Inhibition of Azoxymethane-Induced Colorectal Cancer by CP-31398, a TP53 Modulator, Alone or in Combination with Low Doses of Celecoxib in Male F344 Rats

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Abstract

Tumor suppressor p53 plays a major role in colorectal cancer development. The present study explores the effects of p53-modulating agent CP-31398 alone and combined with celecoxib on azoxymethane-induced aberrant crypt foci (ACF) and colon adenocarcinomas in F344 rats. Maximum tolerated doses were 400 and 3,000 ppm for CP-31398 and celecoxib, respectively. ACF and tumor efficacy endpoints were carried out on azoxymethane-treated 7-week-old rats (48 per group) fed the control AIN-76A diet. Two weeks after carcinogen treatment, rats were fed the diets containing 0, 150, or 300 ppm CP-31398, 300 ppm celecoxib, or 150 ppm CP-31398 plus 300 ppm celecoxib. ACF and colon adenocarcinomas were determined at 8 and 48 weeks after azoxymethane treatment, respectively. Dietary CP-31398 was shown to suppress mean colonic total ACF by 43% and multicrypt ACF by 63%; dietary CP-31398 at 150 and 300 ppm was shown to suppress mean colonic total ACF by 30.4% (P < 0.02) and 44% (P < 0.005), respectively, and adenocarcinoma multiplicity by 51% (P < 0.005) and 65% (P < 0.0001), respectively. Dietary celecoxib suppressed colon adenocarcinoma incidence (60%; P < 0.0003) and multiplicity (70%; P < 0.0001). Importantly, combination of low-dose CP-31398 and celecoxib suppressed colon adenocarcinoma incidence by 78% and multiplicity by 90%. Rats that were fed the high-dose CP-31398 or a combination of low-dose CP-31398 and celecoxib showed considerable enhancement of p53 and p21WAF1/CIP expression, apoptosis, and reduced tumor cell proliferation in colonic tumors. These observations show, for the first time, that CP-31398 possesses significant dose-dependent chemopreventive activity in a well-established colon cancer model and that a combination of low-dose CP-31398 and celecoxib significantly enhanced colon cancer chemopreventive efficacy. [Cancer Res 2009;69(20):8175–82]

Introduction

Epidemiologic and experimental studies indicate that the risk of developing colon cancer may be attributable to genetic and environmental factors, including endogenously occurring promoting agents (1–3). The p53 tumor suppressor protein is involved in DNA damage repair, genomic instability, cell cycle arrest, and apoptosis through transcriptional regulation of genes implicated in these pathways (4–10). Although mutations affecting p53 are present in >50% of all cancers, including colon cancer (4, 5), “stress-induced” nonmutational activation of p53 may occur very early in cancer progression (6–8).

Several attempts to restore mutant p53 as a growth suppressor included microinjection of monoclonal antibody 421, COOH-terminal peptide of p53, and small molecules such as CP-31398 and PRIMA-1 (11–14). CP-31398 can stabilize p53, protect against thermal denaturation, and maintain monoclonal antibody 1620 epitope conformation in newly synthesized p53 (11). CP-31398 stabilizes wild-type p53 in cells by inhibiting Mdm2-mediated ubiquitination and degradation (15). In a chromatin immunoprecipitation assay, CP-31398 promotes binding of mutant p53 to p53 response elements in vitro (16). Other studies using the purified p53 core domain have shown that CP-31398 can restore DNA-binding activity to mutant p53 in vitro (17). Moreover, small-molecule modulators of p53, including CP-31398, appear to suppress growth of human colon tumor xenografts (18) and prevent UVB-induced squamous skin cancer in mice by restoring mutant p53 function (19). Recently, we have shown that CP-31398 administered in the diet suppressed APCmin intestinal tumors in a dose-dependent manner by up-regulating p53 protein levels and downstream signaling molecules (20).

The role of cyclooxygenase-2 (COX-2) in colon carcinogenesis is well established (21, 22). Previously, we have shown chemopreventive effects by the COX-2 selective inhibitor celecoxib in rodent models of colon cancer (23, 24). Of particular interest is the observation that COX-2 metabolites, particularly electrophilic prostaglandins, appear to impair p53 protein function; COX-2 inhibition by celecoxib increases the nuclear localization of functionally active p53 (25). However, although efficacy has been attributed to COX-2 inhibitors, five clinical trials showed that three different COX-2 inhibitors caused an increased rate of myocardial infarction, leading to concerns about the broader applicability of selective COX-2 drugs. It is important, therefore, to develop strategies that target both COX-2 inhibition and up-regulation of p53 in a clinical setting.

