Celecoxib and Curcumin Synergistically Inhibit the Growth of Colorectal Cancer Cells

Shahar Lev-Ari,1,2 Ludmila Strier,2 Diana Kazanov,2 Liora Madar-Shapiro,2 Hadas Dvory-Sobol,1,2 Ilya Pinchuk,2 Brigitte Marian,3 Dov Lichtenberg,2 and Nadir Arber1,2

Abstract

Purpose: Multiple studies have indicated that cyclooxygenase-2 (COX-2) inhibitors may prevent colon cancer, which is one of the leading causes of cancer death in the western world. Recent studies, however, showed that their long-term use may be limited due to cardiovascular toxicity. This study aims to investigate whether curcumin potentiates the growth inhibitory effect of celecoxib, a specific COX-2 inhibitor, in human colon cancer cells.

Experimental Design: HT-29 and IEC-18-K-ras (expressing high levels of COX-2), Caco-2 (expressing low level of COX-2), and SW-480 (no expression of COX-2) cell lines were exposed to different concentrations of celecoxib (0–50 μmol/L), curcumin (0–20 μmol/L), and their combination. COX-2 activity was assessed by measuring prostaglandin E2 production by enzyme-linked immunoassay. COX-2 mRNA levels were assessed by reverse transcription-PCR.

Results: Exposure to curcumin (10–15 μmol/L) and physiologic doses of celecoxib (5 μmol/L) resulted in a synergistic inhibitory effect on cell growth. Growth inhibition was associated with inhibition of proliferation and induction of apoptosis. Curcumin augmented celecoxib inhibition of prostaglandin E2 synthesis. The drugs synergistically down-regulated COX-2 mRNA expression. Western blot analysis showed that the level of COX-1 was not altered by treatment with celecoxib, curcumin, or their combination.

Conclusions: Curcumin potentiates the growth inhibitory effect of celecoxib by shifting the dose-response curve to the left. The synergistic growth inhibitory effect was mediated through a mechanism that probably involves inhibition of the COX-2 pathway and may involve other non-COX-2 pathways. This synergistic effect is clinically important because it can be achieved in the serum of patients receiving standard anti-inflammatory or antineoplastic dosages of celecoxib.

Colorectal cancer is one of the leading causes of cancer death among both men and women in the western world (1). Several 

in vitro, in vivo, and clinical studies have previously indicated that celecoxib (Pfizer, New York, NY), a specific cyclooxygenase-2 (COX-2) inhibitor, may prevent colorectal cancer (2, 3). However, the long-term use of celecoxib may be limited due to cardiovascular toxicity (4). The molecular mechanism responsible for the chemopreventive action of celecoxib is not entirely understood. Multiple lines of evidence from 

in vitro studies and animal models of intestinal polyposis indicate that celecoxib exerts its effect by inhibiting the COX-2 isoenzyme (5). Other studies, however, suggest that celecoxib may also reduce the formation of polyps by COX-2–independent mechanisms, such as by blocking the antiapoptotic kinase (Akt) activity (6) or inducing apoptosis by suppressing the ability of the PPARγ receptor complex to bind to DNA (7). We have recently shown that a low dose of celecoxib (10 μmol/L) was sufficient to inhibit the growth of transformed cells, but not of normal cells, in vitro (8).

The search for new chemopreventive compounds with minimal toxicity is of particular interest in phytochemicals. Curcumin is a diferuloylmethane derived from the plant Curcuma longa. It is a potent antioxidant that possesses both anti-inflammatory and antitumor activities (9, 10). Its chemopreventive efficacy has been shown in several 

in vitro and animal models (11, 12). The chemopreventive properties of curcumin have been attributed, at least in part, to its ability to inhibit COX-2 (13). Recent studies indicate that other targets, such as the inhibition of the activation of transcription factors activator protein and nuclear factor κB (14) or the down-regulation of epidermal growth factor receptor (15) and Her-2 (16), may also be involved in the chemopreventive effect of curcumin.

Numerous studies showed that COX-2 has a central role in the development of colorectal cancer including antiapoptotic effects, increased invasiveness, and promotion of angiogenesis (5). The rationale for combining curcumin and celecoxib was that both drugs inhibit COX-2 by different mechanisms: curcumin down-regulates COX-2 mRNA and protein levels...
(13, 17) whereas celecoxib inhibits COX-2 directly by binding to its active site (18).

