

Inhibition of 5-lipoxygenase by MK886 augments the antitumor activity of celecoxib in human colon cancer cells

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Abstract

Cyclooxygenase (COX)-2 and 5-lipoxygenase (5-LOX) are key enzymes involved in arachidonic acid metabolism. Their products, prostaglandins and leukotrienes, are involved in colorectal tumor development. We aimed at evaluating whether combined blocking of the COX-2 and 5-LOX pathways might have additive antitumor effects in colorectal cancer. The expression/activity of COX-2 and 5-LOX were assessed in 24 human colorectal cancer specimens. The effects of the COX-2 inhibitor celecoxib and the 5-LOX inhibitor MK886 on prostaglandin E₂ and cysteinyl leukotriene production, tumor cell proliferation, cell apoptosis, and Bcl-2/Bax expression were evaluated in the Caco-2 and HT29 colon cancer cells. We also investigated the effect of the enzymatic inhibition on mitochondrial membrane depolarization, one of the most important mechanisms involved in ceramide-induced apoptosis. Up-regulation of the COX-2 and 5-LOX pathways was found in the tumor tissue in comparison with normal colon mucosa. Inhibition of either COX-2 or 5-LOX alone resulted in activation of the other pathway in colon cancer cells. Combined treatment with 10 μmol/L celecoxib and MK886 could prevent this activation and had additive effects on inhibiting tumor cell proliferation, inducing cell apoptosis, decreasing Bcl-2 expression, increasing Bax

expression, and determining mitochondrial depolarization in comparison with treatment with either inhibitor alone. The administration of the ceramide synthase inhibitor fumonisin B1 could prevent some of these antineoplastic effects. In conclusion, our study showed that inhibition of 5-LOX by MK886 could augment the antitumor activity of celecoxib in human colorectal cancer. [Mol Cancer Ther 2006;5(11):2716–26]

Introduction

Cyclooxygenase (COX)-2 and 5-lipoxygenase (5-LOX) are key enzymes involved in arachidonic acid metabolism, leading to important bioactive fatty acids known as eicosanoids (1). These arachidonic acid metabolites (i.e., prostaglandins and leukotrienes) are well characterized pathophysiologically in the inflammation process and allergic diseases (2, 3). However, over the past decade, a large body of experimental and preclinical evidence has pointed to arachidonic acid derivatives as important mediators of tumor development (4–6).

COX-2 is an immediate-to-early response gene undetectable in most normal tissues but promptly induced by proinflammatory and mitogenic stimuli in inflamed and neoplastic tissues (7). An increase in COX-2 mRNA levels and protein expression has been shown in both adenomas and adenocarcinomas of the large bowel (8–11). Consistent with this finding, increased levels of prostaglandin E₂ (PGE₂), the predominant prostaglandin produced by the COX-2 pathway, have been found in colorectal tumors when compared with normal colonic epithelium (9, 12, 13). These experimental data have pointed to COX-2 as a potential therapeutic target against colorectal cancer and selective COX-2 inhibitors are currently being assessed for use as a new class of antineoplastic drug (14, 15). Nevertheless, the specific downstream cellular mechanisms of action of these molecules are not well known. Several *in vitro* and *in vivo* studies have shown that selective COX-2 inhibitors such as celecoxib, rofecoxib, and nimesulide, can induce apoptosis, decrease cell proliferation, and inhibit tumor angiogenesis (reviewed in ref. 16). The inhibition of COX activity and thus reduction in tissue concentrations of prostaglandins seem to play a major role in the antitumor efficacy of these molecules. However, it has been suggested that the tumor-suppressive effect of nonsteroidal anti-inflammatory drugs may be related not only to a reduction in prostaglandin synthesis but also to elevation of the prostaglandin precursor arachidonic acid that in turn stimulates the conversion of sphingomyelin to ceramide, a known mediator of apoptosis (17). It has been shown that ceramide-induced apoptosis is mainly due to its ability to produce a collapse of the mitochondrial

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membrane electrochemical potential ($\Delta\psi$) with a consequent increase in mitochondrial permeability (18).

5-LOX metabolites, such as 5-hydroeicosatetraenoic acid, LTB_4 , and cysteinyl leukotrienes (CysLT), are important proinflammatory mediators, which exert effects on several cellular functions, including smooth muscle contraction, bronchial mucus production, and chemotaxis (19). Recently, it has been shown that even these products may contribute to the development of several human tumors, such as pancreatic (20), esophageal (21), and colon cancers (22). In particular, Öhd et al. (23) have shown that the cysteinyl LTD_4 can increase the survival of both non-transformed intestinal epithelial cells and colon cancer cells via activation of its membrane receptor [i.e., the CysLT₁ receptor (CysLT₁R)].

COX-2 and 5-LOX have been reported to be simultaneously up-regulated in colorectal cancer (22, 24). Because these two arachidonic acid-metabolizing enzymes are so closely related in mechanisms of action and substrate, it is likely that blocking one enzymatic pathway may activate the other. In particular, the question is whether the administration of COX-2 inhibitors may lead to a shunt of the arachidonic acid metabolism toward the 5-LOX pathway with an increase in tumorigenic leukotriene production. This phenomenon might explain, at least in part, the observed limited efficacy of COX-2 inhibitors as anticancer agents at doses that exert the only enzymatic inhibition activity (i.e., lower than 30–40 $\mu\text{mol/L}$; refs. 25–30). At higher concentrations, several authors have suggested that mechanisms independent of COX-2 inhibition may be involved in their antitumor effect (30, 31).

The aim of this study was to assess whether the dual inhibition of the COX-2 and 5-LOX pathways in the two human colon cancer cell lines HT29 and Caco-2 might determine an additive effect on inhibition of tumor cell proliferation and induction of apoptosis. In particular, we focused on the possible role of ceramide and the apoptosis-regulating protein Bcl-2 and Bax in mediating the antitumor effect of combination treatment with the COX-2 inhibitor celecoxib and the 5-LOX inhibitor MK886.

Materials and Methods

Patients and Tissue Collection

Tissue samples were obtained from 24 patients (18 males and 6 females; median age, 68.5 years; age range, 58–77 years) who had undergone surgical resections for primary sporadic colorectal adenocarcinoma at the Department of General Surgery, University of Florence (Italy, Florence) between 2002 and 2004. All patients were thoroughly informed about the aims of the study and gave written consent for the investigation in accordance with the ethical guidelines of our University. None of the patients had taken nonsteroidal anti-inflammatory drugs for at least 3 months before surgery. Tumor distribution was as follows: 10 (41.6%) in the proximal colon (up to the splenic flexure), 7 (29.2%) in the distal colon (up to the end of the sigmoid colon), and 7 (29.2%) in the rectum. All tumors were adenocarcinomas; none had any colloid component.

Tumors were classified into four stages according to the American Joint Committee on Cancer staging system (32): stage I (T_1 - T_2 , N_0 , and M_0 ; $n = 3$), stage II (T_3 - T_4 , N_0 , and M_0 ; $n = 14$), stage III (any T , N_{1-2} , and M_0 ; $n = 4$), and stage IV (any T , any N , and M_1 ; $n = 3$).

