Effects of Nonselective Cyclooxygenase Inhibition with Low-Dose Ibuprofen on Tumor Growth, Angiogenesis, Metastasis, and Survival in a Mouse Model of Colorectal Cancer

Min Yao, Wei Zhou, Simren Sangha, Andrew Albert, Albert J. Chang, Thomas C. Liu, and M. Michael Wolfe

Section of Gastroenterology, Boston University School of Medicine and Boston Medical Center, Boston, Massachusetts

ABSTRACT

***Purpose*: To determine whether the nonselective and relatively inexpensive nonsteroidal anti-inflammatory drug ibuprofen would be effective in inhibiting colorectal cancer and might improve mortality in a mouse model.**

***Experimental Design:* The effects of ibuprofen on tumor growth inhibition and animal survival have been examined in both mouse and human colorectal cancer tumor models. Angiogenesis was measured by *in vitro* endothelial cell tube formation and immunohistochemistry.**

***Results:* Ibuprofen significantly inhibited cell proliferation in mouse (MC-26) and human (HT-29) colorectal cancer cell lines. *In vitro* angiogenesis assays also indicated that ibuprofen decreased both cell proliferation and tube formation. The administration of chow containing 1,360 ppm ibuprofen, which achieved an average plasma concentration of ibuprofen lower than the peak level achieved in humans at therapeutic doses, inhibited tumor growth by 40% to 82%. Fewer liver metastases were found in the ibuprofen group compared with the control group. In combination therapy with the standard antineoplastic agents, 5-fluorouracil, or irinotecan (CPT-11), tumor volumes in the groups with ibuprofen ± CPT-11 or 5-fluorouracil were smaller than in the control group. Ibuprofen was similar to the cyclooxygenase-2 selective inhibitor rofecoxib in its ability to suppress tumor growth and improve overall survival.**

***Conclusions:* Ibuprofen, in part by modulating tumor angiogenesis, decreases both tumor growth and metastatic potential in mice. The ibuprofen doses were in the low range of therapeutic human plasma concentrations. Ibuprofen potentiates the antitumor properties of CPT-11 and improves survival of mice without increasing gastrointestinal toxicity.**

Received 8/23/04; revised 10/26/04; accepted 11/11/04.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: M. Michael Wolfe, Boston Medical Center, Section of Gastroenterology, 650 Albany Street, Boston, MA 02118. Phone: 617-638-8330; Fax: 617-638-7785; E-mail: michael.wolfe@bmc.org.

INTRODUCTION

Colorectal cancer is second only to lung cancer as a leading cause of death from malignancy in the United States (1) and up to 50% of patients present at an incurable stage (2). Nearly 130,000 new cases of colorectal cancer were diagnosed in the United States in 1999, resulting in 56,600 associated deaths (3). Approximately 6% of Americans will develop colorectal cancer during their lifetime and 2.6% will die from this disease (4).

Chemotherapeutic modalities to treat refractory colorectal cancer, although associated with significant toxicity, have provided only minimal benefit in improving survival. 5-Fluorouracil (5-FU), included in several regimens, is generally regarded as the most effective single agent for this disease and is often used as first-line therapy despite only partial responses to 5-FU achieved in 15% to 20% of patients (5). The probability of tumor response to 5-FU seems to be somewhat greater for patients with liver metastases when chemotherapy is infused directly into the hepatic artery, but intra-arterial treatment is costly and toxic and does not seem to prolong survival (6, 7). The concomitant administration of folinic acid (leucovorin) improves the efficacy of 5-FU in patients with advanced colorectal cancer by enhancing the binding of 5-FU to its target enzyme, thymidylate synthase. A 3-fold improvement in the partial response rate has been noted when leucovorin is combined with 5-FU (5). The effect of this combination on survival is marginal, with median survival typically 10 to 14 months (8). Irinotecan (CPT-11) is a potent inhibitor of topoisomerase I, a nuclear enzyme involved in the unwinding of DNA during replication (9). Irinotecan has shown antitumor activity against metastatic colorectal cancer when used alone as first-line treatment or as second-line treatment after the failure of 5-FU (5). Prolonged survival was observed when compared with supportive care or infusion of 5-FU and leucovorin as a second-line therapy (4). The Food and Drug Administration has approved the regimen of irinotecan, 5-FU, and leucovorin for the initial treatment of advanced colorectal cancer, and many oncologists have now adopted this regimen as standard care for colorectal cancer (10–12).

Epidemiologic studies have shown a 40% to 50% reduction in mortality from colorectal cancer in individuals taking nonsteroidal anti-inflammatory drugs, which presumably reduce the risk of colorectal cancer at least in part by inhibiting cyclooxygenase (COX), a key enzyme involved in the conversion of arachidonic acid to prostaglandins (13). Two isoforms of COX have been identified. These isoenzymes are regulated differently and exhibit distinct functions. COX-1 is expressed constitutively in many cell types, whereas COX-2 is a primary response gene, of which expression may be induced by trauma, growth factors, tumor promoters, and cytokines. COX-2 was first discovered as an oncogene-responsive COX

and increased COX-2 expression has been found in up to 85% of colorectal adenocarcinomas, but undetectable in normal intestinal mucosa (14, 15). A casual relationship between the activity of COX and colorectal cancer has been suggested, with COX emerging as a molecular target for chemoprevention in colorectal cancer (16).

We have previously shown that the COX-2 selective nonsteroidal anti-inflammatory drug rofecoxib is effective in preventing the metastasis of colorectal cancer to the liver in mice, an effect achieved via multiple mechanisms (17). In the present study, we sought to determine whether the nonselective and relatively inexpensive nonsteroidal anti-inflammatory drug ibuprofen would likewise be effective in decreasing the growth and metastatic potential of colorectal cancer and might improve mortality in a mouse model of metastatic colorectal cancer. Finally, we examined the cellular and molecular mechanisms by which nonsteroidal anti-inflammatory drugs affect colorectal cancer tumor growth.

MATERIALS AND METHODS

Cell Culture. The transplantable mouse colorectal cancer cell line MC-26 (18) was obtained from Dr. K.K. Tanabe (Massachusetts General Hospital, Boston, MA), and the human colorectal adenocarcinoma cell line HT-29 was obtained from American Type Culture Collection (Manassas, VA). MC-26 cells and HT-29 were maintained in DMEM (Life Technologies, Inc, Gaithersburg, MD) and McCoy's 5A medium, respectively, supplemented with 10% FCS plus antibiotics at 37°C in a humidified atmosphere of 95% air/5% CO_2. Human umbilical vein endothelial cells were purchased from Cambrex (Walkersville, MD) and maintained in EGM-2 medium (Cambrex).

Prostaglandin E_2 Assay. Prostaglandin E_2 (PGE_2), the major metabolite of arachidonic acid metabolism, was measured by ELISA (Cayman Chemical, Ann Arbor, MI) using conditional cell culture medium according to the protocol provided by manufacturer. Measurements were made in triplicate in separate experiments.

***In vitro* Cell Proliferation Assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay].** *In vitro* cell proliferation was assessed using a CellTiter 96 AQ One Solution Cell Proliferation Assay kit (Promega, Madison, WI). Cells (1×10^4 to 3×10^4 cells/mL) were plated in 96-well microtiter plates and incubated overnight. Cells were then treated with various concentrations of ibuprofen for 24, 48, or 72 hours. Twenty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium solution were added to each well and the cells were further incubated at 37°C for 1 hour, at which time the reactions were stopped by the addition of 10% SDS. The absorbance at wavelength 490 nm was measured using an EX800 microplate reader (Bio-Tek, Highland, WI).

In vitro Angiogenesis Assay. An *in vitro* angiogenesis assay was done according to the protocol provided by manufacturer (Chemicon, Temecula, CA) and described previously (19, 20). In brief, human umbilical vein endothelial cells were incubated in endothelial cell growth media overnight. Two milliliters of cell suspension (1×10^5 to 2×10^5 cells/mL) were then harvested and loaded on precoated Matrigel basement membrane matrix dishes and cultured in EGM-2 medium containing ibuprofen (1,000 μmol/L) at 37°C for 0.5, 2, 6, or 24 hours, followed by staining with hematoxylin for 20 minutes. Microscopic digital images were taken and cumulative tube length was measured using Spot 3.5.6. Vascular endothelial growth factor (VEGF) levels were measured using an ELISA kit (Assay Design, Ann Arbor, MI).

Immunohistochemistry. To assess angiogenesis in tumor tissues, frozen sections were made and microvessels were stained with anti-mouse CD31 monocolonal antibodies (BD Transduction Laboratories, Lexington, KY). The specimens were incubated with secondary antibody for 1 hour at 37°C and stained by the avidin-biotin peroxide complex method using an avidin-biotin peroxide complex staining kit (Santa Cruz Biotech, Santa Cruz, CA). They were visualized by 3,3′-diaminobenzidine and counterstained with hematoxylin.

