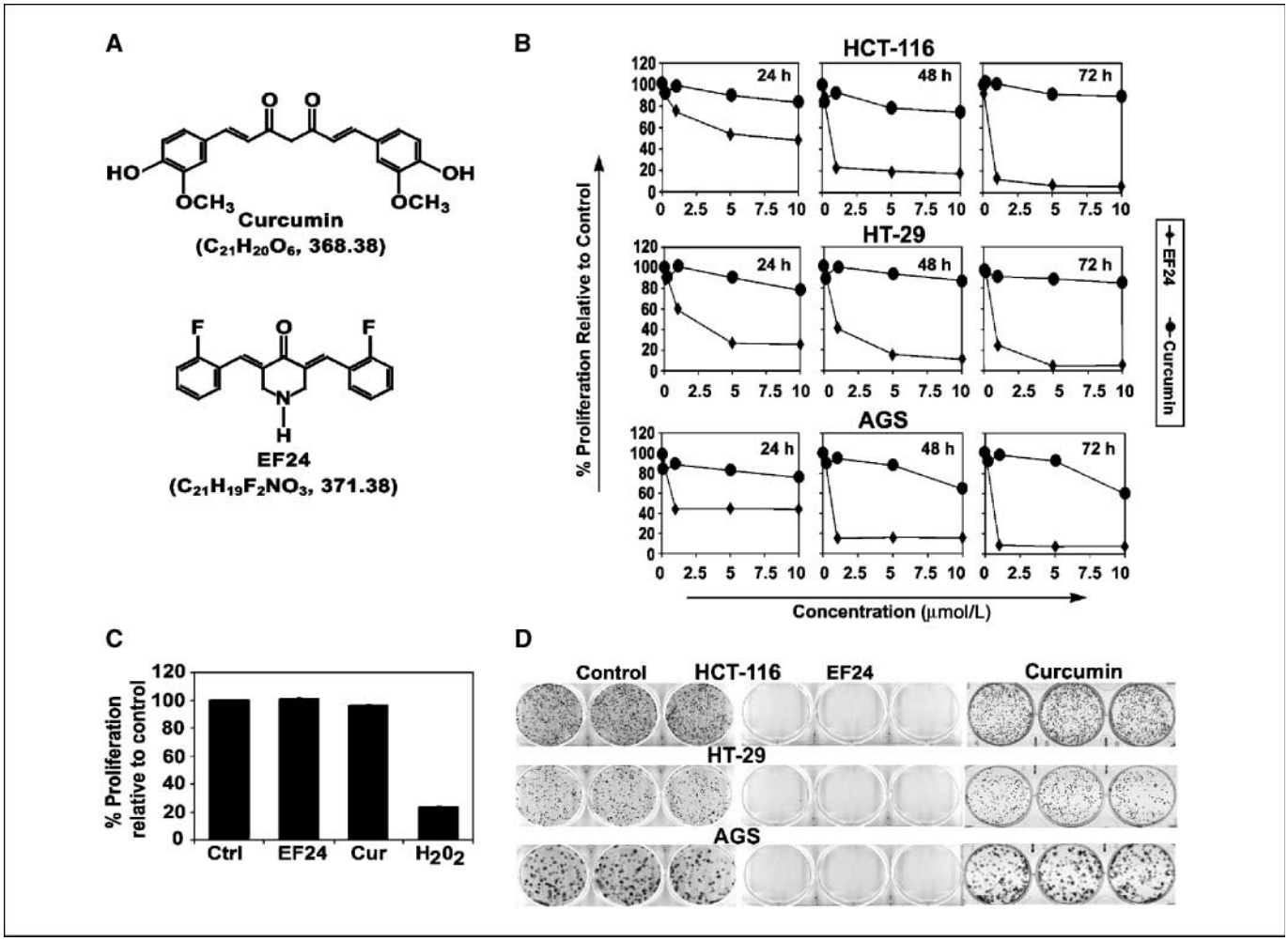


**Figure 1.** EF24 inhibits intestinal cancer cell proliferation. *A,* the topological structures of curcumin (diferuloylmethane) and EF24 (diphenyl difluoroketone). *B,* EF24 inhibits proliferation of HCT-116, HT-29, and AGS cells. These cells were incubated with increasing doses of EF24 (0.01–10 μmol/L) for 24-, 48-, and 72-h periods and analyzed for cell proliferation using hexosaminidase enzyme activity. EF24 treatment resulted in a significant dose-dependent decrease in cell number in all three cells when compared with untreated controls and curcumin. *C,* EF24 does not affect the proliferation of normal mouse embryonic fibroblast cells treated with 5 μmol/L EF24 for 48 h using hexosaminidase enzyme activity. *D,* EF24 inhibits cell growth as indicated by colony formation assays using HCT-116, HT-29, and AGS cells. These cells were incubated with 1 μmol/L EF24 or curcumin for 24 h and subsequently allowed to grow into colonies. As indicated by counts performed 10 d after incubation, EF24, but not curcumin, inhibits colony-forming units. Results are representative of three independent experiments.

control tumors continue to grow during the treatment period reaching a size of 2,000 $mm^3$, tumors in the EF24-treated animals did not grow. No apparent toxicity effects were observed in the EF24-treated animals (data not shown). Furthermore, tumors began to regress in the EF24-treated animals that were allowed to continue living past the 21-day period (data not shown). In our tests, no control animals were allowed to remain alive after 21 days. To evaluate whether EF24 administration affects normal physiology, we treated non–tumor-bearing mice weekly at exactly the same 200 μg/kg body weight dose for 3 weeks. There was no apparent change in liver weight, spleen weight, or body weight in the animals (data not shown). These data imply that EF24 is a potential therapeutic for treatment of colon cancers and that it is relatively nontoxic to mice.