Our current study was undertaken to reconcile potential and otherwise known toxicities by combining two agents, each with different modes of action and proven efficacy, at much lower concentrations than if used individually, to reduce toxicity and enhance efficacy. Specifically, our proposed studies were designed to test whether nontoxic low-dose celecoxib in combination with nontoxic low-dose CP-31398 would provide better protection in a well-established colon cancer model. The effects of CP-31398 and celecoxib on colonic tumor cell proliferation, apoptosis, and expression levels of p53 and p21WAF1/CIP were also determined.
Materials and Methods

Animals, diets, and chemopreventive agents. All animal experiments were done in accordance with NIH guidelines and University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee–approved protocol. Male F344 rats were obtained at age 6 weeks from Harlan Laboratories. Ingredients for the semipurified diets were purchased from Bioserv and stored at 4°C before diet preparation. Diets were based on the modified AIN-76A diet. The semipurified diet includes 20% casein, 52% corn starch, 13% dextrose, 5% corn oil, 5% alphacel, 3.5% AIN mineral mix, 1.2% AIN revised vitamin mix, 0.3% niacin, and 0.2% choline bitartrate (26). CP-31398 and celecoxib were premixed with a small quantity of diet, and then blended into bulk diet using a Hober Mixer. Both control and experimental diets were prepared weekly and stored in a cold room. CP-31398 and celecoxib were provided by the National Cancer Institute chemopreventive drug repository. The agent(s) content in the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution.

Maximum tolerated dose. Experimental design to carry the maximum tolerated dose (MTD) bioassay is shown in Fig. 1A. To estimate the appropriate dose level for the efficacy studies, MTD was determined by feeding male F344 rats CP-31398 and celecoxib in a 8-week toxicity study. MTD was defined as the highest dose that causes no more than a 10% body weight decrement or produces mortality or any external signs of toxicity that would be predicted to shorten the natural lifespan of the animal. At age 7 weeks, groups of male rats (9 per group) were fed the experimental diets containing 0, 100, 200, 400, 800, or 1,600 ppm CP-31398 and 0, 500, 1,000, 2,000, 3,000, and 4,000 ppm celecoxib. Although celecoxib has been extensively studied in animal models by several investigators, no systematic studies have thus far been conducted on MTD of this agent in any animal model. Body weights were recorded twice weekly for 8 to 9 weeks. All animals were monitored daily for signs of toxicity such as ill appearance, circling rashes, tremors, roughened coat, rhinitis, chromatocorrorrea, and prostration. At the end of 6 weeks, mice were sacrificed and their oral cavity, colon, small intestine, stomach, liver, and kidneys were examined for any abnormalities under a dissection microscope.

Experimental protocol for CP-31398–induced colonic aberrant crypt foci inhibition. The experiment was designed to evaluate whether CP-31398 provides protection against the azoxymethane-induced colonic preneoplastic lesions in rats. Experimental design is summarized in Fig. 1B. Based on the MTD, different doses of CP-31398 were administered continuously from 1 week before carcinogen treatment until the end of the study. At age 7 weeks, groups of 12 rats per group (azoxymethane-treated rats) were fed either the control diet or experimental diet containing 0, 100, 200, or 400 ppm CP-31398. At age 8 weeks, rats intended for carcinogen treatment were injected s.c. with azoxymethane (Midwest Research Institute) at a dose rate of 15 mg/kg body weight once weekly for 2 weeks, and those intended for vehicle treatment received an equal volume of normal saline. These dietary regimens were continued until termination of the experiment 8 weeks after the second azoxymethane treatment. Rats were killed by CO2 euthanasia, and all organs were examined grossly. Colonos were evaluated for aberrant crypt foci (ACF). For this evaluation, they were slit open lengthwise from the anus to the cecum and then fixed flat with mucosa on the upper side between filter papers in 10% buffered formalin.