Western Blot Hybridization. Mouse COX-1 and COX-2 monoclonal antibodies were purchased from BD Transduction Laboratories. To extract protein, MC-26 cells were harvested and lysed in radioimmunoprecipitation assay buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 ng/mL 1-phenylmethylsulfonyl fluoride, 66 ng/mL aprotinin). To extract protein from tissues, 0.1 g of tumor tissue was put in 2.0 mL of cold protein extract (radioimmunoprecipitation assay) buffer and homogenized for 1 minute with a Polytron-Aggregate (Kinmatica, Luzern, Switzerland). After removal of cellular debris by centrifugation, total protein extracted from cells or tissues was determined by using bicinchoninic acid protein assay (Pierce, Rockford, IL). Protein was mixed with gel loading buffer (50 mmol/L Tris pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.1% bromphenol blue) and heated for 10 minutes at 100°C. Samples containing 5 to 20 μg protein were loaded onto a 10% to 12% SDS-PAGE gel and then electrophoretically transferred to polyvinylidene difluoride membrane in transfer buffer (25 mmol/L Tris, 190 mmol/L glycine, 20% methanol). The blots were blocked with 7% dry milk for 1 hour at room temperature and incubated with the first antibody overnight. The blots were then washed thrice for 15 minutes each in TBS containing 0.05% Tween 20. The blots were further incubated with the anti-mouse immunoglobulin G antibody (Sigma Chemical Co., St. Louis, MO) for 1 hour at room temperature. After washing thrice, blots were incubated with luminous enhanced chemiluminescence reagent (Pierce Chemicals, Rockford, IL) for 10 to 120 seconds and exposed to Kodak X-ray film. Protein bands were identified by protein size and positive control provided by BD Transduction Laboratories. The amount of protein expression was quantified by Bio-Rad GS 700 Imaging Densitometer.

Blood Sampling and Serum Ibuprofen Concentration. Six-week-old male BALB/C mice were fed with a formulation containing 85, 170, 340, and 1,360 mg ibuprofen/kg chow. Ibuprofen was purchased from Sigma and pellet-formed chow containing ibuprofen was prepared by Harlan Teklad (Madison, WI). Blood was drawn on days 7 and 14 by tail bleeding, and serum was separated from the whole blood and frozen at −70°C until analysis. Serum concentrations of ibuprofen were analyzed by MDS Pharma Services Inc. (Saint-Laurent, Quebec, Canada). The plasma samples were analyzed by a quantified liquid chromatography-tandem mass spectrometry method. Sample preparation was done using protein precipitation.

The precursor-to-product ion transitions used were as follows: ibuprofen at *m/z* 205.2→161.1 and internal standard (phenacetin internal standard) at *m/z* 178.2→148.72. The retention times (t_R) for ibuprofen and internal standard were 1.82 and 1.95 minutes, respectively. A 150-μL aliquot of internal standard working solution (3,000 ng/mL of phenacetin internal standard in acetonitrile) was added to 25 μL of plasma. Each sample was then vortexed and centrifuged for 15 minutes at a speed of 13,000 rpm at 4°C.

After centrifugation, 150 μL of the supernatant were transferred into a high performance liquid chromatography vial, and aliquots of 5 μL were injected using a Perkin-Elmer 200 Series autosampler (Perkin-Elmer, Norwalk, CT). The loading and analytic columns were a Zorbax Extend C18 (4.6 × 12.5 mm, 5 μm d_P; Agilent Technologies, Palo Alto, CA) and a Waters Xterra C18 (3.9 × 20 mm, 3.5 μm d_P; Waters Corporation, Milford, MA), respectively. The loading mobile phase (with gradient loading) consisted of 100% (v/v) 10 mmol/L ammonium acetate/acetonitrile that was held for 1.00 minute. The acetonitrile was increased to 90% at 1.01 minutes and held for 1.00 minute. Original conditions were resumed at 2.40 minutes. Initial conditions for gradient elution were carried out with a mobile phase of 100% (v/v) 0.1% ammonium acetate solution in water/acetonitrile and held for 1.00 minute. The acetonitrile was increased to 70% at 1.50 minutes and held for 0.50 minute, and at 2.10 minutes, the acetonitrile was increased to 90% and held for 1.00 minute. Original conditions were resumed at 3.10 minutes. A flow rate of 1.0 mL/min was used throughout the run with switch events occurring at 1.00 minute (loading) and 2.40 minutes (injecting) for a total run time of 4.00 minutes. The column effluent was analyzed by selected reaction monitoring using a triple quadruple mass spectrometer (Micromass Quattro Ultima, Manchester, United Kingdom) equipped with an electrospray ion source operating in negative ion mode.

Animal Models and Experimental Designs. Six-week-old male BALB/C mice and BALB/C nude mice (T-cell deficiency) were obtained from Taconic (Germantown, NY). We used our previously described mouse colon cancer model as described (17, 21, 22). Briefly, MC-26 model cells were harvested from subconfluent cultures by exposure to trypsin-EDTA (Life Technologies) for 3 minutes, centrifugation at 300 × *g* for 15 minutes at room temperature, and then resuspension in serum-free DMEM or HBSS (Life Technologies) to yield a final concentration of 5×10^6 cells/mL. For the s.c. MC-26 model, using a 27-gauge needle and a 1-mL syringe, 100 μL of MC-26 tumor cells suspension were injected s.c. in the flank of BALB/c mice. For the HT-29 human xenograft colorectal cancer model, tumor slices of ~1.0 mm in diameter, harvested immediately from a xenograft HT-29 tumor, were implanted in the flanks of the animals. All animal studies were conducted using a protocol approved by the Institutional Animal Care and Use Committee of Boston University Medical Center.

To analyze the inhibitory effects of ibuprofen as a single agent on tumor growth, immediately after cells were implanted s.c. into mice on day 1, we randomly divided the mice into two groups and fed them with chow containing either no ibuprofen (control) or ibuprofen at a concentration of 1,360 mg/kg (10 mice/group). In a separate parallel experiment, 0.01% rofecoxib chow (Merck Frosst, Quebec, Canada) was fed to 10 MC-26 cell-bearing mice. In both MC-26 and HT-29 tumor models, tumor size was determined by measuring the longest and shortest diameters of the tumor. Tumor volume (mm^3) was calculated using the standard formula: tumor volume = (shortest diameter)^2 × (longest diameter) × 0.5.

To examine the effects of ibuprofen on experimental colorectal cancer liver metastasis, MC-26 cells (2×10^4 cells/mL) were injected into the subsplenic capsule of six-week-old BALB/c. Mice were randomly divided into a control group and an ibuprofen group (1,360 mg/kg ibuprofen) on day 0 and sacrificed on day 14. The incidence and extent of liver metastasis were then recorded.

To analyze possible synergistic effects of ibuprofen with clinically used standard chemotherapy, mice bearing s.c. MC-26 tumors were given 1,360 mg/kg ibuprofen (dose at which s.c. tumor growth was significantly inhibited) and chemotherapeutic reagents 5-FU (Sigma) plus leucovorin rescue (Sigma) and irinotecan (CPT-11, Pharmacia/Upjohn, Ann Arbor, MI) by i.p. injection. Starting on day 7 after tumor cell inoculation, mice were randomly divided into eight groups as follows:

- Group 1: control; control chow; 0.9% NaCl injected i.p. on days 9, 11, 15, 18, 23, and 28; and 5% glucose i.p. on days 8, 12, 15, 19, 22, and 25.
- Group 2: ibuprofen alone; ibuprofen (1,360 mg/kg) chow; 0.9% NaCl i.p. on days 9, 11, 15, 18, 23, and 28; and 5% glucose i.p. on days 8, 12, 15, 19, 22, and 25.
- Group 3: 5-FU/leucovorin alone; control chow; 5-FU 30 mg/kg and leucovorin 50 mg/kg i.p. on days 9, 11, 15, 18, 23, and 28; and 5% glucose i.p. on days 8, 12, 15, 19, 22, and 25.
- Group 4: irinotecan alone; control chow; irinotecan 30 mg/kg on days 8, 12, 15, 19, 22, and 25; and 0.9% NaCl on days 9, 11, 15, 18, 23, and 28.
- Group 5: ibuprofen/5-FU/leucovorin; ibuprofen (1,360 mg/kg) chow; 5-FU 30 mg/kg and leucovorin 50 mg/kg on days 9, 11, 15, 18, 23, and 28; and 5% glucose on days 8, 12, 15, 19, 22, and 25.
- Group 6: ibuprofen/irinotecan; ibuprofen (1,360 mg/kg) chow; irinotecan 30 mg/kg on days 8, 12, 15, 19, 22, and 25; and 0.9% NaCl on days 9, 11, 15, 18, 23, and 28.
- Group 7: 5-FU/leucovorin/irinotecan; control chow; 5-FU 30 mg/kg and leucovorin 50 mg/kg on days 9, 11, 15, 18, 23, and 28; and irinotecan 30 mg/kg on days 8, 12, 15, 19, 22, and 25.
- Group 8: ibuprofen/5-FU/leucovorin/irinotecan; ibuprofen (1,360 mg/kg) chow; 5-FU 30 mg/kg and leucovorin 50 mg/kg on days 9, 11, 15, 18, 23, and 28; and irinotecan 30 mg/kg on day 8, 12, 15, 19, 22, and 25.