The current study shows that curcumin synergistically augments celecoxib inhibition of cancer cell growth in vitro, and that COX-2 activity is almost totally inhibited by combining both drugs. This synergistic effect is clinically important because it can be achieved in the serum of patients receiving standard anti-inflammatory or antineoplastic doses of celecoxib.

Materials and Methods

Cell culture and reagents

Human colorectal cancer cell lines (HT-29, SW-480, and Caco-2) were obtained from the American Type Culture Collection. Normal enterocytes, derived from the rat ileum (IEC-18 cells) and transformed by c-K-ras oncogene (IEC-18-K-ras cells) as previously described (19), were also used. Curcumin (97% purity) was purchased from Merck (Whitehouse Station, NJ); celecoxib was provided by Pfizer; and rofecoxib was provided by Merck. The different cell lines were grown and maintained in DMEM (Biological Industries, Israel) supplemented with 5% FCS, 1% penicillin, and 1% streptomycin at 37°C, in an atmosphere of 95% oxygen and 5% CO₂ (full medium).

Cell growth inhibition assay

The colorectal cancer cells (HT-29, IEC-18-K-ras, SW-480, and Caco-2) were plated in duplicate at a density of 3 × 10⁴ in 12-well plates containing 1 mL of full medium. Celecoxib, curcumin, or 0.1% DMSO (the drug vehicle) was added at the selected concentrations to the culture medium 24 hours after plating. The number of viable cells was determined in duplicate by a Coulter counter 72 hours later. All experiments were repeated at least thrice and yielded similar results.

Cell viability assay

The CRC cells (2.5 × 10³/well) were seeded in 96-well plastic plates and incubated at 37°C in full medium containing the test drugs. After 48 and 72 hours, cell viability was assessed by the ability of metabolically active cells to reduce tetrazolium salt to colored formazan. The absorbance of the samples was measured with an ELISA reader (wavelength, 450 nm; reference wavelength, 630 nm). Each measurement was done in triplicate. The data are mean values from three different experiments.

Apoptosis assays

Apoptosis was determined by two independent methods.

Flow cytometric analysis. HT-29 cells were plated at a density of 5 × 10⁴ per 10 cm dish with curcumin, celecoxib, and their combination at selected concentrations for 72 hours. The adherent and nonadherent cells were collected during the exponential growth and counted. A total of 1 × 10⁶ to 2 × 10⁶ cells were washed in PBS and the pellet was fixed in 3 mL ethanol for 1 hour at 4°C. The cells were pelleted and resuspended in 1 mL PBS and incubated for 30 minutes with 0.64 mg/mL RNase at 37°C. They were stained with 45 μg/mL propidium iodide at least 1 hour before analysis by flow cytometry using a standard protocol for cell cycle distribution and cell size (20).

Necrotic cells were excluded by counting cells following staining with trypan blue before fixation. All experiments were done thrice and gave similar results. Data acquisition was done on a FACScan and analyzed by CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). All fluorescence and laser light scatter measurements were made with linear signal processing electronics. Data for at least 10,000 cells were collected for each data file.

Fluorescence microscopy. HT-29 cells were plated at a density of 5 × 10⁴ per 10 cm dish with curcumin, celecoxib, and their combination at selected concentrations for 72 hours. Apoptotic HT-29 cells were detected by nuclear morphologic changes using propidium iodide staining. Cells were washed twice with PBS and fixed for 15 minutes at room temperature with 4% paraformaldehyde in PBS. The fixative was removed by aspiration, and the monolayer was washed twice in PBS. DNA was incubated with 0.15 mg/mL RNase for 15 minutes and stained with 5 μg/mL propidium iodide at room temperature. Excess propidium iodide stain was removed, and the monolayer was thoroughly washed with PBS. The coverslip was mounted with glycerol. The stained nuclei were viewed under a 63× objective using a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Protein extraction and Western blotting