Cancer tissue (from the edge of the tumor) and adjacent normal mucosa (at least 10 cm from the tumor) were excised from each surgical specimen. The samples were washed in PBS. They were frozen at -80°C for Western blot analysis and frozen at -20°C for PGE₂ and CysLT production evaluation until processing. Other samples were fixed in 4% formaldehyde and embedded in paraffin for immunohistochemical analysis.

Cell Culture and Drugs

Experiments were done on the Caco-2, HT29, LoVo, and HCT116 human colon cancer cell lines and the COS-7 cells (African green monkey kidney cells). The HT29, LoVo, and HCT cells were a gift from Dr. Claudia Casini Raggi (Department of Clinical Physiopathology, University of Florence). The Caco-2 and COS-7 cells were purchased from Interlab Cell Line Collection (Genoa, Italy). The cells were cultured as described previously (10). The COX-2 inhibitors celecoxib and rofecoxib were provided by Pfizer (Milan, Italy) and Merck (Rome, Italy), respectively. The COX-2 inhibitor nimesulide was purchased from Alexis Biochemical (San Diego, CA). The 5-LOX-activating protein inhibitor MK886, the CysLT₁R antagonist LY171883, the ceramide synthase inhibitor fumonisin B1 (FB1), and the epidermal growth factor (EGF) were purchased from Cayman Chemical Co. (Ann Arbor, MI).

Immunohistochemistry

Four-micron-thick sections were cut from formalin-fixed and paraffin-embedded tissue blocks. They were mounted on poly-L-lysine-coated slides, dewaxed in xylene, and rehydrated through a graded series of ethanol. After deparaffinization, the sections were treated with 3% hydrogen peroxide in methanol solution for 20 minutes to block endogenous peroxidase activity. Antigen retrieval was done for 5-LOX antigen by incubating the sections in 10 mmol/L citrate buffer (pH 6) in a microwave oven for 5 minutes, whereas for COX-2, antigen retrieval was carried out by heating in EDTA buffer (pH 8) in a microwave oven for 5 minutes. Thereafter, slices were cooled down to room temperature and then washed in PBS [0 and 1 mol/L (pH 7.4)]. The slides were then incubated with the primary antibody directed against 5-LOX or against COX-2 for 18 hours overnight at 4°C . The following antibodies were used: anti COX-2 (goat polyclonal; 1:150; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti 5-LOX (goat polyclonal; 1:50; Santa Cruz Biotechnology). The slices were then washed again in PBS and incubated with secondary antibody [rabbit anti-goat IgG horseradish peroxidase conjugated (Zymed Laboratories, San Francisco, CA)] for 10 minutes. Detection of the antibody complex was done by using the 3,3'-diaminobenzidine tetrahydrochloride-plus kit substrate for horseradish peroxidase (Zymed Laboratories). As a negative control for COX-2 and 5-LOX staining, tissue sections were treated with normal serum instead of

each primary antibody. Counterstaining was done with hematoxylin. The extent of COX-2 and 5-LOX staining was recorded using a three-grade system, based on the percentage of tumor epithelial cells stained: grade 0, 1% to 20%; grade 1, 21% to 70%; and grade 2, >70%.

PGE₂ and CysLT Measurement

Normal mucosa and tumor samples were processed as described previously (9). Supernatants of the Caco-2 and HT29 cells were prepared according to the method described previously (10). Drugs (10 μmol/L celecoxib or MK886) were added 30 minutes before PGE₂ and CysLT level determination. After 24 hours of incubation, the supernatants were collected and 500 μL supernatants of tissue homogenates and 100 μL supernatants of the cells were used for PGE₂ and CysLT level determination using enzyme immunoassay kits (Cayman Chemical). PGE₂ enzyme immunoassay detected both PGE₂ and PGE₂-ethanolamide but cannot distinguish between them. The immunoassay was sensitive to 15 pg/mL PGE₂/PGE₂-ethanolamide production. CysLT immunoassay detected LTC₄, LTD₄, and LTE₄. They are collectively referred to as CysLTs and their production evaluation can be considered as a reliable indicator of 5-LOX activity. Assays were done according to the manufacturer's instructions. Protein concentration in the tissue samples and in the cells was determined according to the method described by Lowry et al. (33). Bovine serum albumin was used as the standard. Determinations were done in quintuplicate. PGE₂ values were expressed as microgram per milligram protein in the tissue samples and picogram per microgram protein in the cells. CysLTs were expressed as nanogram per microgram protein in the tissue samples and picogram per microgram protein in the cells.

Cell Proliferation Assay

Caco-2 and HT29 cell proliferation was determined by the [³H]thymidine incorporation assay according to the method described previously (10). The experiments were done in quintuplicate and the values were expressed as disintegrations per minute per well.

Caspase-3 Activity Determination

The activity of caspase-3 was determined by use of a fluorescent substrate according to the method described by Stennicke and Salversen (34). The Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC; Bachem AG, Bubendorf, Switzerland) was used as a fluorescent substrate for caspase-3 (35). Briefly, the cells were seeded in six-well plates. After 24 hours, the cells were washed in a serum-free medium and then incubated with 1 mL medium containing 1% FCS in the absence or presence of test drugs for 24 hours at 37°C. The medium was then removed and the cells were scraped and homogenized with 10 mmol/L HEPES (pH 7.4) containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, 42 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 1 μg/mL pepstatin A. The homogenate was then centrifuged at 10,000 g for 10 minutes. The caspase-3 activity was determined in the supernatant by fluorimetric assay using the substrate, Ac-DEVD-AMC.

Supernatants containing 250 μg total protein were incubated with 40 μmol/L of the caspase-3 substrate Ac-DEVD-AMC for 60 minutes at 37°C. At the end of incubation, substrate cleavage was determined fluorimetrically (Spectrofluor JY3 D, Jobin Yvon, Paris, France) with a λ excitation at 380 nm and a λ emission at 460 nm. Determinations were done in quintuplicate. Data were expressed as arbitrary units per milligram proteins. One unit enzyme activity was defined as the amount of the enzyme required to release 40 micromoles Ac-DEVD-AMC for 60 minutes at 37°C.

Flow Cytometry

The cells were treated with test drugs as describe above. For determination of apoptosis, approximately 0.5 × 10⁶ cells per assay were washed in Annexin binding buffer containing 125 mmol/L NaCl, 10 mmol/L HEPES/NaOH (pH 7.4), and 5 mmol/L CaCl₂. The cells were then stained with Annexin V-FITC kit (Beckman Coulter, Inc., Miami, FL). After 15 minutes, samples were diluted 1:5 and measured by flow cytometric analysis on a Coulter XL flow cytometer (Coulter XL, Coulter Cytometry, Hialeah, FL). Cells were analyzed by forward and side scatter, and Annexin-fluorescence intensity was measured in FL-1. Determinations were done in triplicate and data were expressed as percentage of total cells counted.