In a separate survival study to compare ibuprofen with rofecoxib, MC-26 cell-bearing mice were randomly divided into three groups (15 mice /group): control, ibuprofen (1,360 ppm), and rofecoxib (0.01%). Mice were closely monitored and studies were terminated when all mice in the control group had died. The causes of death included spontaneous death due to tumor burden or drug toxicity or sacrifice by the investigators within 24 hours of determination that a mouse was demonstrating ill behavior. The date and number of animal in each group were recorded for survival analysis.

Statistical Analysis. One-way ANOVA was done for comparing tumor volume and weight and followed by Tukey's procedure for pairwise comparison. Student's *t* test was done to analyze MTT values, densitometric values of Western blot bands, CD31 microvessel staining, and tube length of *in vitro* angiogenesis assay among different conditions compared with data obtained under control conditions. Survival analysis was done using the Kaplan-Meier method and log-rank tests. Statistical significance was assigned if $P < 0.05$.

RESULTS

Cell Proliferation, Cyclooxygenase Expression, and Prostaglandin E_2 Production. No significant differences in COX-1 and COX-2 concentrations among the cells treated with various concentrations of ibuprofen at 24 hours were detected (Fig. 1*A* and *B*). In contrast, the production of PGE_2, the major product of the arachidonic acid pathway, was significantly reduced in cells treated with various concentrations of ibuprofen (Fig. 1*C*). Maximum inhibition of MC-26 cell PGE_2 levels was detected when cells were incubated in an ibuprofen concentration of 1,000 μmol/L, at which time PGE_2 levels were diminished by 70.1 % compared with control (80.0 versus 268.5 pg/mL; $P < 0.01$). A similar pattern was seen when HT-29 cells were cultured in medium containing ibuprofen. After 24 hours, PGE_2 levels were diminished by 81.1% compared with control (18.0 versus 95.6 pg/mL; $P < 0.01$; Fig. 1*C*).

Ibuprofen at concentrations of 125, 250, 500, and 1,000 μmol/L decreased MC-26 cell proliferation by 1.3%, 5.8%, 11.3%, and 39.8% ($P < 0.01$) at 72 hours, respectively, compared with cells incubated in the absence of ibuprofen (Fig. 2*A*). In HT-29 cells, after 72 hours of treatment maximum inhibition of cell proliferation was detected using 1,000 μmol/L ibuprofen, at which time MTT levels were diminished by 26.7% compared with control ($P < 0.01$; Fig. 2*B*).

***In vitro* Angiogenesis.** To assess the effects of ibuprofen on *in vitro* neovascularization, MTT and tube formation assays were done in human umbilical vein endothelial cells (Fig. 3). After 24 hours of treatment, maximum inhibition of cell proliferation was detected using 1,000 μmol/L ibuprofen, at which time MTT levels were diminished by ~80%, compared with control ($P < 0.01$; Fig. 3*A*). Using the tube formation assay, the 6-hour tube lengths were 155.4 ± 21.3 μm in control and 94.0 ± 24.9 μm in 1,000 μmol/L ibuprofen (40% reduction; $P < 0.01$). The 24-hour tube lengths were 76.2 ± 13.5 μm in control and 9.40 ± 6.95 μm in 1,000 μmol/L ibuprofen (88% reduction, $P < 0.01$; Fig. 3*B* and *C*). VEGF levels were slightly reduced in the presence of 100 μmol/L ibuprofen, and the addition of 10 μmol/L PGE_2 attenuated this effect. However, although VEGF concentrations were significantly decreased by 1,000 μmol/L ibuprofen, 10 μmol/L PGE_2 did not alter the reduction (Fig. 3*D*).

Effect of Ibuprofen on Tumor Growth and Liver Metastasis. Figure 4 depicts the plasma concentration of ibuprofen in mice fed with ibuprofen-containing chow. Halsas

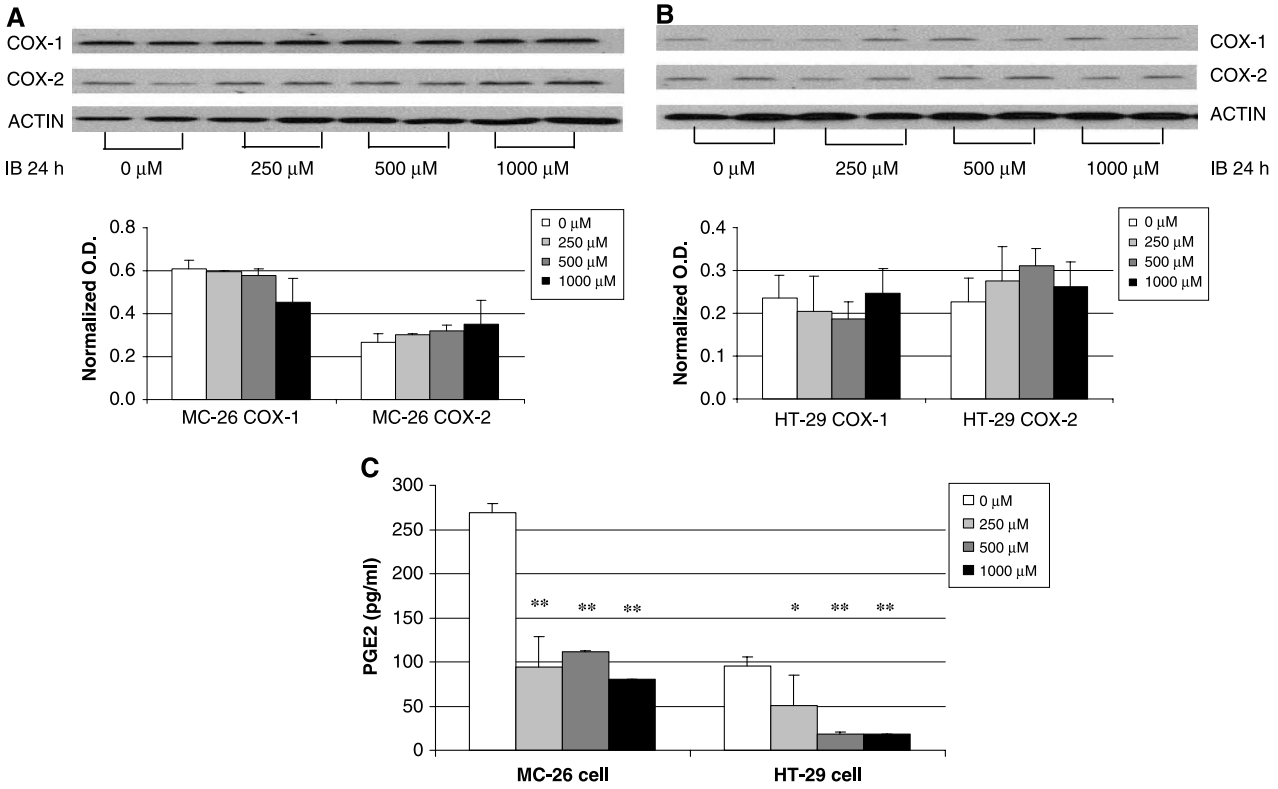


Fig. 1 Effects of ibuprofen on COX-1 and COX-2 expression and PGE_2 production. Cells (1×10^5 cells/mL) were seeded in plates and incubated overnight. MC-26 cell (*A*) and HT-29 cell (*B*) were then treated with ibuprofen for 24 hours at various concentrations. Conditional medium was collected and cells harvested. COX-1 and COX-2 protein expression was determined by Western blot hybridization analysis. PGE_2 (*C*) was quantified by ELISA. *, $P < 0.05$ versus vehicle; **, $P < 0.01$ versus vehicle.