**EF24 inhibits the signaling of tumor cell survival.** Survival signals that originate from extracellular cues result in the activation of the phosphatidylinositol 3-kinase (PI3K)/AKT and/or the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways. The PI3K pathway plays a crucial role in the survival of various cell types (24). Activated AKT phosphorylates numerous proteins that have been implicated in the control of the cell cycle to ultimately stimulate cell growth and inhibit apoptosis (25). Western blot analyses of HCT-116 cell lysates showed a significant dose- and time-dependent decrease in the EGF-mediated AKT phosphorylation in cells treated with EF24 (Fig. 4*A*; Supplementary Fig. S3). MAPKs are also activated by a range of extracellular signals via protein phosphorylation cascades that relay mitogenic signals to the nucleus, thereby modulating the activity of transcription factors (26–29). The two best-characterized isoforms [$p42^{mapk}$ (ERK-2) and $p44^{mapk}$ (ERK-1)] are directly activated by phosphorylation on specific tyrosine and threonine residues by the dual-specificity ERK kinase. Western blot analyses of HCT-116 cell lysates showed a significant dose-dependent decrease in the EGF-mediated ERK phosphorylation in cells treated with EF24 (Fig. 4*B*). In addition, EF24 significantly inhibited the tumor xenograft AKT and ERK phosphorylation (Fig. 4*C* and *D*). These data suggest that EF24 is a potent inhibitor of tumor cell survival through suppression of PI3K and ERK-MAPK pathways.

**EF24 inhibits expression of cancer-promoting genes and angiogenesis.** COX-2, a key rate-limiting enzyme in prostaglandin production, is overexpressed at multiple stages of colon carcinogenesis (30–32). Knockdown of COX-2 activity by either nonsteroidal anti-inflammatory drugs or by deletion of the *COX-2* gene results in the suppression of intestinal polyposis in $APC^{min/+}$ mice (30–32). Previous studies have shown increased levels of COX-2 mRNA in colorectal adenomas, adenocarcinomas, and colon cancer cell lines (33, 34). Therefore, we next determined the effects of EF24 treatment on COX-2 expression. EF24 treatment reduced COX-2 mRNA and protein levels in HCT-116 cells (Fig. 5*A* and *B*). Interestingly, protein levels of both COX-2 and VEGF were rapidly reduced within 2 hour of treatment with EF24, suggesting the presence of posttranslational control mechanisms in regulating expression of these genes.

