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Suppression of Familial Adenomatous Polyposis by CP-31398, a TP53 modulator, in APC^{min/+} Mice¹

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Abstract

p53 mutations occur in a large number of human malignancies. Mutant p53 is unable to affect downstream genes necessary for DNA repair, cell cycle regulation, and apoptosis. The styrylquinazoline CP-31398 can rescue destabilized mutant p53 expression and promote activity of wild-type p53. The present study examines chemopreventive effects of CP-31398 on intestinal adenoma development in an animal model of familial adenomatous polyposis (FAP). Effects were examined at both early and late stages of adenoma formation. Effects of CP-31398 on early-stage adenomas were determined by feeding 7-week-old female C57B/6J-APC^{min} (heterozygous) and wild-type C57BL/6J mice with American Institute of Nutrition (AIN)-76A diets containing 0, 100, or 200 ppm CP-31398 for 75 days. To examine activity toward late-stage adenomas, CP31398 administration was delayed until 15 weeks of age and continued for 50 days. During early-stage intervention, dietary CP-31398 suppressed development of intestinal tumors by 36% ($p < 0.001$) and 75% ($p < 0.0001$), at low and high dose, respectively. During late-stage intervention, CP-31398 also significantly suppressed intestinal polyp formation, albeit to a lesser extent than observed with early intervention. Adenomas in treated mice showed increased apoptotic cell death and decreased proliferation in conjunction with increased expression of p53, p21^{WAF1/CIP}, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP). These observations demonstrate for the first time that the p53-modulating agent CP-31398 possesses significant chemopreventive activity *in vivo* against intestinal neoplastic lesions in genetically-predisposed APC^{min/+} mice. Chemopreventive activity of other agents that restore tumor suppressor functions of mutant p53 in tumor cells is currently under investigation.

Introduction

Colorectal cancer is a leading cause of cancer death in Western countries, including the United States. About 145,290 new cases of colorectal cancer and 56,290 related deaths are expected in 2008 (1). Epidemiological and experimental studies indicate that the risk of developing colon cancer may be attributable to genetic and environmental factors, including endogenously occurring promoting agents (2). Progression of normal epithelium to colon cancer is a multistep process involving accumulation of multiple genetic alterations (3,4). The p53 tumor suppressor protein is involved in DNA damage repair, cell cycle arrest, and apoptosis through transcriptional regulation of genes implicated in these pathways and by direct interaction with other proteins (5,6). Although non-mutational activation of p53 may occur very early during cancer progression (7), mutations that inactivate p53 are present in over 50% of all cancers,

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including colon cancer, giving rise to aggressive cancers that are difficult to treat by chemotherapy or ionizing radiation (7,8). p53 mutations alter the structure and thermal stability of the protein (9,10), affecting its ability to bind to p53 response elements and regulate transcription of downstream genes (11). In addition, we and others have shown that increased levels of electrophilic lipids bind with p53 and block its nuclear translocation, leading to reduced p53 activity (12).

Several attempts to restore mutant p53 as growth suppressor included micro-injection of monoclonal antibody 421, C-terminal peptide of p53, and small molecules such as CP-31398 and PRIMA1 (13–19). Early on, we determined that dietary CP-31398 protects against chemically induced early colonic neoplastic lesions (20). More recently, Wang *et al.* showed that p53 modulators suppress growth of human colon tumor xenografts (21). Similarly, Tang *et al.* found that CP-31398 suppressed UAB-induced squamous skin cancer in mice by restoring mutant p53 function (22). In this regard, CP-31398 can stabilize p53, protect against thermal denaturation, and maintain monoclonal antibody 1620 epitope conformation in newly synthesized p53 (16). CP-31398 stabilizes wild-type p53 in cells by inhibiting Mdm2-mediated ubiquitination and degradation (18,23). In a chromatin immunoprecipitation (ChIP) assay, CP-31398 promotes binding of mutant p53 to p53 response elements *in vivo* (24). Other studies using the purified p53 core domain have shown that CP-31398 can restore DNA-binding activity to mutant p53 *in vitro* (25). Given the multifunctional properties of p53 in cell pathway regulation, it is difficult to determine the exact mechanism by which CP-31398 and other p53 modulators affect p53-induced growth arrest or apoptosis.

As part of a study of p53 modulating agents we identified a tolerable dose of CP-31398 and demonstrated its efficacy in a well-established (*APC*^{min/+}) model of mouse intestinal neoplasia. We also determined the effects of CP-31398 on intestinal tumor proliferation, apoptosis, and levels of p53, p21^{WAF1/CIP}, cleaved caspase-3, and cleaved PARP.