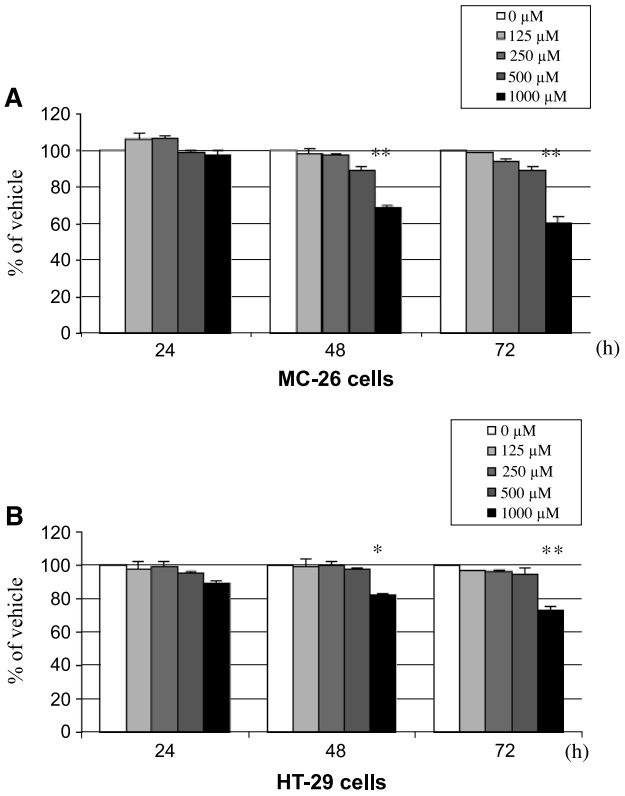


Fig. 2 Effects of ibuprofen on cell proliferation *in vitro* (MTT assay). Cells (1×10^4 to 3×10^4 cells/mL) were plated in 96-well microtiter plates and incubated overnight. MC-26 (*A*) and HT-29 (*B*) cells were then treated with ibuprofen for 24, 48, and 72 hours at various concentrations. *, $P < 0.05$ versus vehicle; **, $P < 0.01$ versus vehicle.

et al. (23) reported that following the administration of 300 mg of ibuprofen (in capsule form) to fasting humans, plasma ibuprofen concentrations were ~27,000 ng/mL at peak concentration. We determined that mice fed chow containing 1,360 mg/kg ibuprofen achieved a plasma ibuprofen concentration of approximately between 18,760 ± 7,270 and 20, 978 ± 5,966 ng/mL (Fig. 4), which is below the level obtained after administration of 300 mg of the drug to humans. Gross necroscopic examination revealed no evidence of gastroduodenal mucosal injury in mice who consumed the chow containing ibuprofen. We therefore used chow containing 1,360 mg/kg ibuprofen during the *in vivo* studies assessing tumor growth and metastasis.

MC-26 cells were injected s.c. in BALB/C mice on day 0. On day 1, mice were randomly divided into three groups with 10 mice per group: control chow, chow containing ibuprofen 1,360 mg/kg, and chow containing 0.01% rofecoxib. Both Ibuprofen and rofecoxib significantly inhibited tumor growth in mice. The tumor volumes were 39.9 ± 19.6, 166.0 ± 144.0, 276.5 ± 227, and 573.1 ± 376 mm^3 on days 10, 14, 17, and 21, respectively, in the control group. In contrast, in the ibuprofen group, the tumor volumes were 21.9 ± 17.3, 59 ± 57, 48 ± 57, and 101 ± 161 mm^3 on days 10, 14, 17, and 21, respectively, corresponding to 47.4%, 64.4%, 82.6%, and 82.3% decreases in tumor volume (Fig. 5*A*). In the rofecoxib group, the tumor volumes were 29.6 ± 23.7, 63 ± 69, 50 ± 66.9, and 64 ± 69 mm^3 on days 10, 14, 17, and 21, respectively, corresponding to 25.1%, 62.1%, 81.8%, and 88.3% decreases in tumor volume. The average body weight was significantly lower on day 21 in the control group (20.3 ± 2.6 g) than in ibuprofen- (24.1 ± 1.73 g) or rofecoxib-treated mice (23.9 ± 1.36 g), corresponding to 16% and 15.1% body weight reductions, respectively, in the control group ($P < 0.01$).

To determine whether the above antitumor effects of ibuprofen are reproducible in human colorectal cancer cells, the HT-29 human colorectal cancer xenograft model was established by the s.c. implantation of tumor fragments, ~1.0 mm in diameter, into the flanks of nude mice. Treatment started on day 1 and was completed on day 42 (week 7). Figure 5 shows that tumor volume in the ibuprofen group on day 42 was reduced by 40% compared with control mice (164.4 ± 99.3 versus 410.9 ± 233.2 mm^3, $P < 0.05$; Fig. 5*B*). However, in contrast to MC-26 cells, no significant difference in body weight was detected between the two groups of mice with implanted HT-29 cell tumors throughout the period of investigation.

The effects of ibuprofen on the metastatic potential of colorectal cancer were examined in an experimental liver metastasis model established by the subsplenic capsule injection of MC-26 cells (17). As depicted in Fig. 5*E*, visible metastatic hepatic nodules were more prevalent and larger in the control group than in ibuprofen-fed mice. Immunohistochemical analysis of frozen tumor sections showed a decrease in the number of CD31 positive microvessels in mice fed chow containing ibuprofen. The number of microvessels was 37.7 ± 15.1/field in the control group ($n = 8$) and 26.4 ± 10.3/field ($n = 7$) in the ibuprofen group, representing approximately a 30% reduction in the latter group of animals ($P = 0.059$, Fig. 6). The reduction in the number of microvessels in the ibuprofen group was associated with a decrease in PGE_2 concentration in tumor extracts compared with the control group ($P < 0.05$, Fig. 6).

Effect of Ibuprofen and Other Antineoplastic Agents in the Treatment of Colorectal Cancer. From day 7 to day 28, mice received combination therapy, and the study was continued until all control mice had died (day 50). As shown in Fig. 7*A*, tumor growth was significantly inhibited in all groups of animals receiving ibuprofen, 5-FU, or CPT-11, either alone or in various combinations, compared with the control group (1,491.3 ± 862 mm^3). The maximal inhibition was found in the group with a combination of ibuprofen, CPT-11, and 5-FU/leucovorin (110.1 ± 80 mm^3), representing a 92.6% reduction in tumor volume ($P < 0.01$). The tumor volume in this group was significantly smaller than in the groups of mice treated with ibuprofen alone (517 ± 377 mm^3) and 5-FU alone (499 ± 234 mm^3; $P < 0.01$).

The effects of the various treatment regimens on body weight were evaluated on day 28 (Fig. 7*C*). In only three of the animal groups (ibuprofen alone, CPT-11 alone, and the combination of ibuprofen and CPT-11) did body weight remain stable (average body weight of 23.1 ± 1.72 g). In addition to significant weight loss in the control group (19.65 ± 1.24 g), body weight decreased in all animals receiving 5-FU/leucovorin. The average body weight in these groups was only 79.8% of groups not receiving 5-FU (18.44 ± 1.88 g versus 23.1 ± 1.72 g; $P < 0.05$).