COX-2 expression was significantly lower in EF24-treated nude mice tumor xenografts when compared with the control tumors

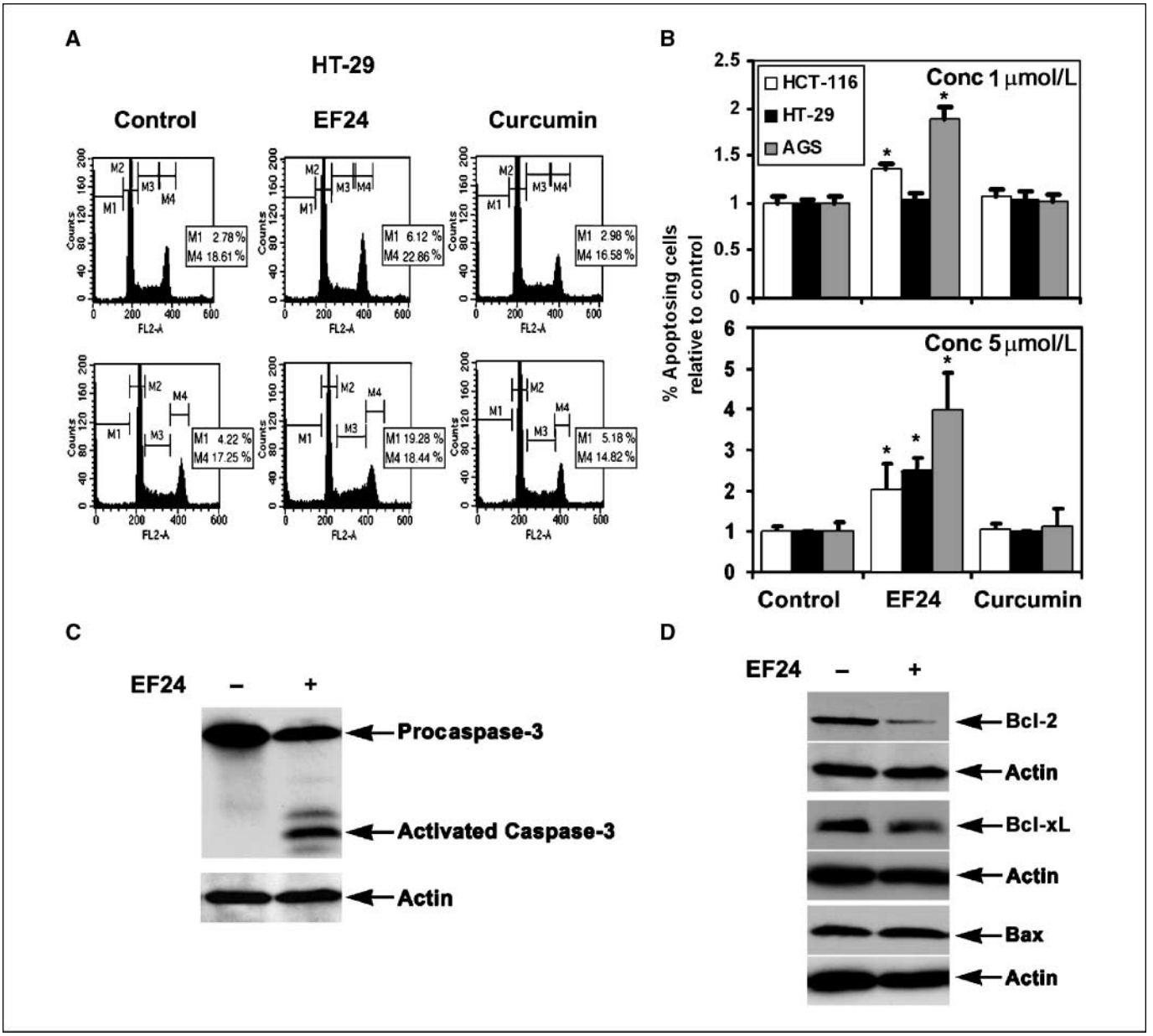


**Figure 2.** EF24 induces cancer cell apoptosis. *A,* DNA content (propidium iodide) and cell cycle analysis of EF24-treated cells. HT-29 cells were treated with EF24 for 24 and 48 h. *M1,* sub-$G_1$-$G_0$ peak; *M2,* $G_1$ phase; *M3,* S phase; *M4,* $G_2$-M phase. Apoptosis was measured as the percentage of cells containing hypodiploid amounts of DNA (sub-$G_1$-$G_0$ peak). EF24 treatment leads to increased number of cells in the $G_2$-M phase after 24 h as indicated by the increase in M4 peaks compared with control. Increase in M1 peaks at 48 h indicates that EF24 induces apoptosis after 48 h. Graphs are representative of data collected from at least three experiments. *B,* EF24 treatment induces apoptosis in HCT-116, HT-29, and AGS cells. HCT-116, HT-29, and AGS cells incubated with 1 μmol/L (*top*) and 5 μmol/L (*bottom*) of EF24 were analyzed for apoptosis. EF24 treatment increased number of apoptotic cells compared with either curcumin-treated or untreated control. *, $P < 0.001$. *C,* EF24 induces caspase-3, an apoptosis mediator. Lysates from HCT-116 cells incubated with 5 μmol/L