Materials and Methods

Animals, diets, CP-31398

Heterozygous female Min (*C57BL/6J-APC*^{min/+}) and wild-type *C57BL/6J* female mice were obtained at 5 weeks of age from Jackson Laboratories (Bar Harbor, ME). Ingredients for the semi-purified diets were purchased from Dyets, Inc. (Bethlehem, PA) and stored at 4°C prior to diet preparation. Diets were based on the modified AIN-76A diet. The high-fat semi-purified diet includes 21.3% casein, 43.5% corn starch, 12% dextrose, 12% corn oil, 5% alphacel, 3.5% AIN mineral mix, 1.2% AIN revised vitamin mix, 0.3% *D, L*-methionine, and 0.2 choline bitartrate (26). CP-31398 was premixed with a small quantity of diet, then blended into bulk diet using a Hobart Mixer (Troy, OH). Both control and experimental diets were prepared weekly and stored in a cold room. CP-31398 was kindly provided by the NCI chemopreventive drug repository (Rockville, MD). Agent 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 (MTD)

To estimate the appropriate dose level for the efficacy study, MTD was determined in female *C57BL/6J* mice by feeding CP-31398 in a 6-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 7 weeks of age, groups of female *C57BL/6J* mice (6/group) were fed experimental diets containing 0, 75, 150, 300, 600, or 1,200 ppm CP-31398, and body weights were recorded twice weekly for 6 weeks. All animals were monitored daily for signs of toxicity such as ill

appearance, circling rashes, tremors, roughened coat, rhinitis, chromodacryorrhea, 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.

Efficacy Study

The experimental protocol is summarized in Fig. 1. Following five days of quarantine, all mice were distributed so that average body weights in each group were about equal (10 *APC*^{min/+} mice in each group and 6 wild-type mice as parallel treatment groups to compare food intake, growth rate, and tumor formation in *APC*^{min/+}). The animals were transferred to a holding room where they were housed individually in plastic cages with filter tops. Laboratory conditions were controlled to maintain a 12-hour light/dark cycle at 50% relative humidity and at $21 \pm 1^\circ\text{C}$. Mice were fed control or experimental diets containing 100 ppm or 200 ppm CP-31398 at either early intervention (7 weeks of age) or late intervention (15 weeks of age) until termination of the study (~75 days for early intervention, 50 days for late intervention). Animals were weighed twice weekly and monitored daily for signs of weight loss or lethargy that might indicate intestinal obstruction or anemia. After necropsy, intestinal tracts were dissected from esophagus to distal rectum, spread onto filter paper, opened longitudinally with fine scissors, and cleaned with sterile saline, then examined under a dissection microscope with 5x magnification for tumor counts. This procedure was completed by two individuals who were blinded to experimental group and genetic status of mice. Colonic and other small intestinal tumors that required further histopathological evaluation to identify adenoma, adenocarcinoma, and enlarged lymph nodes were fixed in 10% neutral-buffered formalin, embedded in paraffin blocks, and processed by routine hematoxylin and eosin staining. In addition, multiple samples of tumors from the small intestines and colons and normal-appearing colonic mucosa were harvested and stored in liquid nitrogen for analysis of p53, cleaved caspase-3, and cleaved PARP expression levels.

Immunohistochemistry

To evaluate the effect of CP-31398 on proliferation, we assessed proliferating cell nuclear antigen (PCNA) expression in large intestinal tumor tissue (delayed intervention) sections by immunohistochemistry, as described (16). Briefly, paraffin-embedded colons from the delayed intervention study were cut longitudinally to five-micron-thick sections and mounted on microscopic slides. After deparaffinization, sections were blocked for endogenous peroxidase activity and incubated with 1% milk. PCNA antibody (Pharmingen, San Diego, CA) was applied at a 1:200 dilution for one hour at room temperature, then washed and incubated with secondary anti-rabbit IgG for 30 min, and then washed and incubated with avidin biotin-complex reagent (Vector Laboratories, Burlingame, CA). After rinsing with PBS, the slides were incubated with the chromogen 3, 3'-diaminobenzidine (DAB) for three minutes, then rinsed and counterstained with hematoxylin. Scoring was performed by two investigators blinded to the identity of the samples who scored at least 30 crypts per colon (light microscopy at $\times 400$ magnification). Cells with a brown nucleus were considered positive. The proliferation index (PI) was determined by dividing the number of positive cells per crypt by the number of cells of the entire crypt or each of its compartments (upper, middle, and lower) and multiplying by 100.