For determination of ceramide, cells were stained for 1 hour at 4°C with 1 μg/mL anti-ceramide antibody (Alexis Biochemical) in PBS containing 1% FCS at a dilution of 1:5 after permeabilization with 0.1% saponin. After three washes with PBS/1% FCS, cells were stained with polyclonal FITC-conjugated goat anti-mouse immunoglobulin-specific antibody (PharMingen, Hamburg, Germany) in PBS/1% FCS at a dilution of 1:50 for 30 minutes. Unbound secondary antibody was removed by washing the cells twice with PBS/1% FCS, and samples were analyzed by flow cytometric analysis on a Coulter XL flow cytometer. FITC-fluorescence intensity was measured in FL-1. Determinations were done in triplicate and data were expressed as percentage of total cells counted.

Western Blot Analysis

Total proteins from tumor tissue and the corresponding normal mucosa were obtained as described previously (9). The Caco-2, HT29, HCT116, LoVo, and COS-7 cells were grown to subconfluence and starved for 24 hours in 0.1% FCS-supplemented medium. After incubation in the absence or presence of drugs for indicated times, cells were washed in PBS and lysed with radioimmunoprecipitation assay buffer [20 mmol/L Tris-HCl (pH 7.1), 150 mmol/L NaCl, 5 mmol/L EDTA, 2% SDS, 0.00125% bromophenol blue, 5% β-mercaptoethanol]. Total proteins (70 μg) as evaluated by using a bicinchoninic acid protein assay from tissue or cultured cells were subjected to Western blotting and immunoblotting analysis as described previously (10). The loading and transfer of equal amounts of proteins were ascertained by either reblotting the membrane with an anti-actin antibody or staining the membrane with Ponceau S. Primary antibodies used were the following: anti-COX-2 goat polyclonal antibody (1:1,000; Cayman Chemical), anti-LOX rabbit polyclonal antibody (1:1,000; Cayman

Chemical), anti-CysLTR₁ rabbit polyclonal antibody (1:1,000; Cayman Chemical), anti-Bcl-2 mouse monoclonal antibody (1:1,000; Santa Cruz Biotechnology), anti-Bax (1:1,000; Santa Cruz Biotechnology), and anti-actin goat polyclonal antibody (1:1,000; Santa Cruz Biotechnology). Binding of each primary antibody was determined by addition of suitable peroxidase-conjugated secondary antibodies [anti-mouse and anti-rabbit antibodies (1:5,000) and anti-goat antibody (1:10,000); Amersham, Braunschweig, Germany]. HCT116 cells do not constitutively express COX-2 (36) and served as COX-2-negative control. LoVo cells have been reported to express 5-LOX (27) and served as 5-LOX-positive control. COS-7 cells do not express CysLTR₁ (22) and served as CysLTR₁-negative control.

Detection of Change in Mitochondrial Transmembrane Potential ($\Delta\psi$)

The change in $\Delta\psi$ occurring during apoptosis was detected by fluorescence-based assay. The Caco-2 cells were cultured on coverslips in DMEM containing the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1, 5 mg/mL; Molecular Probes, Eugene, OR) for 15 minutes at 37°C. This dye has a unique feature: at hyperpolarized membrane potentials (to -140 mV), it forms a red fluorescent J aggregate, whereas at depolarized membrane potentials (to -100 mV), it remains in the green fluorescent monomeric form. Before detection, cells were washed in PBS and placed in an open slide-flow loading chamber that was mounted on the stage of a confocal scanning microscope equipped with an argon laser source and with an elio/neon laser source. The emitted fluorescence was monitored at 488 and 543 nm wavelengths with a Nikon Eclipse TE300 microscope and with a Nikon (Tokyo, Japan) S Flu oil immersion $\times 40$ objective. Image acquisition of the optical sections through the cell was done by a Ropers Scientific (Tucson, AZ) Cool Snap camera.

Statistical Analysis

PGE₂ and CysLT levels, incorporation of [³H]thymidine, caspase-3 activity, and flow cytometric determination of cell apoptosis and ceramide were expressed as mean \pm SE. Differences in PGE₂ and CysLT levels in tumor and normal mucosa specimens and in tumors with and without metastases were analyzed using the Mann-Whitney test. PGE₂ and CysLT levels, [³H]thymidine incorporation, caspase-3 activity, and flow cytometric determination of apoptosis and ceramide in the cells were compared using the paired-value Wilcoxon's test or the Mann-Whitney test, as appropriate. Statistical analysis was done using Stata Statistic software (release 5.0; Stata Corp., College Station, TX). All of the *P*s resulted from the use of two-sided statistical tests; *P*s < 0.05 were considered statistically significant.

Results

COX-2 and 5-LOX Pathways in Human Colorectal Cancer

PGE₂ and CysLT production was significantly higher in the cancer specimens than in the normal mucosa (Fig. 1A and B). These variables were also related to the tumor stage: they were higher in tumors with lymph node or

distant metastases (stage III and IV) than in those tumors without metastases (stage I and II; Fig. 1A and B). Most of the tumors showed extensive immunostaining for COX-2: 7 (29.2%) tumors were grade 0, 11 (45.8%) were grade 1, and 6 (25.0%) were grade 2. COX-2 was found mainly in the tumor epithelial cells (Fig. 1C). It was also occasionally expressed by tumor-infiltrating inflammatory cells and endothelial cells. Normal colon epithelium was negative for COX-2 staining (Fig. 1C). Western blotting confirmed the up-regulation of COX-2 protein expression in 22 (91.6%) cancer specimens when compared with the corresponding normal mucosa (Fig. 1D). Even 5-LOX immunostaining was found in most of the tumors samples: 8 (33.3%) tumors were grade 0, 14 (58.3%) were grade 1, and 2 (8.4%) were grade 2. 5-LOX was observed mainly in the cytoplasm of the tumor epithelial cells (Fig. 1C). Normal colon epithelium was negative for 5-LOX expression (Fig. 1C). However, there was some staining of the nonepithelial interstitial cells (most frequently neutrophilic cells) of normal mucosa. Western blot analysis confirmed the overexpression of 5-LOX protein that had been detected by immunohistochemistry. A higher amount of protein was found in 20 (83.3%) neoplastic tissue specimens when compared with the corresponding normal mucosa (Fig. 1D). Tumor epithelial cells expressing COX-2 stained positive for 5-LOX in the majority of the examined tumor sections. These immunohistochemical findings were confirmed by Western blot analysis: the majority of the carcinomas that showed up-regulation of the COX-2 protein also had high levels of the 5-LOX protein (Fig. 1D).