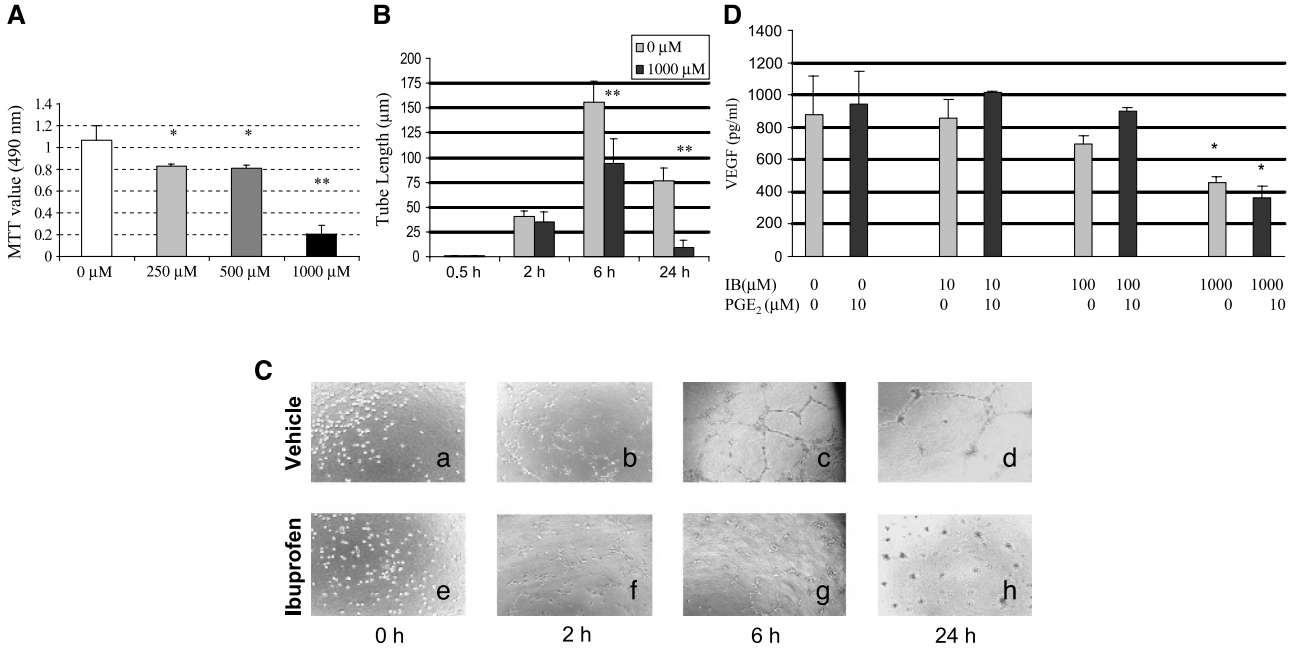


Fig. 3 Effects of ibuprofen on human umbilical vein endothelial cell proliferation and angiogenesis *in vitro*. After 24 hours of ibuprofen treatment, proliferation was assessed in human umbilical vein endothelial cells using MTT assays (*A*). Digital images were taken under the microscope (*C*) and cumulative tube length was measured using Spot 3.5.6 (*B*). VEGF levels were assessed in MC-26 cells measured using ELISA (*D*). *, $P < 0.05$ versus vehicle; **, $P < 0.01$ versus vehicle.

The survival of mice receiving ibuprofen alone, CPT-11 alone, or ibuprofen/CPT-11 was significantly prolonged compared with control mice and mice receiving other treatment regimens. The overall survival rates were 80% in mice receiving ibuprofen alone and CPT-11 alone and 90% in mice treated with ibuprofen/CPT-11 (Fig. 7). In contrast, much like its effects on body weight, survival seemed to be adversely affected by treatment with 5-FU. Some mice in the groups with 5-FU died earlier than those in the control group, and the overall survival rates at day 50 in mice treated with 5-FU alone, ibuprofen/5-FU, 5-FU/CPT-11, and ibuprofen/CPT-11/5-FU were 30%, 20%, 40%, and 30%, respectively (Fig. 7*E* and *F*).

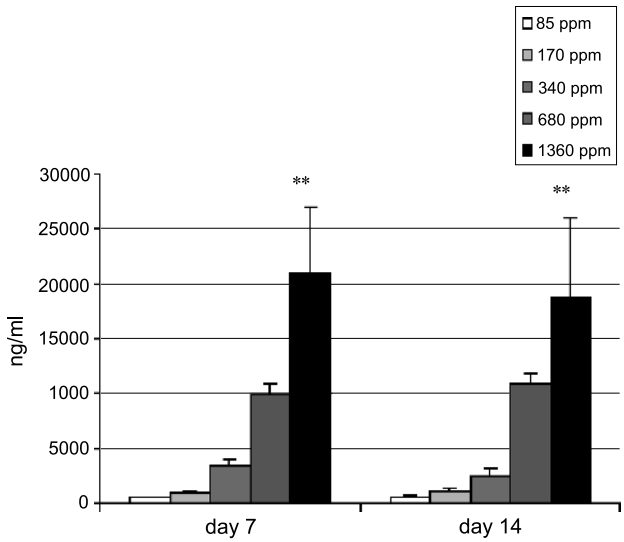


Fig. 4 Ibuprofen plasma concentrations after ingestion of chow containing increasing concentrations of ibuprofen in BALB/c mice. Six-week-old male BALB/C mice were fed with Teklad global 18% protein rodent diet containing ibuprofen at 85 (85 mg ibuprofen/kg diet), 170, 340, and 1,360 ppm. Blood was drawn on days 7 and 14 by tail bleeding. Plasma ibuprofen concentrations were measured by high performance liquid chromatography. **, $P < 0.01$ versus other doses.

Comparison of Ibuprofen with Rofecoxib on Mouse Survival. Using the MC-26 mouse colorectal tumor model, we compared the effects on survival produced by the nonselective COX inhibitor ibuprofen and the selective COX-2 inhibitor rofecoxib. As shown in Fig. 8, the 62-day overall survival rate in the ibuprofen group was nearly identical to the survival rate in the rofecoxib group (93.3%). No mice in the control group survived during the 62 days in this study ($P < 0.0001$, log-rank test). Moreover, no evidence of gastroduodenal mucosal injury in ibuprofen-treated mice was found at the end of the survival study.

DISCUSSION

A growing body of experimental evidence shows the contribution of COX-2 overexpression in colorectal cancer tumorigenesis. Nonselective COX inhibitors, which inhibit both isoforms, have been reported to prevent tumorigenesis. The use of nonselective COX inhibitors is associated with adverse gastrointestinal events, such as upper gastrointestinal bleeding, whereas COX-2 selective inhibitors are thought to exert their anti-inflammatory and antineoplastic properties with diminished toxicity (24–27). Nevertheless, ibuprofen, a generic and relatively inexpensive, nonselective COX inhibitor, is still

widely used in clinical practice. Despite its widespread use throughout the world for over 40 years and the potential benefit of this agent in preventing chemically induced colorectal carcinogenesis in experimental animal (28–30), the possibility of using ibuprofen in the treatment of established colorectal cancer has not been previously examined.

As mentioned previously, peak plasma ibuprofen concentrations in humans administered a single dose of 300 mg (23) are ~27,000 ng/mL or greater. This level is considerably lower than those achieved using ibuprofen doses commonly employed for analgesia or fever-control, which are generally 1.2 to 2.4 g daily in divided doses. Moreover, the use of low-dose ibuprofen is known to be associated with a very low incidence of serious gastrointestinal toxicity. A meta-analysis (31) of three retrospective case-control studies on serious upper gastrointestinal bleeding reported that ibuprofen had the lowest odds ratio (OR) and safest side-effect profile among nonselective nonsteroidal anti-inflammatory drugs [OR, 1.7; 95% confidence interval (95% CI), 1.1-2.5], followed by diclofenac (OR, 4.9; 95% CI, 3.3-7.1), naproxen (OR, 9.1; 95% CI, 6.1-13.7), piroxicam (OR, 13.1; 95% CI, 7.9-21.8) and ketoprofen (OR, 34.9; 95% CI, 12.7-96.5). Most importantly, the risk ratio for ibuprofen at a total dose of 1,200 mg/d or lower was only 1.1 (95% CI, 0.6-2.0), comparable to a control population.

The dose of ibuprofen used in the present animal study was even lower than those employed in humans, which likely accounts for the absence of gastrointestinal mucosal injury. Previous pharmacokinetic data (32, 33) in healthy humans have shown that long-term treatment with high-dose ibuprofen (14 mg/kg) achieved peak plasma concentrations (C_{max}) of 65,000 ng/mL. Moderate doses of 7 mg/kg achieved C_{max} of 34,000 ng/mL, and low doses of 2.6 mg/kg achieved C_{max} of 15,800 ng/mL. In our study, mice fed a 1,360 ppm ibuprofen diet achieved concentrations between 18,760 ± 7,270 and 20, 978 ± 5,966 ng/mL (Fig. 4). The concentrations achieved in our animal study were thus comparable to the lower portion of the therapeutic range in humans.