Apoptosis

Large Intestinal tumor tissues from delayed intervention were fixed in 10% formalin for 24 hours and then embedded in paraffin. Sections, about $5 \mu\text{M}$, were cut and mounted on slides, rehydrated, and stained using the terminal dUPT-mediated nick end labeling (TUNEL) method. Briefly, slides were incubated with 3% H_2O_2 in PBS for 5 minutes, rinsed, and then incubated

in TdT buffer (140 mM cacodylate (pH 7.2), 30 mM Tris HCl, and 1 mM CoCl₂) for 15 minutes at room temperature. TdT reaction mixture [0.2 unit/μl TdT, 2 mM biotin-11-dUTP, 100 mM cacodylate, 2.5 mM CoCl₂, 0.1 mM DTT and 0.05 mg/ml BSA] was added, and the slides were incubated for an additional 30 minutes at 37°C. After blocking with 2% BSA and incubation with avidin-biotin peroxidase complexes, the TUNEL reaction was visualized by chromogenic staining with DAB, and slides were counterstained by malachite green. Stained apoptotic epithelial cells (a minimum of 10 microscopic fields/section) were counted manually in a single-blind fashion.

Western blot analysis of p53, p21^{WAF1/CIP}, cleaved caspase-3, and cleaved PARP

Intestinal polyps isolated from individual mice were combined to obtain sufficient tissue (6–8 samples/group). Normal-appearing intestinal mucosal samples were homogenized in 1:3 vol. of 100 mM Tris-HCl buffer (pH 7.2) with 2 mM CaCl₂. After centrifugation at 100,000 g for one hour at 4°C, the resulting separations were subjected to 8% SDS-PAGE for p53 and cleaved PARP, and 12% for p21 and cleaved caspase-3. The proteins were electroplated onto polyvinylidene fluoride (PVDF) nitrocellulose membranes as described previously (26). These membranes were blocked for one hour at room temperature with 5% skim milk powder and probed with primary antibodies for one hour. The primary antibodies p53, p21, cleaved caspase-3, and cleaved PARP (Santa Cruz Biotech., Santa Cruz, CA) were used at 1:500 dilutions. Blots were washed three times and incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotech.) at 1:2500 dilutions for one hour. The membranes were washed three times and incubated with Super-Signal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) for five minutes, exposed to Kodak XAR5 photographic film, and developed to detect proteins. Intensities of each band were scanned by a computing densitometer. α -Tubulin (Ab-1) mouse monoclonal antibody (Oncogene, San Diego, CA) was used at 1:1000 dilution as the internal standard for all Western blots.

Statistical analyses

All results are expressed as means $\pm$ SEM and were analyzed by Student's *t*-test. Differences were considered significant at the $P < 0.05$ level.

Results

MTD and Dose Selection for Efficacy Studies

The administration of CP-31398 at 600 ppm and 1,200 ppm in the diet for 6 weeks reduced body weight of mice by 14% and 42%, respectively, as compared to control diet-fed mice. At doses up to 300 ppm, CP-31398 did not affect body weight. Based on these findings, 100 and 200 ppm CP-31398 were selected for efficacy studies. At these doses, CP-31398 had no apparent adverse effects on mice during either the 75-day (early intervention) or 50-day (late intervention) efficacy studies. Body weights of all mice fed diets containing 100 or 200 ppm were comparable; however, body weights of mice fed the control diet were significantly lower (data not shown). This was due mainly to increased small intestinal tumor burden in controls that resulted in impaired food absorption and anemia. Chronic administration of CP-31398 produced no signs of toxicity or any gross changes indicative of toxicity in the organs examined.