Effects of Celecoxib and MK886 on PGE₂, CysLT, and Ceramide Production

Both the Caco-2 and the HT29 colon cancer cells constitutively express COX-2 and 5-LOX (Fig. 1E). The administration of 10 μ mol/L celecoxib to the Caco-2 and the HT29 cells produced a significant decrease in PGE₂ production, whereas CysLT levels increased by $\sim 100\%$ in both cell lines (Fig. 2A and B). The administration of 10 μ mol/L MK886 reduced the basal levels of CysLTs and induced a significant increase in PGE₂ production in the two cell lines (Fig. 2A and B). Combined treatment with celecoxib and MK886 could prevent both the celecoxib-induced increase in CysLT production and the MK886-induced increase in PGE₂ production in the two cell lines (Fig. 2A and B). The administration of 10 μ mol/L celecoxib or MK886 did not affect either COX-2 or 5-LOX protein expression in both cell lines (data not shown). Treatment with 10 μ mol/L celecoxib, MK886, or FB1 alone did not affect ceramide formation in the two cell lines in comparison with untreated cells (data not shown). The simultaneous administration of celecoxib and MK886 determined a significant increase in ceramide levels (Fig. 3A and B) and this effect was prevented by the administration of FB1, a ceramide synthase inhibitor (Fig. 3A and B).

Effects of Celecoxib, MK886, LY171883, and FB1 on Cell Proliferation

We found higher protein levels of the LTD₄ receptor CysLT₁ in 19 (79.1%) tumor specimens than in the

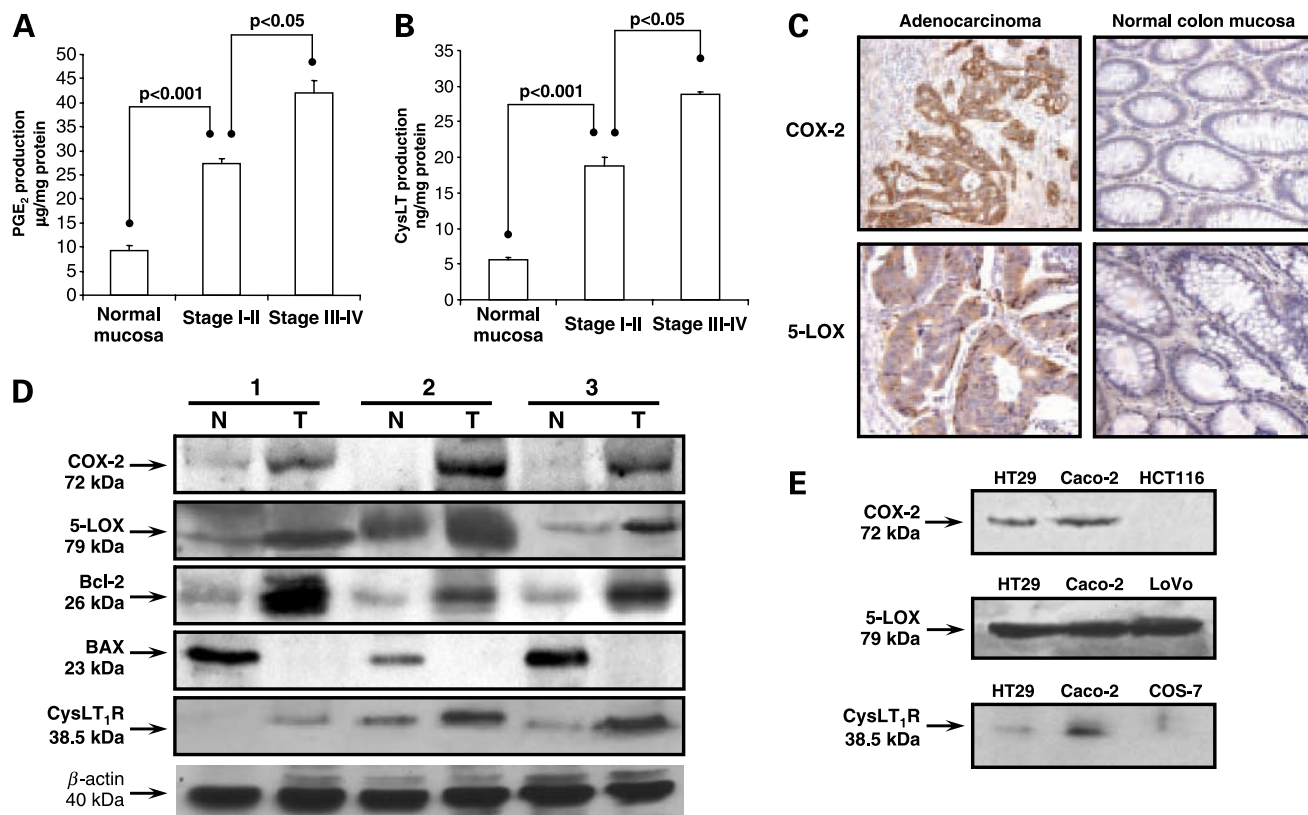


Figure 1. **A** and **B**, PGE₂ and CysLT levels, respectively, in 24 normal mucosa specimens, 17 nonmetastatic (stage I and II) tumors, and 7 metastatic (stage III and IV) tumors. *Columns*, mean of five determinations; *bars*, SE. **C**, COX-2 and 5-LOX immunohistochemical staining. Expression of COX-2 (grade 2) and 5-LOX (grade 2) in two representative paired human cancer specimens and adjacent normal colon mucosa. Hematoxylin counterstain. Original magnification, $\times 200$. **D**, Western blot analysis. Expression of COX-2, 5-LOX, Bcl-2, Bax, and CysLT₁R in representative paired adenocarcinoma and adjacent normal mucosa from three patients. Samples were normalized for protein loading (70 μ g) by reblotting the membrane-bound protein with an anti-actin antibody. *N*, normal mucosa; *T*, tumor; *kDa*, kilodalton. **E**, Western blot analysis. Expression of COX-2, 5-LOX, and CysLT₁R in the HT29 and Caco-2 colon cancer cells. Samples were normalized for protein loading (70 μ g) by Ponceau S staining of the membrane-bound protein. HCT116 and COS-7 served as COX-2- and CysLT₁R-negative controls, respectively. LoVo served as 5-LOX-positive control.

corresponding normal mucosa (Fig. 1D). Moreover, CysLT₁R was found to be constitutively expressed in the Caco-2 and HT29 cells (Fig. 1E). These findings suggested a possible role of LTD₄ in colorectal carcinogenesis. Therefore, we examined the effect of celecoxib, MK886, and the CysLT₁R antagonist LY171883 on the proliferation of the Caco-2 and HT29 cells. Basal proliferation was very low in both cell lines and was not significantly affected by the administration of 10 μ mol/L celecoxib, MK886, LY171883, or FB1 (Fig. 4). Thus, cells were stimulated with 10 ng/mL EGF. This treatment determined a significant increase in cell proliferation in both cell lines (Fig. 4). The EGF-stimulated cell proliferation was decreased by the administration of 10 μ mol/L celecoxib, MK886, or LY171883, whereas FB1 at 10 μ mol/L determined an increase in cell growth (Fig. 4). The dual inhibition of COX-2 and 5-LOX by celecoxib and MK886 caused a significant additive effect in reducing EGF-stimulated cell growth in the Caco-2 and HT29 cells (Fig. 4). Even combined treatment with celecoxib and LY171883 produced a greater reduction in cell growth after EGF stimulation than the individual drugs (Fig. 4).