Exponentially growing HT-29 cells were treated with curcumin, celecoxib, and their combination at selected concentrations for 72 hours. The cells were collected and washed thrice in ice-cold PBS as described earlier. The cell pellets were resuspended in lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 2 mmol/L EDTA, 6 mmol/L 6-mercaptoethanol, 1% NP40, 0.1% SDS and 10 mmol/L NaF, plus the protease inhibitors leupeptin 10 μg/mL, aprotinin 10 μg/mL, and 0.1 mmol/L phenylmethylsulfonylfluoride]. The protein concentration of each sample was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). For Western blotting, samples containing 50 μg of total cell lysate were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis. Proteins were transferred to Hybond-C membranes (Amersham, Arlington Heights, IL) in transfer buffer (25 mmol/L Tris, 190 mmol/L glycine, 20% methanol) using a Trans Blot transfer apparatus at 70 mA for 12 to 18 hours at room temperature. The membranes were blocked with blocking buffer (PBS/0.2% Tween 20/0.5% gelatin) for 1 hour at room temperature and subsequently washed thrice for 5 minutes in washing buffer (PBS/0.05% Tween 20). The membranes were incubated with monoclonal human anti–COX-1, and anti-actin antibodies for 1 hour at room temperature. The membranes were washed as described above and incubated with anti-goat secondary antibodies (1:2,000) for 1 hour at room temperature. Additional washes were carried out as previously described, and immune detection was done using the ECL Western blotting detection system (Amersham). Intensities of the different proteins were quantified by densitometric scanning. All experiments were repeated at least thrice and yielded similar results.

Reverse transcription-PCR

HT-29 cells were treated with curcumin, celecoxib, and their combination for 72 hours as indicated. mRNAs were extracted and equal amounts were transcribed to cDNA from a kit (Promega, Madison, WI). cDNAs were taken from samples at various times and used as DNA templates for PCR. Primers used for COX-2 were 5'-TTC AAA TGA GAT TGT GGG AAA AT-3' and 5'-AGA TCA TCT CCT GAG TAT CTT-3'. Glyceraldehyde-3-phosphate dehydrogenase was used to ensure equal loading. Two milliliters were used as the template and 1 mL of each primer was used for each cDNA sample. The samples went through 30 rounds of PCR. They were separated on 1% agarose gel and visualized by ethidium bromide. Difference in RNA expression was analyzed by densitometric scanning.

Measurement of prostaglandin E₂ concentration

HT-29 cells were treated for 72 hours with curcumin, celecoxib, and their combination as indicated. Prostaglandin E₂ (PGE₂) concentration in the medium, as released by the cells, was determined by a commercially available PGE₂-specific enzyme-linked immunonassay (R&D Biosystems, Abingdon, United Kingdom) according to the protocol of the manufacturer.

Lipid peroxidation assay

The antioxidative properties of celecoxib, curcumin, and their combination were analyzed as previously described (21). Copper-induced peroxidation was monitored at 37°C by continuous recording of absorbance at 245 and 268 nm using a Kontron (UVikon 933) double-beam spectrophotometer equipped with a 12 position automated sample changer. Measurements were carried out in quartz cuvettes.
(optical pathway, 1 cm) after addition of CuCl₂ (final concentration, 100 μmol/L) and PBS [mmol/L NaCl 146, 3.3 mmol/L NaH₂PO₄, 3.3 mmol/L Na₂HPO₄, pH 7] to a solution containing 30 μL serum and 720 μmol/L of sodium citrate (final volume, 1.5 mL). Serum was obtained by centrifugation (1,000 × g) of venous blood collected from healthy individuals after 12 hours of fasting. The absorbance at 245 nm is attributed to deionic hydroperoxides and 7-ketocholesterol whereas the absorbance at 268 nm is attributed mostly to dienals and other final products of decomposition of hydroperoxides. This method of assessing the susceptibility of serum lipids to peroxidation ex vivo has been shown to strongly correlate with copper-mediated oxidation of low-density lipoprotein as reported by Schnitzer et al. (22). Different concentrations of celecoxib and curcumin were added to 50-fold diluted serum at time 0. The kinetics of oxidation was analyzed as previously described in terms of (a) the maximal rate of accumulation of absorbing products (Vₘₐₓ); (b) the time at which the rate of peroxidation achieved its maximal value (tₘₐₓ); this variable is in good correlation with the "lag" preceding oxidation and reflects the resistance of serum lipids to oxidative stress; and (c) the maximal accumulation of absorbing products (Aₘₐₓ), which correlates with the total concentration of oxidizable lipids.

### Statistical analysis

The results were measured as mean ± SD. To evaluate the difference between treatment with each of the drugs and treatment with their combination, the one-way ANOVA test was done using an SPSS Fig. 1. Celecoxib and curcumin inhibit cell growth of cancer cell lines in a dose-dependent matter. The cell lines were exposed for 72 hours to different concentrations of curcumin and celecoxib as indicated. Points, mean values from three individual experiments done in duplicate; bars, SD. A, effect of curcumin on cell lines growth. B, effect of celecoxib on cell lines growth.