Effects on Intestinal Tumor Formation

APC^{min/+} mice spontaneously develop intestinal tumors, essentially all of which (>95%) occur in the small intestine. In this study, on average, mice developed 17–58 (early intervention) or 25–67 (delayed intervention) tumors/mouse in the small intestine, but only 0–3 tumors/mouse in the colon. All histopathologically classified tumors in the small intestine, as well as those

in the colon, were adenomas (adenomatous polyps), with no evidence of local invasion of the lamina propria. Figure 2A summarizes the chemopreventive effect of CP-31398 administered at 100 ppm or 200 ppm on tumor multiplicity in the small intestine. In the early intervention study, the low and high dose of CP-31398 significantly suppressed polyp formation by 39% ($p < 0.001$) and 75.6% ($p < 0.0001$), respectively, compared with controls. In the delayed intervention study, low and high doses of CP-31398 significantly suppressed small intestinal polyp formation by 23% ($p < 0.04$) and 48% ($p < 0.001$), respectively, compared with controls. Data were also analyzed for the colon alone. In the early intervention study, the mean number of tumors/mouse was 1.2 in controls, 0.7 in the low-dose group, and 0.2 in the high-dose group. Delayed intervention found multiplicity of 1.6, 0.9, and 0.5 in control, low- and high-dose groups, respectively. Although both 100 ppm and 200 ppm CP-31398 reduced colon tumor multiplicity, only the higher dose produced statistically significant inhibition (Figure 2B). Also, we observed statistically significant difference between the mice fed 100 ppm and 200 ppm CP-31398 in small intestinal tumor multiplicity in both interventions and in colon tumors of early intervention (Figure 2A and 2B).

Effects on Tumor Cell Proliferation and Apoptosis

Figure 3A and Figure 4 summarize the effects of CP-31398 on tumor cell proliferation in the late intervention study as measured by PCNA overexpression. CP-31398 dose-dependently suppressed proliferation. In the 100 ppm group, proliferation was diminished by 24.5% ($p < 0.002$) and in the 200 ppm group, by 37% ($p < 0.0001$). Figure 3B and Figure 4 show the effects of CP-31398 on tumor cell apoptosis. Compared to controls, low and high doses of CP-31398 induced a 2.2 and 3.8-fold increase in intestinal tumor cell apoptosis.

Modulation of p53, p21^{CIP/WAF1}, cleaved caspase-3, and cleaved PARP

Expression levels of p53, p21^{CIP/WAF1}, cleaved caspase-3, and cleaved PARP are important indicators of cell growth arrest and apoptosis. As shown in Figure 5A, CP-31398 dose-dependently induced expression of p53 protein in intestinal tumor tissues in both early and late intervention protocols. In addition, tumors in mice fed CP-31398 showed significant induction of p21^{CIP/WAF1} expression when compared to controls. However, while CP-31398 effected a dose-dependent increase in p21^{CIP/WAF1} during early intervention, no such effect was seen in late intervention (Figure 5A). Figures 5B and 5C show the proteolytic cleavage of PARP and caspase-3 activation, two hallmarks of apoptosis, in intestinal tumors. Augmented cleaved PARP and caspase-3 were clearly observed in mice fed CP-31398, as compared to control diet-fed mice.

Discussion

p53 mutations are common in many human cancers, including colorectal cancer (8,27). Restoring mutant p53 function and/or enhancing wild-type p53 by genetic means suppresses growth of various tumor types (11,12,27). The identification of CP-31398 and other small molecules such as PRIMA-1 that activate mutant p53 could constitute an effective pharmacological approach for cancer prevention/treatment (28–32). While CP-31398 has been extensively studied in *in vitro* models (13–19,28,29), only a few studies have assessed the tumor inhibitory potential of CP-31398 *in vivo* (20–22).

In this study, we evaluated the toxicity, optimal dosing, tumor inhibition, and effectiveness on selected molecular targets of CP-31398. Our results are the first to show that CP-31398 effectively suppresses intestinal tumor formation whether given early or late in polyp formation. It is important to note, however, that CP-31398 showed a more pronounced effect on tumor suppression when administered early during tumor development, suggesting its potential usefulness as a chemopreventive agent. The present results further corroborate the

anticarcinogenic effects of CP-31398 against UVB-induced skin carcinogenesis in mice and chemically induced colon carcinogenesis in rats (20,22). In the skin model, CP-31398 was administered either IP or topically; in the present study, CP-31398 was administered in the diet. By all routes of administration, CP-31398 showed antitumor effects. Compared with previous studies in the *APC^{min/+}* model, the efficacy of CP-31398 in this study is dramatic and comparable to nonsteroidal antiinflammatory drugs (*e.g.*, celecoxib, sulindac) and other agents (26,35–38). *APC^{min/+}* mice are an appropriate model for human colon cancer, as the mechanism of *APC* gene inactivation mimics that observed in FAP patients and most sporadic human colon adenomas (34,35).