Quantification of ACF. Topographic analysis of the colonic mucosa was done according to Bird (27) as routinely performed in our laboratory (28, 29). After a minimum of 24 h, fixed colons were stained with 0.2% methylene blue solution for 5 to 10 min, placed mucosal side up on a microscopic slide, and viewed under a light microscope. Total number of ACF in the entire colon was determined in every 2 cm section of the colon, starting from the distal (taken as 0 cm) to the proximal end of the colon. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, increased distance from lamina to basal surfaces of cells, and easily discernible pericryptal zone. The parameters used to assess the aberrant crypts were incidence and multiplicity. Aberrant crypt multiplicity was determined as the number of crypts in each focus and categorized as containing up to four or more aberrant crypts/focus.

Chemoprevention of azoxymethane-induced colorectal adenocarcinoma by CP-31398 alone or in combination with celecoxib. The experimental protocol is summarized in Fig. 1C. Two dose levels of CP-31398 or 300 ppm celecoxib and a single low-dose combination of CP-31398 and celecoxib were evaluated for their chemopreventive efficacy. Based on MTD and ACF studies, we selected 150 and 300 ppm CP-31398 and 300 ppm celecoxib (<8% MTD), considering these doses to be safe for long-term administration. Studies were designed to determine the efficacy of CP-31398 and celecoxib alone or in combination administered in the diet during the post-initiation stage of azoxymethane-induced colon carcinogenesis.

Figure 1. Experimental protocol for the evaluation of MTD and chemopreventive efficacy of CP-31398 and celecoxib in a rat colon cancer model. A, MTD assay; groups of rats (n = 9) were fed the control diet (AIN 76A) or diets containing 100, 200, 400, 800, and 1,600 ppm CP-31398 and 0, 500, 1,000, 2,000, 3,000, or 4,000 ppm celecoxib administered at age 7 wk for 8 to 9 wk to identify the optimal dose. B, ACF assay; groups of rats (n = 12) were fed the control diet (AIN 76A) or diets containing 100, 200, or 400 ppm CP-31398 at age 7 wk; 1 wk later, rats were given 15 mg azoxymethane (AOM)/kg body weight s.c. once weekly for 2 wk; 8 wk after azoxymethane treatment, rats were killed and colons were histologically evaluated for ACF as described in the text. C, colon tumor efficacy assay; groups of rats (n = 36) were fed the control diet (AIN 76A) at age 7 wk; 1 wk later, rats were given 15 mg azoxymethane (AOM)/kg body weight s.c. once weekly for 2 wk. After 2 wk, azoxymethane-treated rats were fed the control diet, 150 and 300 ppm CP-31398, 300 ppm celecoxib, or 150 ppm CP-31398 plus 300 ppm celecoxib for 48 wk to assess colorectal adenocarcinomas.

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Animals: Male F344 Rats. MTD: 9 rats/group; ACF: 18 rats/group; Tumor Efficacy (48 rats/group) Carcinogen: Azoxymethane (AOM), 15 mg/kg Body Weight
treatment groups were cut longitudinally to 5-immunohistochemistry (24). Paraffin-embedded colons from different and celecoxib on tumor cell proliferation by BrdUrd incorporation using for formalin and processed by standard methods for histopathologic conditions with a 12-h light and dark cycle at 50% relative humidity and 21°C. At age 8 weeks, animals intended (46 rats per group, 36 azoxymethane-treated and 12 vehicle treated) for carcinogenesis treatment received 2 weekly s.c. injections of azoxymethane (15 mg/kg body weight). Vehicle-treated groups (12 rats per group) received an equal volume of normal saline instead of azoxymethane. Two weeks after the second injection of azoxymethane or normal saline, rats were placed on control diet or diets containing two doses of CP-31398 and 300 ppm celecoxib or low-dose combination of both agents as outlined in Fig. 1C. Body weights were recorded every 2 weeks until the 16th week and then every 4 weeks until termination of the experiment 50 weeks after the last azoxymethane treatment. Eighteen hours before the termination, 6 to 8 rats from each group were administered 50 mg bromodeoxyuridine (BrdUrd)/kg body weight i.p. to assess cell proliferation. Moribund animals were killed and necropsied. Two rats in the control diet and one rat in the low-dose CP-31398 developed ear duct tumors and these moribund rats were killed 4 weeks before scheduled termination. All organs, including intestine, were examined grossly under the dissecting microscope. Colon tumors with a diameter >0.4 cm were cut into halves; one half was quickly frozen in liquid nitrogen and stored at -80°C until analyzed for expression and activity of COX isozymes and markers of apoptosis and cell proliferation. Remaining portions of tissues were fixed in 10% neutral buffered formalin and processed by standard methods for histopathologic evaluation (27).