The administration of FB1 prevented cell growth inhibition induced by the combined treatment with celecoxib and MK886 (Fig. 4).

Effects of Celecoxib, MK886, LY171883, and FB1 on Cell Apoptosis

We first evaluated whether celecoxib, MK886, or LY171883 was involved in inducing the early events of the apoptotic process (i.e., caspase-3 activation). Caspase-3 is activated once cytochrome *c* is released from the mitochondria and caspase-9 is activated as a consequence of an apoptotic stimulus. Treatment of both Caco-2 and HT29 cells with 10 μ mol/L celecoxib, MK886, LY171883, or FB1 did not significantly affect caspase-3 activation in comparison with untreated cells (Fig. 5). When celecoxib and MK886 were combined, caspase-3 activation increased by $\sim 100\%$ in comparison with treatment with either inhibitor alone (Fig. 5). Similar results were obtained when celecoxib was administered in combination with LY171883 (Fig. 5). The administration of FB1 prevented caspase-3 activation induced by combined treatment with celecoxib and MK886 (Fig. 5). We found similar results on caspase-3

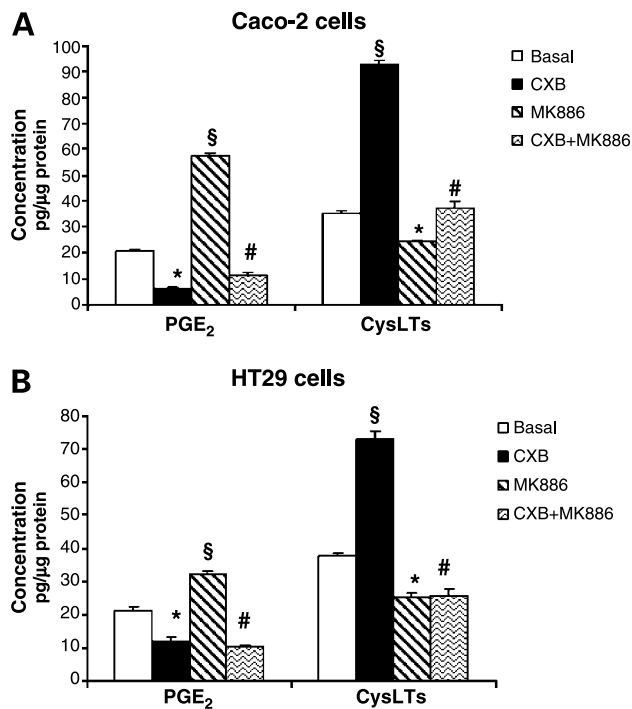


Figure 2. A and B, variations in PGE₂ and CysLT concentration after treatment with 10 μmol/L celecoxib (CXB), MK886, or celecoxib + MK886 in the Caco-2 and HT29 cells, respectively. Columns, mean of five determinations; bars, SE. *, significant decrease compared with basal condition ($P < 0.05$); §, significant increase compared with basal condition ($P < 0.05$); #, significant decrease compared with celecoxib or MK886 treatment ($P < 0.05$).

activation when MK886 was administered in combination with two other types of COX-2 inhibitor (i.e., rofecoxib and nimesulide; Table 1).

Flow cytometric detection of cell apoptosis confirmed our findings on caspase-3 activation. No apoptotic effects were observed after the administration of 10 μmol/L celecoxib, MK886, LY171883, or FB1 alone (Table 2). When celecoxib was combined with MK886 or LY171883, we found a significant increase in the number of apoptotic cells (Table 2). This effect was prevented by the administration of FB1 (Table 2).

Effects of Celecoxib, MK886, and LY171883 on Bcl-2 and Bax Expression

The Bcl-2 protein family plays a pivotal role in the regulation of apoptosis. Their major site of action is on the regulation of cytochrome *c* release from the mitochondria into the cytosol. Western blot analysis showed significantly higher levels of the antiapoptotic protein Bcl-2 in 23 (95.8%) cancer specimens than in the corresponding normal mucosa (Fig. 1D). On the contrary, the expression of the proapoptotic protein Bax was lower in 20 (83.3%) tumor specimens than in normal mucosa (Fig. 1D). Therefore, we evaluated the effects of COX-2 and 5-LOX inhibitor treatment on the expression of these two apoptosis-related proteins. The administration of 10 μmol/L celecoxib or

MK886 alone did not affect Bcl-2 levels in the Caco-2 and HT29 cells (Fig. 6A). Dual treatment with celecoxib and MK886 determined a decrease in Bcl-2 protein level in both cell lines (Fig. 6A). Likewise, treatment with 10 μmol/L celecoxib or MK886 alone had no effects on Bax expression in the two cell lines (Fig. 6B), whereas combined treatment with the two inhibitors produced a significant increase in Bax levels (Fig. 6B).

The administration of 10 μmol/L LY171883 alone did not affect Bcl-2 or Bax expression in the two cell lines (Fig. 6A and B), whereas combination treatment with 10 μmol/L celecoxib determined a decrease in Bcl-2 expression and an increase in Bax levels (Fig. 6A and B).

Effects of Celecoxib, MK886, and FB1 on Mitochondrial Depolarization

Mitochondria are believed to be the targets in ceramide-mediated apoptosis. Ceramide can produce a collapse of $\Delta\psi$, thus allowing the release of cytochrome *c* into the cytosol. Therefore, we investigated the effects of celecoxib, MK886, and FB1 on mitochondrial membrane charge in the Caco-2 cells through the quantification of JC-1 uptake. The shift in membrane charge was observed as disappearance of fluorescent red-orange-stained mitochondria (large negative $\Delta\psi$) and an increase in fluorescent green-stained

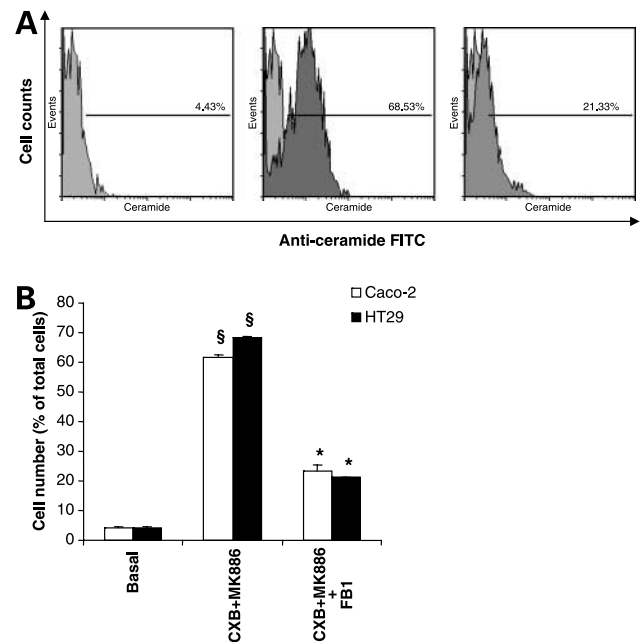


Figure 3. Variations in ceramide formation after combined treatment with 10 μmol/L celecoxib + MK886 or celecoxib + MK886 + FB1. A, representative histograms of ceramide detection by flow cytometry in the Caco-2 cells: basal condition (left, light gray), cells treated with celecoxib + MK886 (middle, dark gray), and cells treated with celecoxib + MK886 + FB1 (right, gray). B, columns, mean of three determinations of ceramide-positive cells given in percentage of total cells; bars, SE. Treatment of the Caco-2 and HT29 cells with celecoxib, MK886, or FB1 alone did not produce a significant increase in ceramide production when compared with untreated cells (data not shown). §, significant increase compared with basal condition ($P < 0.05$); *, significant decrease compared with treatment with celecoxib + MK886 ($P < 0.05$).