The mechanisms through which p53 inhibits cell proliferation and induces apoptosis have been studied in *in vitro* models (5–8). In the present study, CP-31398 suppressed tumor cell proliferation and induced apoptosis in conjunction with upregulation of p53 and its downstream effector p21. These results are consistent with previous studies demonstrating an increase in p53 target genes by CP-31398, as well as in p53 reporter gene expression in cancer cells (11, 13,29,39).

The importance of p53 mutations in colon cancers is well established (8,27). However, in the *APC^{min/+}* model, p53 mutations are likely a late event in tumor development. This suggests that restoration of mutant p53 function plays a minor role in the tumor inhibitory activity of CP-31398 seen in this study. On the other hand, activation of wild-type p53 by CP-31398 might be a major mechanism leading to suppression of tumor growth in *APC^{min/+}* mice. In this regard, activation of wild-type p53 by CP-31398 has been demonstrated in other models, both *in vitro* and *in vivo* (16–22). It is also possible that CP-31398 affects additional targets. Regardless of its exact mechanism, the finding that CP-31398 given either early or late in tumor development can suppress tumor growth indicates p53 is a rational target for chemoprevention of colorectal cancer. Ultimately, the combined use of low molecular weight p53 modulating agents acting through different mechanisms (*e.g.*, CP-31398 and PRIMA-1) or combinations with agents targeting other molecular pathways is likely to substantially increase anti-tumor effects. Taken together, these findings support further development of CP-31398 for colon cancer prevention and treatment.

Abbreviations

Ab-1, α -tubulin
 AIN, American Institute of Nutrition
 APC, adenomatous polyposis coli gene
 ChIP, chromatin immunoprecipitation
 CP-31398, p53 modulating agent
 DAB, diaminobenzidine
 FAP, familial adenomatous polyposis
 MTD, maximum tolerated dose
 cleaved PARP, poly (ADP-ribose) polymerase
 PCNA, proliferating cell nuclear antigen
 PI, proliferative index
 TP53, tumor suppression protein 53
 TUNEL, terminal dUTP nick end labeling

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Experimental Design: Evaluation of Efficacy of CP-31398 on APC^{min} mice Intestinal Tumorigenesis

Figure 1.

Experimental protocol for the evaluation of chemopreventive activity of CP-31398 in $APC^{min/+}$ mice model. Groups of mice were fed control diet (AIN 76A) or diets containing 100 or 200 ppm of CP-31398 administered at seven weeks of age for 75 days (Early intervention) or 15 weeks of age for 50 days (Delayed intervention). (Detailed information has been given in Materials and Methods).

Figure 2.

Figure 2A. Effect of CP-31398 on intestinal tumor formation in *APC^{min/+}* mice. Number of intestinal tumors (Mean $\pm$ SEM, N=10), in *APC^{min/+}* mice administered control and experimental diets containing 100 and 200 ppm CP-31398 either early intervention (seven-weeks of age) or late intervention (fifteen weeks of age). Significance between control and treatment groups were analyzed by t-test.

Figure 2B. Effect of CP-31398 on colon tumor formation in *APC^{min/+}* mice. Number of colon tumors (Mean $\pm$ SEM, N=10), in *APC^{min}* mice administered control and experimental diets and significance between control and treatment groups were analyzed by t-test.

Figure 3. Effect of 100 and 200 ppm CP-31398 (administered as late intervention) on large intestinal tumor cell proliferation (3A) and apoptotic index (3B) in *APC^{min/+}* mice. Representative data are presented as Mean $\pm$ SEM and significance was analyzed by *t*-test.

Figure 4. Immunohistochemical staining of cell proliferation (PCNA) and apoptosis (TUNEL) in colonic tumors of mice exposed 0, 100 or 200 ppm of CP-31398 (delayed intervention). Representative DAB-stained (brown colored) cells either PCNA or TUNEL are depicted at X400 magnifications.

Figure 5.

Expression levels of p53, p21, cleaved PARP, and cleaved Caspase-3 protein from tumor samples of mice administered with 100 ppm and 200 ppm CP-31398 either early or late intervention protocols. Intestinal tumor tissue lysates were homogenized in lysis buffer and were subjected to SDS-PAGE followed by Western blotting as described in Materials and Methods. Membranes were probed for p53, p21^{cip1}, cleaved caspase-3 and cleaved PARP specific primary antibodies and then peroxidase-conjugated appropriate secondary antibodies. Proteins were visualized with enhanced chemiluminescence detection system.