Suppression of Familial Adenomatous Polyposis by CP-31398, a TP53 Modulator, in APC\textsuperscript{min/+} Mice

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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. 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 C57BL/6-J\textsuperscript{APCmin} (heterozygous) and wild-type C57BL/6J mice with American Institute of Nutrition-76A diets containing 0, 100, or 200 ppm of CP-31398 for 75 days. To examine activity toward late-stage adenomas, CP-31398 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, p21WAF1/CIP, cleaved caspase-3, and cleaved poly(ADP-ribose) polymerase.

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). 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 (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 nonmutational activation of p53 may occur very early during cancer progression (7), mutations that inactivate p53 are present in more than 50% of all cancers, 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 translocalization, leading to reduced p53 activity (12).

Several attempts to restore mutant p53 as growth suppressor included microinjection of monoclonal antibody 421, COOH-terminal peptide of p53, and small molecules such as CP-31398 and PRIMA-1 (13–19). Early on, we determined that dietary CP-31398 protects against chemically induced early colon neoplastic lesions (20). More recently, Wang and colleagues (21) showed that p53 modulators suppress growth of human colon tumor xenografts. Similarly, Tang and colleagues (22) found that CP-31398 suppressed UAB-induced squamous skin cancer in mice by restoring mutant p53 function. 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 assay, CP-31398 promotes binding of mutant p53 to p53 response elements in vitro (24). Other studies using the purified p53 core domain have shown that CP-31398 can restore DNA-binding activity to mutant p53 in vivo (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 showed its efficacy in a well-established (APC\textsuperscript{min/-}) model of mouse intestinal neoplasia. We also determined the effects of CP-31398 on intestinal tumor proliferation, apoptosis, and levels of p53, p21WAF1/CIP, cleaved caspase-3, and cleaved poly(ADP-ribose) polymerase (PARP).

Materials and Methods

Animals, diets, and CP-31398. Heterozygous female Min (C57BL/6-J\textsuperscript{APCmin/+}) and wild-type C57BL/6J female mice were obtained at 5 wk of age from The Jackson Laboratory. Ingredients for the semipurified diets were purchased from Dyets, Inc. and stored at 4°C before diet preparation. Diets were based on the modified American Institute of Nutrition (AIN)-76A diet. The high-fat semipurified 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
Suppression of Intestinal Tumors by CP-31398

Experiment Design: Evaluation of Efficacy of CP-31398 on APC\textsuperscript{min/+} mice Intestinal Tumorigenesis

Early Intervention

- Control diet
- 0 or 100 or 200 ppm CP-31398 in diet
- Sacrifice Histopathology

Control diet
- 65 d
- 15 d
- 50 d
- 22 d
- Sacrifice Histopathology

Delayed Intervention

- 6 wk
- 0 or 100 or 200 ppm CP-31398 in diet

Control diet
- 6 wk
- 0 or 100 or 200 ppm CP-31398 in diet

CP-31398

Figure 1. Experimental protocol for the evaluation of chemopreventive activity of CP-31398 in APC\textsuperscript{min/+} mice model. Groups of mice were fed control diet (AIN-76A) or diets containing 100 or 200 ppm of CP-31398 administered at 7 wk of age for 75 d (Early Intervention) or 15 wk of age for 50 d (Delayed Intervention). Detailed information has been given in Materials and Methods.
0.05 mg/mL bovine serum albumin (BSA) was added, and the slides were incubated for an additional 30 min 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 per section) were counted manually in a single-blend fashion.

Western blot analysis of p53, p21WAF1/CIP, cleaved caspase-3, and cleaved PARP. Intestinal polyps isolated from individual mice were combined to obtain sufficient tissue (six to eight samples per group). Normal-appearing intestinal mucosal samples were homogenized in 1:3 volume of 100 mmol/L Tris-HCl buffer (pH 7.2) with 2 mmol/L CaCl2. After centrifugation at 100,000 × g for 1 h 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 difluoride nitrocellulose membranes as described previously (26). These membranes were blocked for 1 h at room temperature with 5% skim milk powder and probed with primary antibodies for 1 h. The primary antibodies p53, p21, cleaved caspase-3, and cleaved PARP (Santa Cruz Biotechnology) were used at 1:500 dilution. Blots were washed thrice and incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at 1:2,500 dilution for 1 h. The membranes were washed thrice and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.) for 5 min, 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) was used at 1:1,000 dilution as the internal standard for all Western blots.

Statistical analyses. All results are expressed as mean ± SE 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 and 1,200 ppm in the diet for 6 weeks reduced body weight of mice by 14% and 42%, respectively, compared with control diet–fed mice. At doses up to 300 ppm, CP-31398 did affect body weight. Based on these findings, 100 and 200 ppm of 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

![Figure 2. A, effect of CP-31398 on intestinal tumor formation in APCmin/+ mice. Number of intestinal tumors in APCmin/+ mice administered control and experimental diets containing 100 and 200 ppm of CP-31398 either early intervention (7 wk of age) or late intervention (15 wk of age). Columns, mean (n = 10); bars, SE. Significance between control and treatment groups was analyzed by t test. B, effect of CP-31398 on colon tumor formation in APCmin/+ mice. Number of colon tumors in APCmin/+ mice administered control and experimental diets. Columns, mean (n = 10); bars, SE. Significance between control and treatment groups was analyzed by Student’s t test.](image)

![Figure 3. Effect of 100 and 200 ppm of CP-31398 (administered as late intervention) on large intestinal tumor cell proliferation (A) and apoptotic index (B) in APCmin/+ mice. Columns, mean; bars, SE. Significance was analyzed by t test.](image)
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<sub>min/+</sub>* mice spontaneously develop intestinal tumors, essentially all of which (>95%) occur in the small intestine. In this study, on average, mice developed 17 to 58 (early intervention) or 25 to 67 (delayed intervention) tumors per mouse in the small intestine, but only 0 to 3 tumors per 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 2 summarizes the chemopreventive effect of CP-31398 administered at 100 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 per 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-dose, and high-dose groups, respectively. Although both 100 and 200 ppm of CP-31398 reduced colon tumor multiplicity, only the higher dose produced statistically significant inhibition (Fig. 2B). In addition, we observed statistically significant difference between the mice fed 100 and 200 ppm of CP-31398 in small intestinal tumor multiplicity both in interventions and in colon tumors of early intervention (Fig. 2A and B).

Effects on tumor cell proliferation and apoptosis. Figures 3A and 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). Figures 3B and 4 show the effects of CP-31398 on tumor cell apoptosis. Compared with 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<sub>WAF1/CIP1</sub>, cleaved caspase-3, and cleaved PARP. Expression levels of p53, p21<sub>WAF1/CIP1</sub> cleaved caspase-3, and cleaved PARP are important indicators of cell growth arrest and apoptosis. As shown in Fig. 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<sub>WAF1/CIP1</sub> expression when compared with controls. However, although CP-31398 effected a dose-dependent increase in p21<sub>WAF1/CIP1</sub> during early intervention, no such effect was seen in late intervention (Fig. 5A). Figure 5B and C shows 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 compared with 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 pharmacologic approach for cancer prevention/treatment (28–33). Although 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 i.p. 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* 

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 up-regulation of p53 and its downstream effector p21. These results are consistent with previous studies showing an increase in p53 target genes by CP-31398, as well as in p53 reporter gene expression in cancer cells (11, 13, 29, 39).

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**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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