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 2. Effect of curcumin, celecoxib, and their combination on the growth of cancer cell lines expressing high levels of COX-2. The cell lines were exposed for 72 hours to different concentrations of celecoxib, curcumin, and their combination as indicated. Columns, values from three individual experiments done in duplicate mean; bars, SD. Differences in cell growth after exposure to curcumin and celecoxib separately and to their combination were determined using the one-way ANOVA test. *, significant differences (P < 0.05). A, HT-29 cells; B, IEC-18-K-ras cells.
software package (SPSS, Inc., Chicago, IL). Statistical significance ($P < 0.05$) was established by the post hoc Tukey’s pairwise comparison.

**Results**

**Celecoxib and curcumin synergistically inhibited cell growth.**

The effect of celecoxib and curcumin on cell growth of three human colon carcinoma cell lines (HT29, SW-480, and Caco-2) and the c-K-ras–transformed rat intestinal epithelial cell line (IEC-18-K-ras) was assessed alone and in combination. An inhibitory effect of curcumin and celecoxib on cell growth of the cell lines was found to be dose dependent (Fig. 1).

In HT-29 cells that express high levels of COX-2 protein, treatment with either celecoxib (5-10 μmol/L) or curcumin (10 μmol/L) resulted in a minor (20-30%) inhibition of cell growth (Fig. 2A). When celecoxib and curcumin were combined (5 and 10 μmol/L, respectively), there was an 80% reduction in cell number. This effect was similar to that exerted by a 10-fold higher concentration of celecoxib (50 μmol/L). A similar synergistic effect was observed in another cell line (IEC18-K-ras) that expressed a high level of COX-2 (Fig. 2B). A weaker effect (additive; Fig. 3A and B) was seen in Caco-2 cells that produce low levels of COX-2 (23) and in SW-480 cells that do.
not express COX-2 (24). Cell viability assays confirmed these results (data not shown).

Rofecoxib, up to 20 μmol/L, did not have any additive growth inhibitory effect when added to curcumin (data not shown). HT-29 cells, in which the maximal synergistic effect was observed, were chosen for further studies. These cells express high levels of COX-1 and COX-2 mRNAs and proteins (25).

Celecoxib and curcumin synergistically induced apoptosis. The extent of apoptosis was assessed by flow cytometry analysis following 72 hours exposure of HT-29 cells to the different treatments. The combination of celecoxib (5 μmol/L) and curcumin (15 μmol/L) significantly increased the percentage of cells with subdiploid DNA content, the hallmark of apoptosis (Fig. 4A), compared with treatment with each drug alone. A similar synergistic effect was obtained in IEC-18-K-ras cells. In SW-480 cells that do not express COX-2, there was a subadditive increase in the level of apoptosis (data not shown).

Drug-treated HT-29 cells were examined for morphologic evidence of apoptosis using fluorescence microscopy, following propidium iodide staining. Typical apoptotic features of chromatin condensation and nuclear fragmentation were seen in the treated cells only when they were exposed to both drugs (illustrated in Fig. 4B).

Only the combination therapy involved down-regulation of cyclooxygenase-2 expression. To determine whether the combination effect involves down-regulation of COX-2 synthesis, HT-29 cells were treated with celecoxib, curcumin, and their combination at selected concentrations. The mRNA was isolated and probed with COX-2 cDNA. As shown in Fig. 5A, celecoxib (5 μmol/L) had no effect on the expression of COX-2 mRNA, whereas curcumin (10 μmol/L) had only a minor effect on COX-2 mRNA expression. The two drugs significantly down-regulated COX-2 mRNA expression.

We then investigated whether COX-2 inhibition is required for growth inhibition. PGE2 concentration was evaluated in HT-29 cells as a measure of COX-2 activity. Treatment with low concentrations of celecoxib (5 μmol/L) resulted in 80% inhibition of PGE2 production. Curcumin (10-15 μmol/L) had a dose-dependent effect on PGE2 synthesis. The addition of curcumin to celecoxib almost totally (>95%) diminished PGE2 synthesis (Fig. 5B).

Combination therapy did not alter cyclooxygenase-1 level. Western blot analysis showed that the level of COX-1 was not altered by treatment with celecoxib, curcumin, or their combination (Fig. 6A).