EF24 were analyzed by Western blotting for caspase-3 protein expression levels using rabbit anti–caspase-3 antibody. EF24-treated cells show cleaved (activated) caspase-3, whereas untreated cells have no cleaved caspase-3. *D,* EF24 reduces expression of antiapoptotic proteins Bcl-2 and Bcl-xL in treated cells when compared with untreated cells. Lysates from HCT-116 cells incubated with 5 μmol/L EF24 were analyzed by Western blotting for Bcl-2, Bcl-xL, and Bax protein expression levels. Whereas both Bcl-2 and Bcl-xL were reduced, Bax expression was not affected by EF24 treatment.

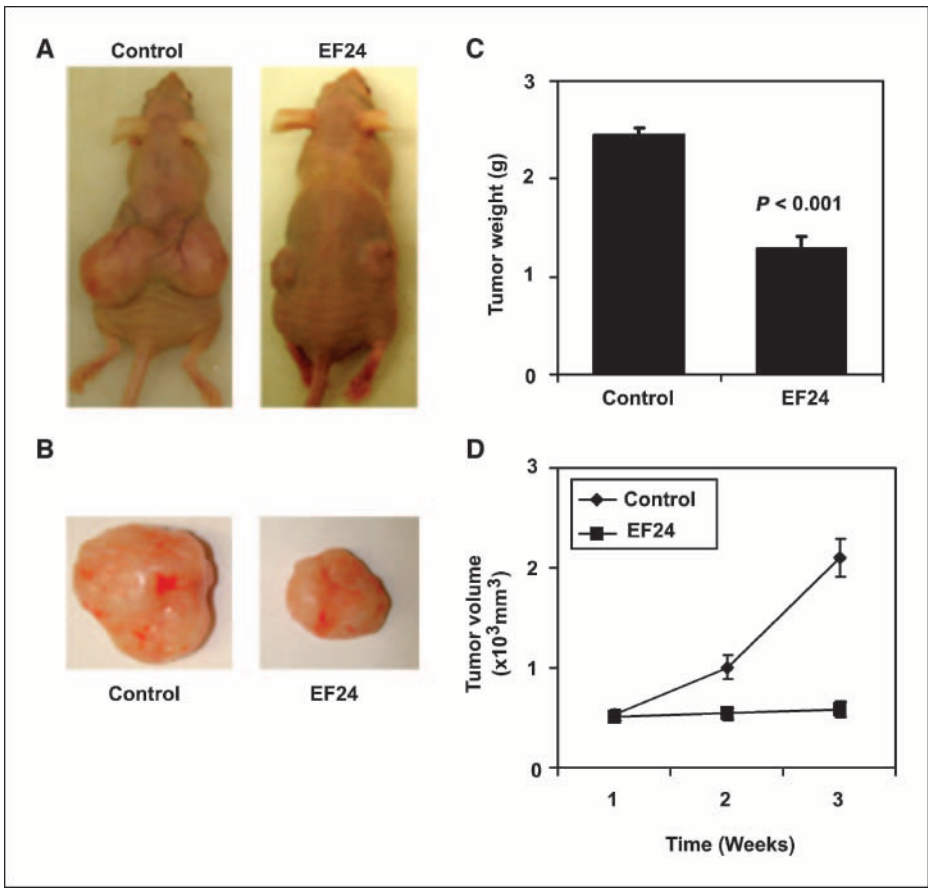


**Figure 3.** EF24 inhibits colon cancer tumor xenograft growth *in vivo*. *A* and *B*, HCT-116 cells were injected to the flanks of nude mice and palpable tumors were allowed to develop for 7 d. Subsequently, EF24 was injected daily i.p. for up to 21 d. On day 22, tumors were excised and subjected to further analyses. Tumor volumes in EF24 given mice were smaller than that of control mice. *C*, EF24 treatment resulted in significantly lower tumor weight when compared with controls. *D*, tumor size was measured every week. There was a significant reduction in tumor size from EF24-treated animals when compared with untreated controls.