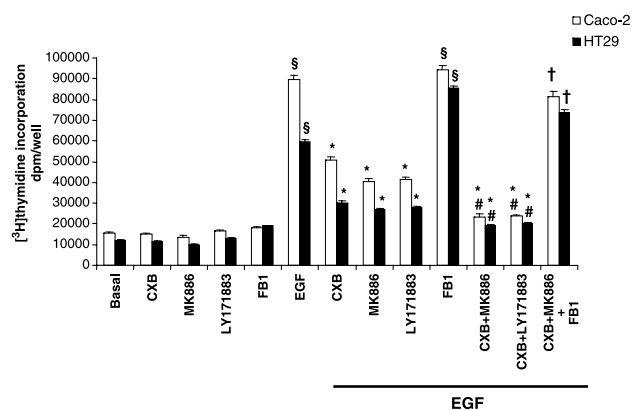


Figure 4. Effects of 10 $\mu\text{mol/L}$ celecoxib, MK886, LY17883, FB1, celecoxib + MK886, celecoxib + LY171883, and celecoxib + MK886 + FB1 on tumor cell proliferation at basal condition and after stimulation with 10 ng/mL EGF. Columns, mean of five different experiments; bars, SE. §, significant increase compared with either unstimulated cells or cell stimulation with EGF ($P < 0.05$); *, significant decrease compared with cell stimulation with EGF ($P < 0.05$); #, significant decrease compared with treatment with celecoxib, MK886, or LY171883 alone ($P < 0.05$); †, significant increase compared with treatment with celecoxib + MK886 ($P < 0.05$).

mitochondria (loss of $\Delta\psi$). Treatment of the Caco-2 cells with 10 $\mu\text{mol/L}$ celecoxib or MK886 alone did not produce a significant mitochondrial membrane depolarization in comparison with untreated cells (Fig. 7). Following combined administration of celecoxib and MK886, most cells underwent mitochondrial membrane depolarization: indeed, cells with red-orange-stained mitochondria dropped to $\sim 10\%$ of total cells (Fig. 7). Treatment with FB1 before COX-2 and 5-LOX inhibitor administration significantly prevented mitochondrial membrane depolarization because $>60\%$ of cells examined maintained red-orange-stained mitochondria (Fig. 7).

Discussion

Our immunohistochemical and Western blot results clearly showed that the two major metabolic pathways of arachidonic acid (i.e., the COX-2 and the 5-LOX ones) are simultaneously up-regulated in human colorectal cancer. We also showed that both PGE₂ and CysLT levels were significantly higher in the tumor tissue than in the normal colon mucosa. Likewise, they were higher in the metastatic tumors (stage III and IV) than in the nonmetastatic ones (stage I and II). These findings confirm the datum that prostaglandins and leukotrienes are involved in colorectal carcinogenesis and, more interestingly, suggest the hypothesis that these products may regulate each other in this process. Because both selective COX-2 and 5-LOX inhibitors are being currently assessed as potential anticancer drugs, the simultaneous up-regulation of the two enzymes in colorectal cancer cells raises the question of whether blocking one pathway may result in an amplification of the other through a shunting of arachidonic acid. Previous studies on experimental models of inflammation (37, 38)

have shown that genetic knockout of 5-LOX or COX-1/COX-2 resulted in activation of the other pathway. Moreover, chemical inhibitors of one pathway may also activate other arachidonic acid-metabolizing pathways (39). Recently, Ye et al. (24) have shown that inhibition of COX-2 may lead to a shunt of arachidonic acid metabolism toward the leukotriene pathway during colonic tumorigenesis promoted by cigarette smoke, whereas suppression of 5-LOX did not induce such a shunt. In the present study, we evaluated the synthesis of PGE₂ and CysLTs in the Caco-2 and HT29 cells after treatment with either MK886 or celecoxib, respectively. We found that inhibition of either 5-LOX or COX-2 led to a shunt of arachidonic acid metabolism toward the other active enzymatic pathway. More interestingly, we found that simultaneous inhibition of the COX-2 and 5-LOX pathways could prevent both the MK886-mediated increase in PGE₂ production and the celecoxib-mediated increase in CysLTs levels in the two cell lines. Therefore, dual blocking of COX-2 and 5-LOX in cancers coexpressing these two enzymes might improve the potential suppressive effects of their enzymatic inhibitors on tumor cell survival.

Inhibition of cell proliferation and promotion of apoptosis are two of the most important mechanisms underlying the antineoplastic effect of nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors. Celecoxib concentrations required to inhibit tumor cell growth in previously published studies (25–30) were in the range of 40 to 100 $\mu\text{mol/L}$. Recently, Maier et al. (30) have shown that activation of caspase-3/caspase-9 and release of cytochrome *c* occurred only after the addition of celecoxib at concentrations between 80 and 100 $\mu\text{mol/L}$ in the Caco-2 cells. Concentrations of celecoxib ≤ 10 $\mu\text{mol/L}$ have been shown to have no effect on either cell growth or death

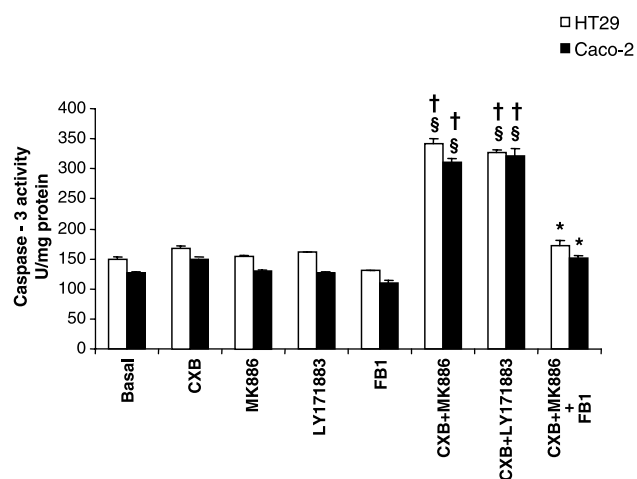


Figure 5. Effects of 10 $\mu\text{mol/L}$ celecoxib, MK886, LY17883, FB1, celecoxib + MK886, celecoxib + LY171883, or celecoxib + MK886 + FB1 on activation of caspase-3. Columns, mean of five different experiments; bars, SE. §, significant increase compared with basal condition ($P < 0.05$); †, significant increase compared with treatment with celecoxib, MK886, or LY171883 alone ($P < 0.05$); *, significant decrease compared with treatment with celecoxib + MK886 ($P < 0.05$).