**BrdUrd assay for cell proliferation.** We assessed the effect of CP-31398 and celecoxib on tumor cell proliferation by BrdUrd incorporation using immunohistochemistry (24). Paraffin-embedded colons from different treatment groups were cut longitudinally to 5-μm-thick sections and mounted on microscopic slides. After deparaffinization, sections were blocked for endogenous peroxidase activity and incubated with 1% milk. BrdUrd antibody (Pharmingen) was applied at a 1:200 dilution for 1 h at room temperature, washed and incubated with secondary anti-rabbit IgG for 30 min, and washed and incubated with avidin-biotin-complex reagent (Vector Laboratories). After rinsing with PBS, the slides were incubated with the chromogen 3,3′-diaminobenzidine for 3 min and then rinsed and counterstained with hematoxylin. Scoring was done by two investigators blinded to the identity of the BrdUrd-positive cells at ×400 magnification. Cells with a brown nucleus were considered positive. The proliferation index was determined by dividing the number of positive cells by the number of negative cells and multiplying by 100.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay for apoptosis.** Sections (5 μm) were cut and mounted on slides, dehydrated, and stained using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method as described in our previous publication (20). Stained apoptotic epithelial cells (a minimum of 10 microscopic fields per section) were counted manually in a single-blinded fashion. Scoring index will be similar to proliferation index as described above.

**p53 and p21<sub>WAF1/CIP1</sub> expression.** Expression of p53 and p21<sub>WAF1/CIP1</sub> in colonic tumor tissues was assessed by standard immunohistochemistry and Western blot methods by applying p53 and p21<sub>WAF1/CIP1</sub> (Santa Cruz Biotechnology) primary antibodies.

**Total COX and COX-2 synthetic activity.** COX activities in colon tumor samples (4-8 per group) were assayed using our previously published method (36). Briefly, the microsomal pellet was suspended in 30 mmol/L potassium phosphate (pH 7.4). To determine total COX activity, a 150 μl reaction mixture containing 12 μmol/L [14C]arachidonic acid (420,000 dpm), 1 mmol/L epinephrine, 1 mmol/L glutathione in 50 mmol/L phosphate buffer, and 25 to 35 μg tumor microsomal protein were incubated at 37°C for 15 min. To determine COX-2 activity, the reaction mixture was preincubated with 150 μmol/L aspirin to block COX-1 activity and modify COX-2 activity to 15(R)-HETE. The reaction was terminated by adding 40 μL of 0.2 mol/L HCl. COX metabolites of arachidonic acid were extracted three times with 0.5 mL ethyl acetate, evaporated to dryness under N<sub>2</sub>, redissolved in chloroform, and subjected to TLC on silica G plates. Metabolites of [14C]arachidonic acid corresponding to prostaglandin E<sub>2</sub>; prostaglandin F<sub>2α</sub>, prostaglandin D<sub>2</sub>, 6-keto-prostaglandin F<sub>1α</sub>, and thromboxane B<sub>2</sub> were detected by their comigration with authentic standards for total COX activity and [14C]-15(R)-HETE for COX-2 activity. Radioactivity was counted with a BioScan 2000 Radiomatic Detector and results were expressed as pmol or nmol/mg protein/min.

**Statistical analyses.** All results are expressed as mean ± SE. Differences in body weights among groups were analyzed by ANOVA. Tumor incidences (percentage of rats with tumors) among dietary groups were compared by the χ<sup>2</sup> method. ACE, tumor multiplicity (number of tumors per rat), protein expression and activities, and proliferative and apoptotic indexes were analyzed by unpaired t test with Welch’s correction. Dose-response effect was analyzed by linear regression (r<sup>2</sup>) analysis. Differences were considered statistically significant at P < 0.05.

**Figure 2.** Effect of dietary CP-31398 (CP; A) and celecoxib (Coxib; B) on body weight gain of male F344 rats. Mean ± SE (n = 10-12).
Results

MTD and dose selection for efficacy studies. Effects of dietary CP-31398 and celecoxib on body weight of male F344 rats are summarized in Fig. 2 and B. Dietary administration of CP-31398 at 100 to 400 ppm for 9 weeks had no significant effect on the body weights of rats. Dietary CP-31398 at 800 and 1,600 ppm for 9 weeks appears to have resulted in no weight gain compared with untreated rats, although the rats in question were active and survived until termination of the experiments.