(Fig. 6*A* and *C*). Immunohistochemistry showed a diffused cytoplasmic staining for COX-2 in the epithelial cells in the control tumors, and the expression was higher subepithelial myofibroblasts (Fig. 6*D*). However, COX-2 staining was significantly reduced in both the epithelial cells and myofibroblasts in EF24-treated tumors.

VEGF and IL-8 are potent inducers of capillary growth into the tumor, and without angiogenesis, tumor growth normally stops at a diameter of about 1 to 2 mm. Prostaglandins and the other tumor-promoting mediators are known to induce the expression of VEGF and IL-8 in epithelial cells. Hence, we also determined the effect of EF24 on expression of these two genes in colon cancer cells. Both VEGF and IL-8 expression were significantly reduced in HCT-116 cells (Fig. 5*A*–*C*). Similarly, VEGF and IL-8 levels were significantly reduced in the EF24-treated tumor xenografts (Fig. 6*A*–*C*). We also determined the effect of EF24 on tumor vascularization by staining for the endothelial-specific antigen CD31. As shown in Fig. 6*D*, EF24 treatment leads to a significant reduction in CD31 staining and to the obliteration of the normal vasculature that is associated with tumor angiogenesis. We also calculated the microvessel density and found it to be significantly decreased following EF24 treatment (Fig. 6*D*).

## Discussion

Our results indicate that the novel curcumin analogue EF24 possesses great potential as a promising anti–colon therapeutic agent. Colon cancer is a common malignancy in economically developed countries, particularly in North America, Europe, and Australia, and has emerged as one of the leading causes of cancer-related deaths in the Western world. The significant morbidity of surgery, radiation, and chemotherapy for colon cancer has led searches for less toxic alternative therapies. Many studies have shown that curcumin suppresses the proliferation of a variety of tumor cells, including breast, colon, oral, lung, melanoma, myeloma, leukemia, and prostate carcinoma (10, 11, 35–43). The data presented in the article show that EF24 selectively inhibits the proliferation of colon cancer cells, suppresses the formation of colon cancer cell colonies, inhibits cell survival signaling, promotes cell cycle arrest and apoptosis, and inhibits cancer-promoting genes. *In vivo*, EF24 decreases tumor size, volume, and microvessel density and suppresses mRNA expression and protein levels of the colon cancer–promoting genes *COX-2, IL-8*, and *VEGF*.

Because curcumin is poorly absorbed through the intestine, more potent and soluble curcumin analogues have been developed (17). Furthermore, as shown in our studies, at low doses, curcumin did not have a therapeutic effect. Similar results were observed in studies on squamous cell carcinomas, where a dose of 3.75 μmol/L curcumin did not affect growth of the cells (44). Consequently, higher doses are required. Synthetic chemical analogues to molecularly targeted chemotherapeutic drugs and chemopreventative photochemical confound a myriad of molecular events in host and tumor tissues. These events include the acquisition of self-sufficient growth signals, insensitivity to signals that usually inhibit proliferation, use of survival pathways to evade apoptosis, initiation of angiogenesis to ensure sufficient oxygen and nutrient