Table 1. Effects of combined treatment with rofecoxib/nimesulide and MK886, LY171883, or FB1 on caspase-3 activity in the HT29 and Caco-2 colon cancer cells

Drugs	Caspase-3 activity (units/mg protein)	
	HT29 cells	Caco-2 cells
None	149.9 ± 2.7	110.3 ± 2.0
Rofecoxib (10 μmol/L)	128.8 ± 8.1	123.1 ± 5.3
Nimesulide (10 μmol/L)	141.6 ± 4.0	128.7 ± 1.1
MK886 (10 μmol/L)	172.8 ± 1.9	142.7 ± 2.8
LY171883 (10 μmol/L)	160.7 ± 1.3	125.8 ± 3.2
FB1 (10 μmol/L)	129.6 ± 1.7	109.9 ± 3.4
Rofecoxib + MK886	309.3 ± 9.2* [†]	330.0 ± 6.2* [†]
Nimesulide + MK886	319.1 ± 12.4* [†]	334.2 ± 4.4* [†]
Rofecoxib + LY171883	307.7 ± 3.4* [†]	297.4 ± 5.0* [†]
Nimesulide + LY171883	304.4 ± 2.1* [†]	300.8 ± 6.3* [†]
Rofecoxib + MK886 + FB1	140.1 ± 1.1 [‡]	147.8 ± 4.9 [‡]
Nimesulide + MK886 + FB1	144.6 ± 5.0 [‡]	147.3 ± 3.2 [‡]

*Significant increase compared with basal condition ($P < 0.05$).

[†]Significant increase compared with treatment with rofecoxib, nimesulide, MK886, or LY171883 alone ($P < 0.05$).

[‡]Significant decrease compared with treatment with rofecoxib/nimesulide + MK886 ($P < 0.05$).

in vitro (29, 40), although they are effective in fully inhibiting COX-2 enzymatic activity and thus prostaglandin synthesis. Some authors have explained this phenomenon by showing that not only COX-dependent mechanisms but also COX-independent mechanisms exist for the antiproliferative and proapoptotic actions of COX-2 inhibitors (41). These COX-2-independent mechanisms mainly become apparent at high celecoxib concentrations (31). In the present study, we addressed the hypothesis that concomitant activation of the 5-LOX pathway during COX-2 inhibition may explain, at least in part, the limited efficacy of celecoxib as an anticancer agent at low concentrations. We first evaluated the effects of celecoxib and MK886 on inhibition of tumor cell proliferation. Dual treatment with these enzymatic inhibitors determined a greater effect in inhibiting EGF-induced cell growth than treatment with either inhibitor alone. Then, we focused on the effects of COX-2/5-LOX inhibitors on induction of apoptosis. In agreement with previously published data (30), we found that the administration of 10 μmol/L celecoxib did not have any significant effect on caspase-3 activation and apoptosis. Even 10 μmol/L MK886 were ineffective to activate caspase-3 and induce cell death. This finding was in contrast with previously reported results, which showed an apoptotic effect of MK886 at concentrations >5 μmol/L in prostate cancer cells (42) and at the concentration of 10 μmol/L in the human colon cancer cell COLO205 (27). These differences may be explained by the different type of tumor cell tested in the first study and by the fact that the COLO205 colon cancer cells express 5-LOX but not COX-2 in the second study. The cell lines used in our study constitutively expressed both COX-2 and 5-LOX. Interestingly, combined treatment of both Caco-2 and HT29 cells

with celecoxib and MK886 produced a superadditive proapoptotic effect with an increase in caspase-3 activation by ~100% and an increase in the number of apoptotic cells by ~5-fold in comparison with treatment with either inhibitor alone. We obtained similar results on caspase-3 activation even through the combined treatment of the two cell lines with MK866 and two other COX-2 inhibitors (i.e., rofecoxib and nimesulide). These findings suggest that MK886 acts independently of the type of COX-2 inhibitors used and provide further evidence that the reduced proapoptotic activity of celecoxib at low doses is mainly due to the concomitant activation of the 5-LOX pathway during COX-2 inhibition.

Three recent studies have shown previously a cooperative effect of COX-2 and 5-LOX inhibitors in suppressing colon, pancreatic, and esophageal carcinogenesis in animal models (24, 43, 44). However, the precise molecular mechanisms underlying this antitumor effect have not been investigated. In the present study, we aimed at identifying which apoptosis-related protein(s) was involved in the increased induction of apoptosis following combined inhibitory treatment. Bcl-2 inhibition has been reported as one of the most important mechanisms underlying the apoptotic effect of both COX-2 and 5-LOX inhibitors (26, 29, 45–47). According to caspase-3 activation results, we showed that either celecoxib or MK886 alone at the dose of 10 μmol/L did not affect Bcl-2 expression in the Caco-2 and HT29 cells. On the contrary, combined treatment with the two inhibitors determined a marked decrease in Bcl-2 levels. These findings may be explained by the fact that celecoxib and MK886 can contemporarily reduce the production of PGE₂ and LTD₄, these eicosanoids being able to directly up-regulate Bcl-2 expression (23, 45). We also investigated the possible involvement of Bax. Even this proapoptotic protein has been reported to mediate the

Table 2. Effects of CXB, MK886, LY171883, FB1, CXB + MK886, CXB + LY171883, or CXB + MK886 + FB1 on HT29 and Caco-2 cell apoptosis as determined by Annexin V test using flow cytometry

Drugs	Apoptotic cells (% of total)	
	HT29 cells	Caco-2 cells
None	2.3 ± 1.2	3.0 ± 0.3
CXB (10 μmol/L)	7.7 ± 1.3	6.7 ± 0.5
MK886 (10 μmol/L)	4.0 ± 2.4	6.2 ± 0.3
LY171883 (10 μmol/L)	4.6 ± 1.8	6.6 ± 0.6
FB1 (10 μmol/L)	3.1 ± 0.3	2.2 ± 1.1
CXB + MK886	34.7 ± 1.5* [†]	34.1 ± 0.1* [†]
CXB + LY171883	33.6 ± 0.4* [†]	35.1 ± 1.0* [†]
CXB + MK886 + FB1	8.6 ± 1.9 [‡]	3.5 ± 2.3 [‡]

*Significant increase compared with basal condition ($P < 0.05$).

[†]Significant increase compared with treatment with CXB, MK886, or LY171883 alone ($P < 0.05$).

[‡]Significant decrease compared with treatment with CXB + MK886 ($P < 0.05$).