Rats fed celecoxib at 500 to 3,000 ppm for 8 weeks showed body weight gain compared with the control diet group. At 4,000 ppm celecoxib, rats had slightly lower body weight gain during weeks 2 to 4 but had significant body weight loss beginning at week 6 (P < 0.05) to week 8 (P < 0.01) albeit with no significant toxicity or animal death. Based on these studies, we determined a MTD for CP-31398 of 400 ppm and for celecoxib of >3,000 ppm but <4,000 ppm in male F344 rats. Chronic administration of CP-31398 and celecoxib at their respective MTDs, individually or together, produced no outward signs of toxicity nor any gross changes indicative of toxicity in the organs examined.

Effect of CP-31398 on azoxymethane-induced colonic ACF. The effects of dietary CP-31398 on azoxymethane-induced colonic ACF formation are shown in Fig. 3A and B. Azoxymethane-treated rats fed diets containing 100, 200, and 400 ppm CP-31398 showed a significant decrease in the number of total ACF/colon (18-43%; P < 0.05-0.0005) compared with azoxymethane-treated rats fed a control diet (mean ± SE, 168 ± 10 colon ACF), including, on average, 31 of one crypt foci, 78 of two crypt foci, 35 of three crypt foci, and 24 of four or more crypt foci (Fig 3A). Thus, significant dose-dependent inhibition of total ACF suppression, particularly of ACF containing four or more crypt foci (17-63%; P = 0.08-0.0001), was seen using dietary CP-31398 (Fig. 3B).

CP-31398 decreased incidence and multiplicity of colon adenocarcinomas. The effects of dietary CP-31398 on azoxymethane-induced colon adenocarcinomas are shown in Fig. 4A and B. Azoxymethane-treated rats fed a control diet formed colon tumors at an incidence (percentage of rats with colon adenocarcinomas) of 74% and multiplicity (number of adenocarcinomas/colon) of 1.42 ± 0.21 (mean ± SE; n = 36). In contrast, azoxymethane-treated rats fed CP-31398 at 150 ppm inhibited colon adenocarcinoma incidence by 32% (P < 0.02) and multiplicity by 51.4% (P < 0.005); the corresponding values for CP-31398 at 300 ppm were 44% (P < 0.001) and 64.8% (P < 0.0003). A linear regression analysis of CP-31398 dose against tumor multiplicity yielded a statistical coefficient of r = 0.994 (P < 0.0001). Dietary celecoxib at 300 ppm suppressed colon adenocarcinoma incidence by 62% and multiplicity by 75.3% in agreement with previous studies concerning the effects of celecoxib on colon adenocarcinomas (23, 31). However, combining low-dose CP-31398 (150 ppm) and celecoxib (300 ppm) significantly suppressed colon adenocarcinoma incidence by 77.6% (P < 0.0001) and multiplicity by 89.8% (P < 0.0001), which was statistically better than treatment with celecoxib at 300 ppm alone (Fig. 4B). Administration of CP-31398, celecoxib, or a combination of both significantly reduced colon tumor volume compared with control (data not shown). Thus, the combination approach using low doses of celecoxib with a low-dose p53-modulating agent such as CP-31398 provided significant additive tumor-inhibitory effects.

Effect of CP-31398 on colonocyte proliferation and apoptosis.

The effect of CP-31398 and celecoxib as potential chemopreventive agents was assessed by cell kinetics analyses. Figure 5A (top) shows BrdUrd incorporation in azoxymethane-induced colon tumors of rats fed CP-31398 alone or in combination with celecoxib. Figure 5C summarizes cell proliferation results as measured by BrdUrd-positive cells. Administration of dietary CP-31398 at 150 ppm inhibited the proliferation index by ~25%, but this inhibition did not reach statistical significance. However, dietary CP-31398 at 300 ppm significantly suppressed the proliferative index in azoxymethane-induced colon tumors (42%) compared with control diet. Celecoxib at 300 ppm significantly suppressed BrdUrd incorporation in colon tumor cells (~52%). Importantly, the combination of CP-31398 at 150 ppm and celecoxib at 300 ppm inhibited colon tumor cell proliferation by ~64%. Although combinational agents–induced inhibition of colon tumor cell proliferation is more compared with celecoxib alone, this inhibition did not reach statistical significance (P = 0.064). Figure 5B represents the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay on colon tumor cell apoptosis.