A direct comparison of rofecoxib and ibuprofen in the present study using our mouse colorectal cancer model showed that specific COX-2 inhibition and nonselective COX inhibition prolonged survival to a similar degree (Fig. 8). The rofecoxib blood levels achieved in mice fed rofecoxib-containing chow at the concentration employed in this study were equivalent to those levels attained following 25-mg daily dosing in humans, the full therapeutic dose normally prescribed chronically for the treatment of osteoarthritis. In contrast, as stated above, the blood levels achieved in response to the administration of chow containing ibuprofen at a concentration of 1,360 ppm corresponded to the lower therapeutic range in humans. It is thus possible that the benefit of nonsteroidal anti-inflammatory drug therapy in the prevention and treatment of colorectal cancer and

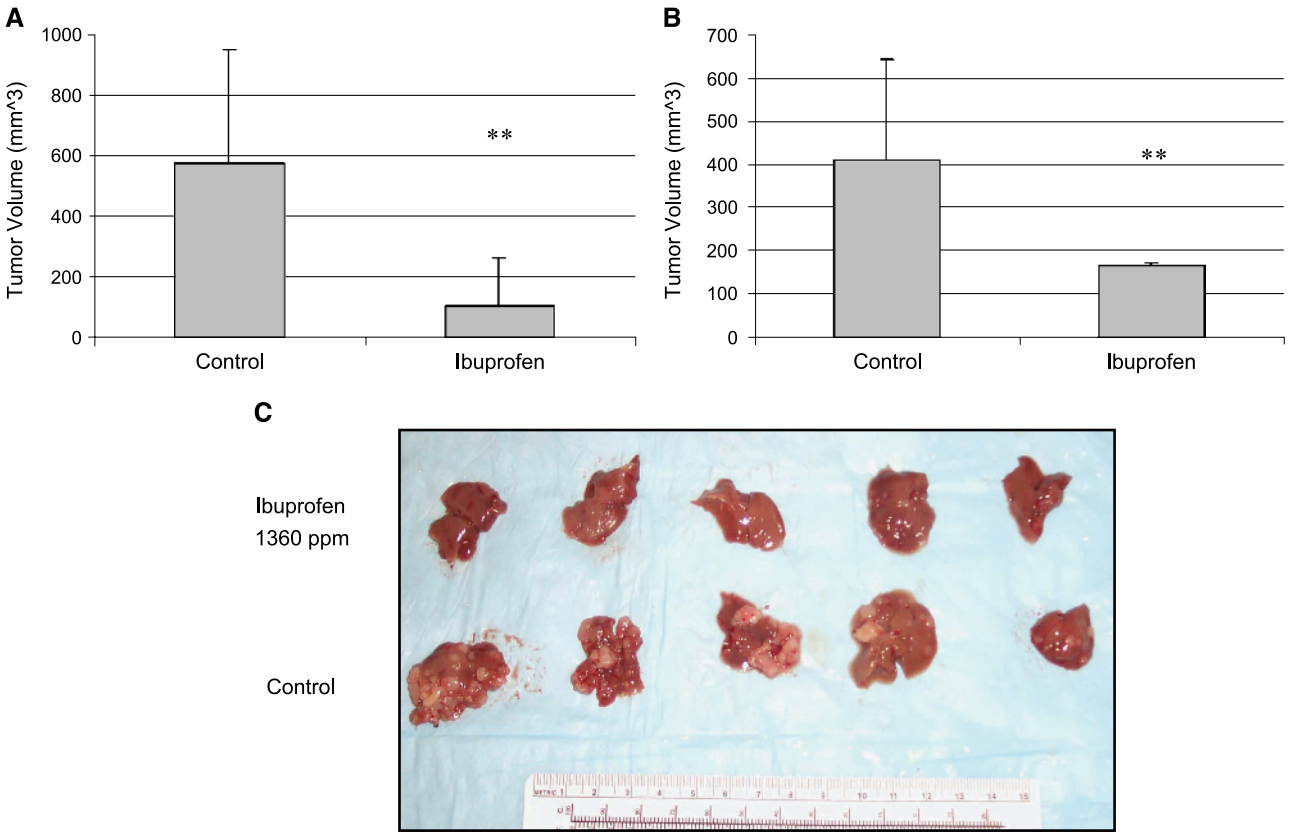


Fig. 5 Effects of ibuprofen on tumor growth *in vivo* and liver metastasis. The inhibitory effect of ibuprofen on tumor growth was determined in two colorectal cancer models using MC-26 cells (*A*) and HT-29 cells (*B*). The extent of liver metastasis was then recorded (*C*). *, $P < 0.05$; **, $P < 0.01$.

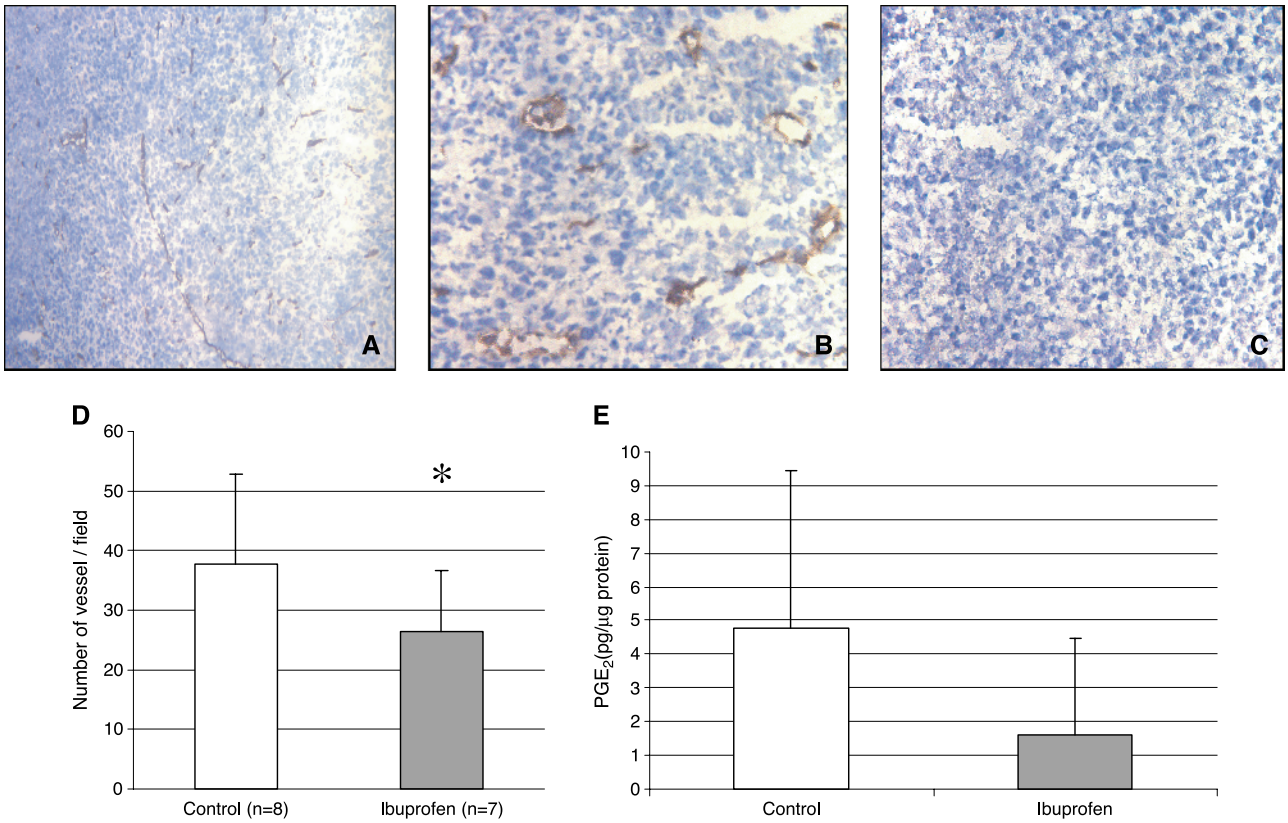
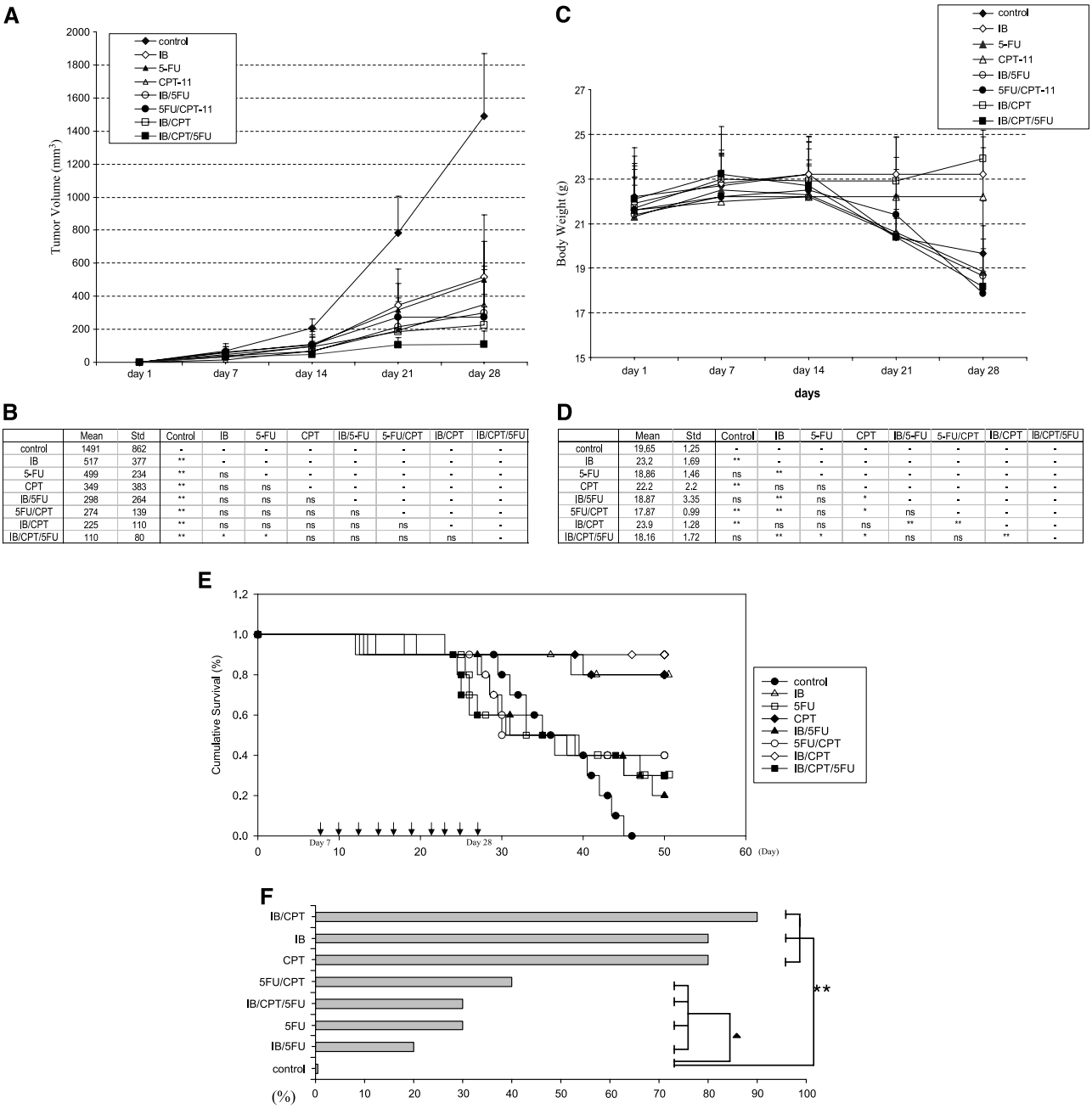