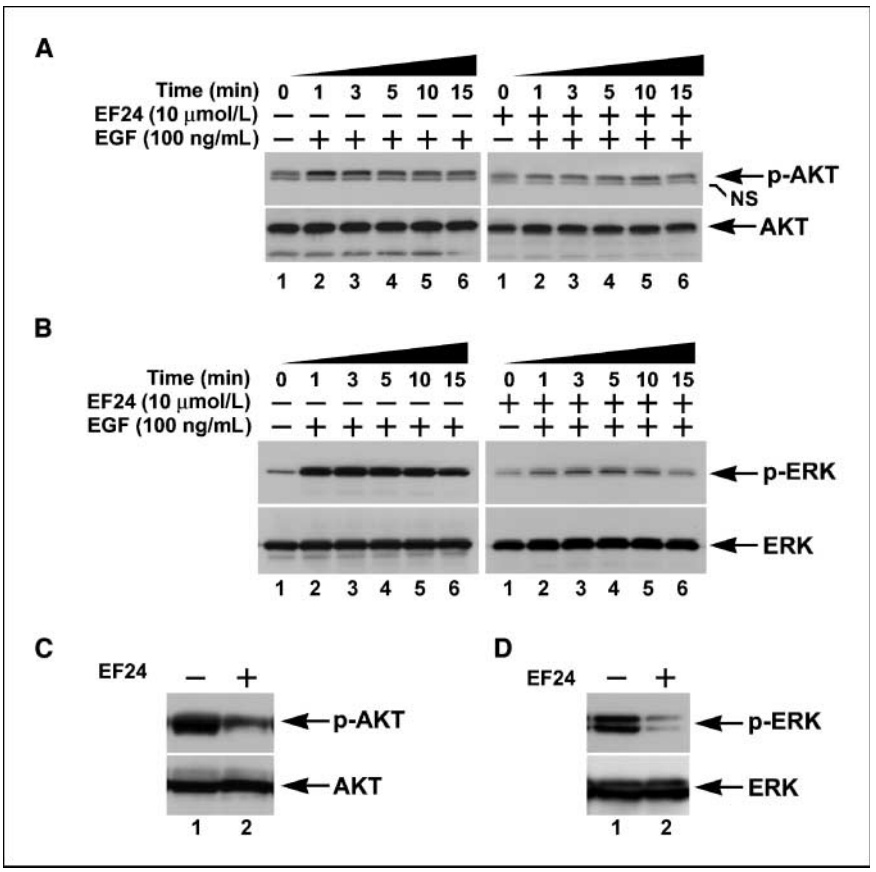


**Figure 4.** EF24 inhibits AKT and ERK activation required for cell survival. *A* and *B,* HCT-116 cells were pretreated with 10 μmol/L EF24 for 2 h followed by treatment with EGF (100 ng/mL) for the indicated time (0–15 min). Lysates from EF24 treatment showed significant reduction in the EGF-mediated AKT and ERK phosphorylation at all time points. *C* and *D,* AKT and ERK phosphorylation was significantly lower in tumor xenografts from EF24-treated animals when compared with controls.

supply, and attainment of the ability to invade and metastasize (45). EF24 molecule interferes with the progression of cancer by disrupting many of the characteristic cancer-promoting events more effectively than curcumin. EF24 inhibits proliferation of HCT-116 colon cancer cells, promotes apoptosis, prevents angiogenesis of tumors in mice xenografts, and decreases tumor growth. In cell cycle analyses, EF24 was observed to increase the number of cells in the $G_2$-M phase at 24 hours. At the same time, there are