![Figure 3](image_url)

**Figure 3.** Inhibitory effect of CP-31398 on azoxymethane-induced colonic ACF formation in male F344 rats. **A,** effect of CP-31398 on mean colon ACF/rat. **B,** effect on the colonic multicrypt foci (four or more). Mean ± SE (n = 12).
Modulation of p21<sub>WAF1/CIP1</sub> and p53. Expression levels of p53 and of p21<sub>WAF1/CIP1</sub> are important indicators of cell growth arrest and apoptosis. As shown in Fig. 6A and B, CP-31398 dose-dependently induced expression of p21<sub>WAF1/CIP1</sub> protein in colonic tumor tissues (23–48%); however, limited expression of p21<sub>WAF1/CIP1</sub>, presumably p53-induced, was observed in tumor tissues of control diet–fed rats. Induction of p21<sub>WAF1/CIP1</sub> expression in the colonic tumors of rats fed 300 ppm celecoxib was observed in ~68% tumor cells, whereas colonic tumors of rats fed a combination of CP-31398 (150 ppm) and celecoxib (300 ppm) showed >85% of p21<sub>WAF1/CIP1</sub> overexpression. Similarly, p53 expression in rats fed CP-31398 resulted in a dose-dependent enhancement of p53 in colonic tumors compared with those from rats fed a control diet (Fig. 6A and B). Further, celecoxib at 300 ppm also induced significant expression of p53 in colonic adenocarcinomas. Significant synergistic induction of both p53 and p21 protein expression was observed in rats fed with low doses of CP-31398 and celecoxib (Fig. 6A and B).

**Discussion**

Restoration of mutant p53 function and/or enhancement of wild-type p53 expression by genetic means has been shown to suppress growth of various tumor types (10, 18, 32). The identification of CP-31398 and other small molecules such as PRIMA-1 that rescue/activate mutant p53 could constitute an effective pharmacologic approach for cancer prevention and treatment (33–38). Although CP-31398 has been extensively studied in *in vitro* models (9, 11–15, 18–20), only a few studies have shown the tumor-inhibitory potential of CP-31398 *in vivo* (18–20). Recently, we have shown ~75% suppression of intestinal polyps by dietary CP-31398 (200 ppm) in APC<sup>min</sup> mice (20). The azoxymethane-induced colon carcinogenesis model in F344 rats is well established and has been used to develop chemopreventive agents for clinical trials (39), including celecoxib (40). Our primary objective was to test whether low-dose CP-31398 would further augment very low dose effects of celecoxib on colonic adenocarcinoma development.

Dose selections of CP-31398 for tumor efficacy studies are based on a 9-week MTD assay. In the present study, effects of 800 and 1,600 ppm CP-31398 on the body weight of rats showed that high-dose levels completely retarded weight gain albeit without significant toxic symptoms or survival and/or food intake differences for 9 weeks compared with controls. We have determined the MTD for dietary CP-31398 in male F344 rats to be >400 ppm, with an applied dose of CP-31398 in colon adenocarcinoma equal to
∼ 30% to 60% MTD. Also, we have evaluated MTD of celecoxib in the present study primarily due to the fact that to date there is no published preclinical data for this agent. As shown in Fig. 2B, celecoxib administered at 4,000 ppm in diet showed 11.2% body weight suppression without visible organ toxicities in rats. When compared with other selective COX-2 inhibitors and traditional nonsteroidal anti-inflammatory drugs, celecoxib tolerability is very high (4, 22–24). Previous studies from our laboratory and others frequently used ∼ 1,500 ppm in the diet. In the present study, we used 300 ppm celecoxib (∼ 8% MTD), a significantly lower dose (23, 24). Use of low-dose celecoxib is strongly preferred based on human clinical trials showing that prolonged higher doses of celecoxib are associated with increased prothrombotic effects and risk of cardiovascular disease (40, 42–44).