Celecoxib does not augment curcumin antioxidative properties. The antioxidative properties of celecoxib and curcumin were assessed using the ex vivo procedure as described in Materials and Methods. Curcumin was found to be a very potent antioxidant even at low (5 μmol/L) concentrations, whereas celecoxib had no antioxidative activity (Fig. 6). Furthermore, the addition of celecoxib did not alter the antioxidative properties of curcumin (Fig. 6).

Discussion

The phytochemical curcumin synergistically augments the growth inhibitory effect of celecoxib in human colon cancer cell lines in vitro. This observed synergistic effect may be clinically important, as it can be achieved in the serum of patients receiving standard anti-inflammatory doses of celecoxib.

One of the lessons learned from cancer research in recent years is that combinatorial strategies in cancer therapy can provide dramatic improvement in safety and efficacy over monotherapy regimens, especially if the drugs differ in their mode of action. Several combinations of nonsteroidal anti-inflammatory drugs with other chemopreventive drugs have previously been investigated (26, 27). For instance, it was found that green tea enhances the effect of sulindac, a classic nonsteroidal anti-inflammatory drug that inhibits the activities of both COX-1 and COX-2 isoenzymes (28).

Curcumin is commonly consumed (as turmeric spice) at high quantities (up to 100 mg/d) in certain countries. Moreover, a recent clinical trial showed that curcumin is not toxic even at very high doses of 8 g/d (29). Therefore, the
development of a therapeutic regimen that includes a natural herb that has a very low profile of side effects, in combination with a low dose of a selective COX-2 inhibitor that does not suppress COX-1 activity, is a major step forward. Because the combination of celecoxib and curcumin did not alter COX-1 protein levels, it is reasonable to assume that this therapeutic approach may be devoid of the side effects associated with traditional nonsteroidal anti-inflammatory drugs, the new COX-2 inhibitors, or even the recently reported cardiovascular toxicity of these agents.

Aggarwal et al. (30) reported a similar findings in HT 29 cells; however, in their study, very high and potentially toxic dosages of curcumin (50 μmol/L) and SC236 (75 μmol/L), a COX-2 inhibitor that is structurally analogous to celecoxib, were used.

Our current results show that in the presence of low concentrations of curcumin (10-15 μmol/L), a physiologic concentration of celecoxib (5 μmol/L) is sufficient to inhibit cell growth by inhibiting proliferation and inducing apoptosis through the COX-2 and non–COX-2 pathways. This effect is similar to that achieved with a 10-fold higher concentration of celecoxib (50 μmol/L) when administered alone. The clinical importance of this effect lies in the fact that it can be achieved in the serum of patients treated with standard anti-inflammatory (200-400 mg) or antineoplastic (400-800 mg) doses of celecoxib (31). This may pave the way for a novel strategy to prevent and treat colorectal cancer, given that this approach will involve a regimen with a low profile of side effects.

The synergistic effect was seen in HT-29 and IEC18-K-ras cells that express high levels of COX-2. Only the combined modality regimen reduced the level of COX-2 mRNA and almost entirely diminished PGE2 production. At the same time, a significant additive growth inhibition was seen in colorectal cancer cell lines that expressed low or no COX-2 activity (e.g., Caco-2 and SW-480). Moreover, rofecoxib, up to 20 μmol/L, did not have any additive growth inhibitory effect when added to curcumin. This suggests that the combination effect in inhibiting cell growth may involve the non–COX-2 pathway. Interestingly, recent studies have suggested several non–COX-2 pathways that are targeted by both curcumin and celecoxib: Akt (32, 33), nuclear factor κB (34, 35), cyclin D1 (36, 37), and nitric oxide synthase (38, 39). Additional experiments will be required to identify the precise underlying mechanism.

Curcumin can effectively scavenge oxygen free radicals that play an important role in carcinogenesis (40). In the current study, we showed that the synergistic effect is not attributed to the potentiation of the antioxidant effect of curcumin by celecoxib (Fig. 6).

In summary, curcumin synergistically augments the growth inhibitory effect of celecoxib. The underlying mechanism most probably involves down-regulation of COX-2 mRNA expression. This synergistic effect is clinically important because it can be achieved in the serum of patients receiving standard anti-inflammatory or antineoplastic dosages of celecoxib.

References


Cancer Therapy: Preclinical
Celecoxib and Curcumin Synergistically Inhibit the Growth of Colorectal Cancer Cells