Fig. 6 Effect of ibuprofen on the angiogenesis *in vivo*. Mice bearing MC-26 tumor were sacrificed and tumor harvested on day 21. Frozen sections were made and microvessels were stained with anti-mouse CD31 monocolonal antibodies using the avidin-biotin peroxide complex method. Angiogenesis was determined by microscopic counting of the CD31 stained microvessels with 100× (*A*) and 400× (*B*) objectives. The same staining procedure was done without addition of primary antibody as a negative control (*C*). Data were analyzed using the one-tail *t* test (*D*). PGE_2 was detected in tumor tissues using enzyme immunoassay (*E*). *, $P = 0.059$.

other gastrointestinal malignancies extends beyond selective COX-2 inhibition and that additional benefit might be derived by inhibiting both COX isoforms. Furthermore, our observations are consistent with the notion that the lack of gastrointestinal toxicity was due to the low ibuprofen concentrations required to achieve an antineoplastic effect, compared with the markedly higher concentrations, associated with greater toxicity, needed to attain analgesic and antipyretic effects in humans. Additionally, because it is available without prescription, ibuprofen is considerably less expensive than selective COX-2 inhibitors. Using the on-line pricing site of a major pharmacy (http://www.CVS.com), we found the following prices listed for a one-month supply (30 capsules) of these medications: ibuprofen 600 mg ($9.99), rofecoxib 25 mg ($95.59), and celecoxib 200 mg ($92.99). Thus, the prolonged use of this relative inexpensive drug might confer significant health benefits with a considerable cost saving.

In our studies examining various treatment regimens, although tumor growth was significantly inhibited in each therapeutic group, each group treated with 5-FU manifested severe toxicity associated with remarkable body weight loss. A survival analysis showed that some of the 5-FU treated mice, even with a low tumor burden, were starting to die by the third week of treatment (week 4 of the study). These mice had an even shorter survival than the ones in the control group. In contrast, the survival of mice receiving ibuprofen alone, CPT-11 alone, or ibuprofen/CPT-11 combination was significantly prolonged compared with control mice and mice receiving other treatment regimens.

Multiple molecular and cellular mechanisms are involved in exerting the anti-inflammatory and antitumor effects of nonsteroidal anti-inflammatory drugs. These mechanisms are usually divided into two major categories: COX-dependent (13) and independent pathways (34–36). It has been long assumed that the antiproliferative effects of nonsteroidal anti-inflammatory drugs are dependent on inhibition of COX activity and prostaglandin synthesis. COX-2 selective inhibitors are able to induce cell differentiation and cell apoptosis (37) and inhibit angiogenesis (38) and cell invasion/migration (39, 40). Using rofecoxib, we recently showed (17), by Western blot hybridization analysis of primary tumors, decreased protein expression of COX-2, β-catenin, cyclin D1, VEGF, matrix metalloproteinase-2, and matrix metalloproteinase-9. These observations indicate that multiple mechanisms are involved in exerting the beneficial effects of COX-2 selective inhibition on tumor growth and metastasis. In the present study, we focused on the effects of ibuprofen on tumor angiogenesis and, like selective COX-2 inhibition, showed the marked inhibitory



B

	Mean	Std	Control	IB	5-FU	CPT	IB/5-FU	5-FU/CPT	IB/CPT	IB/CPT/5FU
control	1491	862	-	-	-	-	-	-	-	-
IB	517	377	**	-	-	-	-	-	-	-
5-FU	499	234	**	ns	-	-	-	-	-	-
CPT	349	383	**	ns	ns	-	-	-	-	-
IB/5FU	298	264	**	ns	ns	ns	-	-	-	-
5FU/CPT	274	139	**	ns	ns	ns	ns	-	-	-
IB/CPT	225	110	**	ns	ns	ns	ns	ns	-	-
IB/CPT/5FU	110	80	**	*	*	ns	ns	ns	ns	-

D

	Mean	Std	Control	IB	5-FU	CPT	IB/5-FU	5-FU/CPT	IB/CPT	IB/CPT/5FU
control	19.65	1.25	-	-	-	-	-	-	-	-
IB	23,2	1,69	**	-	-	-	-	-	-	-
5-FU	18,86	1,46	ns	**	-	-	-	-	-	-
CPT	22.2	2.2	**	ns	ns	-	-	-	-	-
IB/5FU	18.87	3.35	ns	**	ns	*	-	-	-	-
5FU/CPT	17.87	0.99	**	**	ns	*	ns	-	-	-
IB/CPT	23.9	1.28	**	ns	ns	ns	**	**	-	-
IB/CPT/5FU	18.16	1.72	ns	**	*	*	ns	ns	**	-

Fig. 7 Effect of ibuprofen alone and in combination with other chemotherapeutic drugs on tumor growth, body weight, and animal survival. Treatment started on day 7 after tumor cell inoculation and ended on day 28 and tumor volume (*A*) and body weights (*C*) were determined once weekly for 4 weeks. The date and animal number in each group were recorded for survival analysis (*E*). The study was terminated after none in control were alive. *B* and *D,* statistical results for pairwise-comparisons between any two groups on day 28 using ANOVA. *F,* overall survival rate on day 50. *, $P < 0.05$; **, $P < 0.01$; ns, $P > 0.05$.

effect of ibuprofen not only on prostaglandin synthesis and tumor/endothelial cell proliferation but also on angiogenesis both *in vitro* and *in vivo*. PGE_2 levels were decreased in tumor extracts in ibuprofen-treated mice compared with control mice, and the reduction of PGE_2 level correlated with a decreased number of tumor microvessels. The addition of 10 μmol/L PGE_2 reversed the VEGF reduction induced by 100 μmol/L ibuprofen, but not by 1,000 μmol/L, consistent with the notion that the antiangiogenic effect of ibuprofen may be mediated in part through COX-independent pathways.