Our results are the first to show that CP-31398 effectively inhibits azoxymethane-induced colonic preneoplastic lesions (ACF) and adenocarcinomas in the rat. These results further corroborate the preventive effects of CP-31398 in UVB-induced skin carcinogenesis SKH-1 mice and intestinal neoplasia in APCmin/− mice (19, 20). In the skin model, CP-31398 was administered either i.p. or topically; in APCmin/− mice and in the present study, CP-31398 was administered in the diet. By different routes of administration, CP-31398 showed profound chemopreventive effects. In the APCmin/− mice studies, tumor suppression by dietary CP-31398 was more pronounced early during tumor development (20). In the present study, dose-dependent suppression of colonic ACF and adenocarcinomas clearly suggests the potential usefulness of CP-31398 in chemoprevention of colon cancer. The efficacy of CP-31398 in this study is comparable with several potential colon cancer chemopreventive agents (e.g., celecoxib, sulindac, curcumin, oltipraz, and nitric oxide–releasing nonsteroidal anti-inflammatory drugs) and other agents (23, 24, 30, 31, 45–47).

Another major objective of the present study was to evaluate low-dose CP-31398 together with low-dose celecoxib in the azoxymethane-induced model of colon adenocarcinoma. Our results show that a low nontoxic dose of CP-31398 profoundly enhanced the chemopreventive properties of low nontoxic dose celecoxib. Remarkably, the combined efficacy of this regimen compares favorably with the outcome efficacy of, for example, high-dose (1500 ppm) celecoxib or nonsteroidal anti-inflammatory drugs applied at >60% MTD, or 4,000 ppm difluoromethylornithine, an ornithine decarboxylase inhibitor in rat colon cancer models (23, 24, 45, 48). Thus, the combination regimen applied in this study supports our previous low-dose combination approaches to suppress colon cancers in a synergistic or additive manner (31, 48, 49).

The importance of p53 mutations in colon cancers is well established (4, 5). However, in the rodent models of colon cancer, p53 mutations likely represent a late event in tumor development. This may suggest that restoration/rescue of mutant p53 function plays a lesser role in the tumor-inhibitory activity of CP-31398. However, as shown here, limited expression of p21WAF1/CIP1, ...

Figure 5. Immunohistochemical staining of cell proliferation (BrdUrd; A) and apoptosis (TUNEL; B) in colonic tumors of rats administered 0, 150, or 300 ppm CP-31398, 300 ppm celecoxib, or 150 ppm CP-31398 plus 300 ppm celecoxib. Representative 3,3'-diaminobenzidine–stained (brown) cells with either BrdUrd or TUNEL are depicted at ×400 or ×200 magnification. Effect of CP-31398 and celecoxib alone or combined on azoxymethane-induced colon tumor cell BrdUrd labeling index (C) and apoptotic index (D) in rats. Mean ± SE (n = 6; 4 rats in the combination group) and significance between control and treatment groups was analyzed by t test.
presumably p53-induced, was observed in tumor tissues of control diet–fed rats. Thus, although nonmutational activation of wild-type p53 appears to play a major role during early colon tumor development (8), further activation by CP-31398 might represent yet another mechanism leading to suppression of tumor growth in rat colon. In this regard, activation of wild-type p53 by CP-31398 has been shown in other models both in vitro and in vivo (11–19). We have shown that dietary CP-31398 inhibited intestinal tumors in APCmin/+ mice concurrent with induction of p53 expression and downstream signaling molecules, leading to both inhibition of proliferation and induction of apoptosis (20). Significantly, an effect by dietary CP-31398 on collateral targets, such as, for example, COX-2 in azoxymethane-induced colon cancers, presents a novel mechanism.

As shown in Supplementary Fig. S1, there is mechanistic rationale to test the combination of a p53-modulating agent with COX-2 inhibitors. We and others have shown that COX metabolites, particularly electrophilic prostaglandins, significantly impair the translocation of p53 to the nucleus and that COX-2 selective inhibitors facilitate this nuclear translocation (25, 50). Thus, the present study provides mechanistic validation for the development of a combination approach of p53 modulators with very low dose COX-2 selective inhibitors such as celecoxib or other nonsteroidal anti-inflammatory drugs. Ultimately, combining low molecular weight p53-modulating agents acting through different mechanisms, or combining agents targeting other molecular pathways such as COX, is likely to substantially increase antitumor effects. Taken together, these findings support further development of CP-31398 alone or in combination with celecoxib for colon cancer prevention and treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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Inhibition of Azoxymethane-Induced Colorectal Cancer by CP-31398, a TP53 Modulator, Alone or in Combination with Low Doses of Celecoxib in Male F344 Rats

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