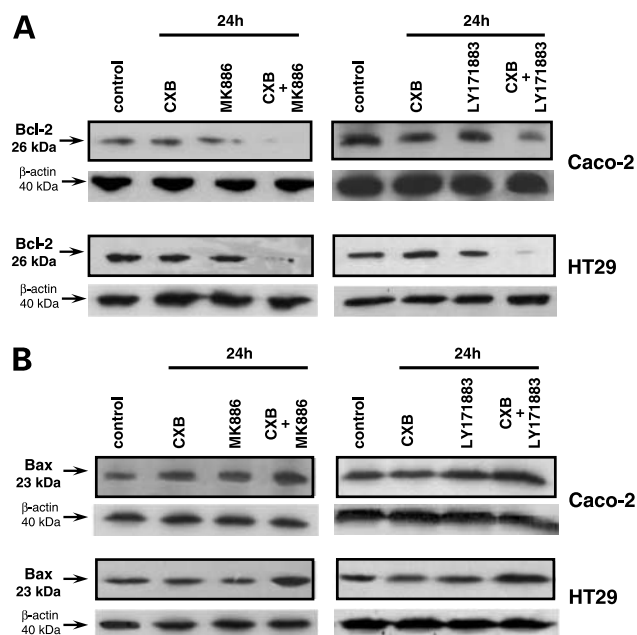


Figure 6. Western blot analysis. **A** and **B**, effects of 10 $\mu\text{mol/L}$ celecoxib, MK886, celecoxib + MK886, LY17883, or celecoxib + LY17883 on Bcl-2 and Bax expression, respectively, in the Caco-2 and HT29 cells. Samples were normalized for protein loading (70 μg) by reblotting the membrane-bound protein with an anti-actin antibody.

apoptotic response of cells to both COX-2 and 5-LOX inhibitors (47, 48). Again, 10 $\mu\text{mol/L}$ celecoxib or MK886 alone did not modify Bax expression, whereas a significant increase in this protein was observed after dual treatment.

Another possible mechanism proposed for nonsteroidal anti-inflammatory drug-mediated apoptosis is the accumulation of arachidonic acid following COX blocking and the consequent stimulation of the conversion of sphingomyelin to ceramide (17). Ceramide is a well-known mediator of apoptosis (17, 18). In particular, it has been shown that ceramide exposure induces mitochondrial cytochrome *c* release in neuronal cells (49) and leads to activation of the intrinsic caspase-cascade pathway at least partially independently of Bcl-2 family protein expression (50). We found that combined treatment of the Caco-2 and HT29 cells with celecoxib and MK886 determined a significant increase in tumor cell ceramide production. Moreover, administration of FB1, an inhibitor of ceramide synthase, could prevent both EGF-induced cell growth inhibition and induction of apoptosis that occur after dual treatment with celecoxib and MK886. We also showed that dual inhibition of COX-2 and 5-LOX led to mitochondrial depolarization in the Caco-2 cells and this effect was prevented by the administration of FB1. Because the collapse of $\Delta\psi$ is considered one of the most important mechanisms underlying ceramide-induced apoptosis, our findings strongly suggest the involvement of ceramide in mediating, at least in part, the antitumor effect of combined treatment with celecoxib and MK886.

Altogether, these results clearly show that the single use of low concentrations of COX-2 or 5-LOX inhibitors might be an inefficient means of suppressing tumor growth, whereas their combination may represent an effective therapeutic strategy to induce cell death through the mitochondrial pathway. Our findings of at least two different proapoptotic mechanisms (i.e., inhibition of both prostaglandin and leukotriene effects on Bcl-2/Bax expression and arachidonic acid-induced production of ceramide) might explain the additive antitumor effect of the simultaneous inhibition of COX-2 and 5-LOX pathways in comparison with blocking of only one.

Of the leukotrienes, LTB_4 and LTD_4 are procarcinogenic in a number of both experimental and human tumors, including colon cancer (6, 22–24). In particular, Öhd et al. (22) have shown the presence of considerably increased CysLT₁R immunoreactivity in colon cancer tissue. This expression has been found to correlate with poor prognosis in their Dukes B colorectal cancer patients. Moreover, LTD_4 has been shown to induce up-regulation of COX-2 and Bcl-2 in both the intestinal epithelial cells Int 407 and the colon cancer cells Caco-2 (23). Our Western blot results confirmed the up-regulation of CysLT₁R in the tumor tissue when compared with normal colon mucosa. We also showed that dual treatment of colon cancer cells with celecoxib and the CysLT₁R antagonist LY17883 determined similar results about cell growth inhibition, caspase-3 activation, and Bcl-2/Bax expression to those obtained after celecoxib and MK886 treatment. These findings suggest that the antitumor effect of MK886 is mainly mediated by the inhibition of LTD_4 production and that this eicosanoid may play a pivotal role in colon carcinogenesis.

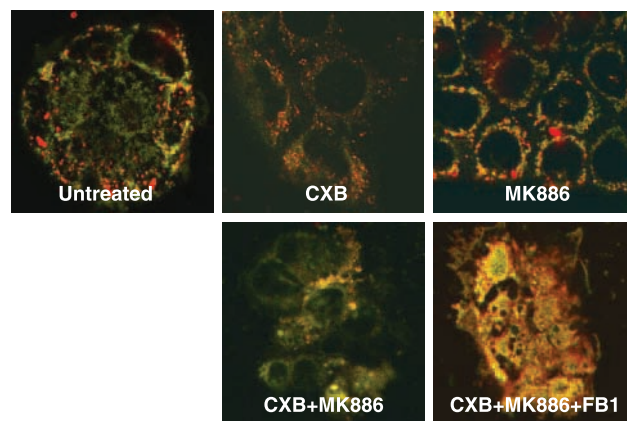


Figure 7. Detection of change in $\Delta\psi$ induced by treatment with 10 $\mu\text{mol/L}$ celecoxib, MK886, celecoxib + MK886, or celecoxib + MK886 + FB1. Dual emission images (488 and 543 nm) are the signal from monomeric (green) and J aggregate (red) JC-1 fluorescence in Caco-2 colon cancer cells. The shift in membrane charge was observed as disappearance of fluorescent red-orange-stained mitochondria (large negative $\Delta\psi$) and an increase in fluorescent green-stained mitochondria (loss of $\Delta\psi$). Each image is representative of at least three independently done experiments with similar results. The total number of cells analyzed was 30 for each treatment. Original magnification, $\times 40$.

In conclusion, our results show that inhibition of one arm of the arachidonic acid cascade may result in amplification of others with potentially untoward effects. In particular, inhibition of the COX-2 pathway can determine the activation of the 5-LOX one with increased production of tumorigenic leukotrienes, such as LTD₄. A combination of low doses of COX-2 and 5-LOX inhibitors would prevent this shunting and provide an additive antitumor activity through at least two different proapoptotic mechanisms. The possibility of augmenting the antitumor activity of celecoxib and thus reducing its therapeutic doses seems important considering the recent withdrawal of rofecoxib therapy due to cardiovascular side effects. However, further studies are needed to translate these *in vitro* studies into a safe and efficacious strategy for human colorectal cancer treatment.

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