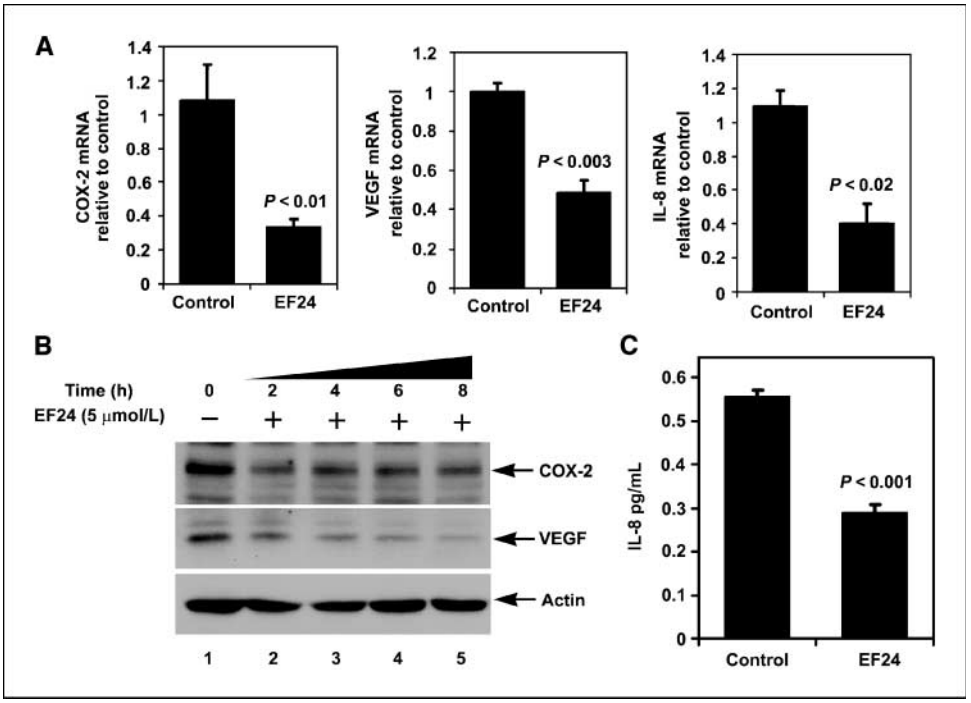


**Figure 5.** EF24 inhibits the expression of cancer-promoting genes. *A,* real-time reverse transcription-PCR analysis of total RNA from HCT-116 cells following 1 μmol/L EF24 treatment for 24 h showed reduction in the expression of COX-2, VEGF, and IL-8 mRNA. *B,* lysates from EF24 treatment caused significant reduction of COX-2 and VEGF protein levels in HCT-116 cells. *C,* medium from EF24-treated cells showed significantly lower levels of IL-8 protein.