In conclusion, ibuprofen, within or below the range of therapeutic human plasma concentrations, decreases both tumor growth and the potential for liver metastasis, at least in part, by modulating tumor angiogenesis in the colorectal cancer mouse model. Ibuprofen potentiates the antitumor properties of CPT-11 and 5-FU in colorectal cancer without

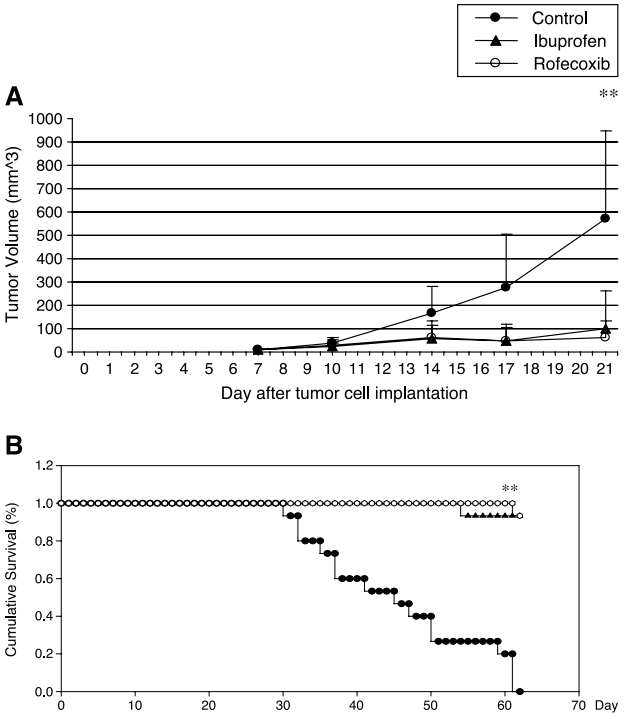


Fig. 8 Effect of ibuprofen in combination with rofecoxib on survival of mouse colorectal tumor model. Mice were randomly divided into three groups: control, ibuprofen, and rofecoxib. Tumor growth was expressed as tumor volume (*A*). The survival study was terminated when all mice in control group had died (*B*). *, $P < 0.05$; **, $P < 0.01$.

increasing gastrointestinal mucosal injury and improves survival of mice treated with CPT-11, but not 5-FU. Ibuprofen may thus prove to be a very effective, safe, and inexpensive adjunct to standard chemotherapy regimens. Further studies in humans will be necessary to determine whether ibuprofen might be beneficial not only in treating established colorectal cancer but also in the prevention of recurrent colorectal polyps and cancer.

REFERENCES

1. Winawer SJ, Fletcher RH, Miller L, et al. Colorectal cancer screening: clinical guidelines and rationale. Gastroenterology 1997;112:594–642.

2. Moertel CG, Fleming TR, Macdonald JS, et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N Engl J Med 1990;322:352–8.

3. Ladabaum U, Chopra CL, Huang G, et al. Aspirin as an adjunct to screening for prevention of sporadic colorectal cancer. A cost-effectiveness analysis. Ann Intern Med 2001;135:769–81.

4. Saltz LB, Cox JV, Blanke C, et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N Engl J Med 2000;343:905–14.

5. Douillard JY, Cunningham D, Roth AD, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet 2000;355:1041–7.

6. Lorenz M, Muller HH, Schramm H, et al. Randomized trial of surgery versus surgery followed by adjuvant hepatic arterial infusion with 5-fluorouracil and folinic acid for liver metastases of colorectal cancer. German Cooperative on Liver Metastases (Arbeitsgruppe Lebermetastasen). Ann Surg 1998;228:756–62.

7. Ekberg H, Tranberg KG, Lundstedt C, et al. Determinants of survival after intra-arterial infusion of 5-fluorouracil for liver metastases from colorectal cancer: a multivariate analysis. J Surg Oncol 1986;31:246–54.

8. Borner MM, SO. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. Advanced Colorectal Cancer Meta-analysis Project. J Clin Oncol 1992;10:896–903.

9. Goldberg RM, Sargent DJ, Morton RF, et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004;22:23–30.

10. Sargent DJ, Niedzwiecki D, O'Connell MJ, Schilsky RL. Recommendation for caution with irinotecan, fluorouracil, and leucovorin for colorectal cancer. N Engl J Med 2001;345:144–5; author reply 46.

11. Van Cutsem E, Douillard JY, Kohne CH. Toxicity of irinotecan in patients with colorectal cancer. N Engl J Med 2001;345:1351–2.

12. Van Cutsem E, Dicato M, Wils J. Recent advances in the management of colorectal cancer. Eur J Cancer 2001;37:2302–9.

13. Sheng H, Shao J, Kirkland SC, et al. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J Clin Invest 1997;99:2254–9.

14. Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (Part II). J Natl Cancer Inst 1998;90:1609–20.

15. Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenesis (part I). J Natl Cancer Inst 1998;90:1529–36.

16. Dannenberg AJ, Zakim D. Chemoprevention of colorectal cancer through inhibition of cyclooxygenase-2. Semin Oncol 1999;26:499–504.

17. Yao M, Kargman S, Lam EC, et al. Inhibition of cyclooxygenase-2 by rofecoxib attenuates the growth and metastatic potential of colorectal carcinoma in mice. Cancer Res 2003;63:586–92.

18. Singh P, Walker JP, Townsend CM Jr, Thompson JC. Role of gastrin and gastrin receptors on the growth of a transplantable mouse colon carcinoma (MC-26) in BALB/c mice. Cancer Res 1986;46:1612–6.

19. Yanamandra N, Kondraganti S, Srinivasula SM, et al. Activation of caspase-9 with irradiation inhibits invasion and angiogenesis in SNB19 human glioma cells. Oncogene 2004;23:2339–46.

20. Strasly M, Doronzo G, Capello P, et al. CCL16 activates an angiogenic program in vascular endothelial cells. Blood 2004;103:40–9.

21. Yao M, Song DH, Rana B, Wolfe MM. COX-2 selective inhibition reverses the trophic properties of gastrin in colorectal cancer. Br J Cancer 2002;87:574–9.

22. Yao M, Lam EC, Kelly CR, Zhou W, Wolfe MM. Cyclooxygenase-2 selective inhibition with NS-398 suppresses proliferation and invasiveness and delays liver metastasis in colorectal cancer. Br J Cancer 2004;90:712–9.

23. Halsas M, Hietala J, Veski P, Jurjenson H, Marvola M. Morning versus evening dosing of ibuprofen using conventional and time-controlled release formulations. Int J Pharm 1999;189:179–85.

24. Wolfe MM. Future trends in the development of safer nonsteroidal anti-inflammatory drugs. Am J Med 1998;105:44–52S.

25. Wolfe MM, Lichtenstein DR, Singh G. Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. N Engl J Med 1999;340:1888–99.

26. Hirata T, Ukawa H, Yamakuni H, Kato S, Takeuchi K. Cyclo-oxygenase isozymes in mucosal ulcergenic and functional responses following barrier disruption in rat stomachs. Br J Pharmacol 1997;122:447–54.

27. Lichtenstein DR, Wolfe MM. COX-2-Selective NSAIDs: new and improved? JAMA 2000;284:1297–9.

28. Wargovich MJ, Chen CD, Harris C, Yang E, Velasco M. Inhibition of aberrant crypt growth by nonsteroidal anti-inflammatory

agents and differentiation agents in the rat colon. Int J Cancer 1995; 60:515–9.

29. Reddy BS, Tokumo K, Kulkarni N, Aligia C, Kelloff G. Inhibition of colon carcinogenesis by prostaglandin synthesis inhibitors and related compounds. Carcinogenesis 1992;13:1019–23.

30. Williams JL, Borgo S, Hasan I, et al. Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) alter the kinetics of human colon cancer cell lines more effectively than traditional NSAIDs: implications for colon cancer chemoprevention. Cancer Res 2001;61: 3285–9.

31. Lewis SC, Langman MJ, Laporte JR, et al. Dose-response relationships between individual nonaspirin nonsteroidal anti-inflammatory drugs (NANSAID) and serious upper gastrointestinal bleeding: a meta-analysis based on individual patient data. Br J Clin Pharmacol 2002;54:320–6.

32. Konstan MW, Krenicky JE, Finney MR, et al. Effect of ibuprofen on neutrophil migration *in vivo* in cystic fibrosis and healthy subjects. J Pharmacol Exp Ther 2003;306:1086–91.

33. Corpetti G, Rosignoli MT, Dionisio P. Comparative bioavailability study of two oral formulations of ibuprofen. Arzneimittelforschung 1998;48:392–5.

34. Kundu N, Smyth MJ, Samsel L, Fulton AM. Cyclooxygenase inhibitors block cell growth, increase ceramide and inhibit cell cycle. Breast Cancer Res Treat 2002;76:57–64.

35. Grosch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. FASEB J 2001;15:2742–4.

36. Babbar N, Ignatenko NA, Casero RA Jr, Gerner EW. Cyclo-oxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. J Biol Chem 2003; 278:47762–75.

37. Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83:493–501.

38. Tsujii M, Kawano S, Tsuji S, et al. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 1998;93: 705–16.

39. Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci U S A 1997;94:3336–40.

40. Cheng J, Imanishi H, Amuro Y, Hada T. NS-398, a selective cyclooxygenase 2 inhibitor, inhibited cell growth and induced cell cycle arrest in human hepatocellular carcinoma cell lines. Int J Cancer 2002; 99:755–61.