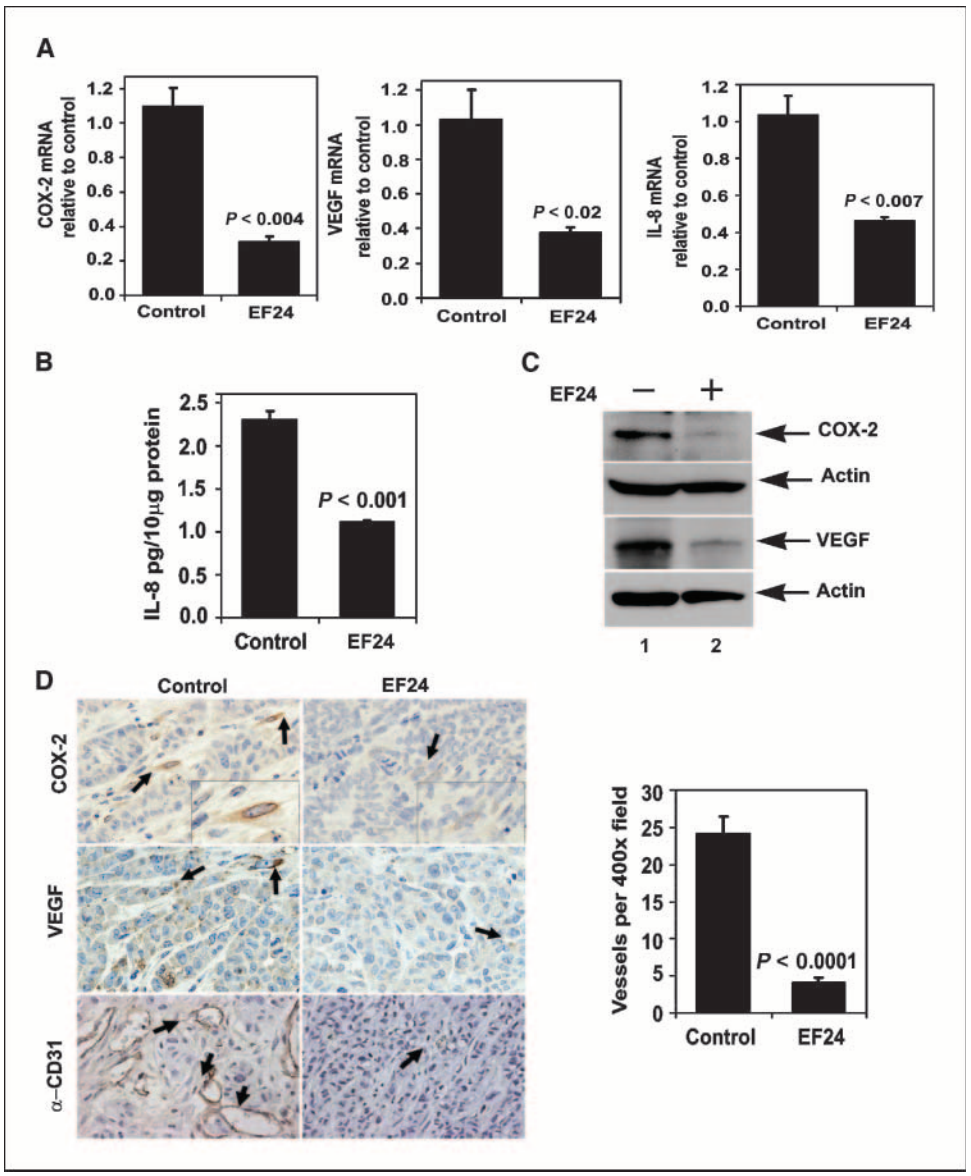


**Figure 6.** EF24 suppresses tumor angiogenesis. *A,* EF24 treatment decreases COX-2, VEGF, and IL-8 mRNA expression in the HCT-116 tumor xenografts. *B,* ELISA analysis of tissue lysates from the EF24-treated mice shows significantly lower levels of IL-8. *C,* Western blot analysis showed that tissue lysates from the EF24-treated animals have significantly lower levels of COX-2 and VEGF proteins. *D,* immunohistochemistry shows that EF24 treatment significantly reduced the expression of COX-2 and VEGF in tumor xenografts. *Arrows,* proteins are stained brown. Inset in the COX-2 immunohistochemistry shows high-power field view of a COX-2–positive stromal cell. Tumor sections were stained for CD31, an endothelial cell–specific surface marker, and the vessel areas were counted. Microvessel density was significantly reduced in the xenografts of EF24-treated animals.

significantly higher levels of apoptosis. These data imply that EF24 treatment leads to mitotic catastrophe in which the proliferating cancer cells undergo cell death but not necessarily an arrest in the $G_2$-M phase of the cell cycle. This was further supported through our observation that the majority of cells were present in the sub-$G_0$ phase at 48 h following treatment.

The effects of EF24 on *in vivo* activity are dependent on the bioavailability of drug at the site of the tumor. Dietary curcumin is poorly absorbed through the intestinal tract. This results in good availability of the compound only to the surface epithelial cells of the intestine and colon but significantly poor availability of the compound to those cells that develop at the deeper tissue locations in the intestine and colon or if the tumor is malignant and is localized in other sites. This has led to the development of analogue with better bioavailability. In our studies, we observed marked suppression of tumor growth in mice xenograft with EF24 treatment. However, further studies are needed to confirm and extend the present study to use EF24 as an effective therapy for colorectal cancer. Absorption and pharmacokinetic properties of EF24 in particular need to be identified in future studies; however, preliminary studies indicate that EF24 seems to have low toxicity in liver, kidney, and spleen and allows mice treated with EF24 to maintain normal weight gain (19). In addition, EF24, like curcumin, seems to mediate its actions through multiple molecular targets, including COX-2, VEGF, and IL-8. Because COX-2 overexpression during colon carcinogenesis causes resistance to apoptosis (46), treatment of colon cancer cells with EF24 may potentially restore susceptibility to apoptosis. Furthermore, IL-8 overexpression has been correlated with the inflammation-related risk of sporadic colorectal cancer (47), so treatment with EF24 may also potentially ameliorate the inflammatory responses that are associated with the development of cancer. Finally, VEGF is important in angiogenesis and promotion of tumor growth, and the ability of EF24 to inhibit VEGF expression is yet another molecular mechanism by which EF24 may function to prevent colorectal cancer.

In this study, we have shown that EF24 suppressed EGF-mediated signaling. However, colon cancer cells, including HCT-116 cells, are known to express autocrine factors such as prostaglandins,

insulin-like growth factors, and progastrin, which activate many cellular pathways (48–53). It would be interesting to determine whether EF24 is equally potent in inhibiting these signal transduction pathways.

In conclusion, our studies show that EF24 treatment of intestinal cancer cells results in growth inhibition *in vitro* and *in vivo*. Our *in vitro* and *in vivo* studies in combination with the observation that EF24 does not affect proliferation of normal human fibroblasts strongly suggest that EF24 has promising potential for use as a therapeutic or chemopreventative agent for intestinal cancer. Similar to curcumin, EF24 also seems to have multiple molecular targets and its enhanced potency in cancer cell lines and xenograft tumors renders it a strong candidate for therapeutic applications for colon cancer as well as other cancers and inflammatory disease states.

## Acknowledgments

Received 10/29/2007; revised 1/9/2008; accepted 1/16/2008.

**Grant support:** Hungarian Research Fund OTKA T048334 (K. Hideg) and NIH grants AT004118 (R.P. Ramanujam), DK62265, and CA109269 (S. Anant).

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We thank Drs. Chinthalapalli V. Rao and James H. Wyche for their helpful discussions during the course of these studies.

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*Cancer Res* 2008;68:1962-1969.