View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

NEOPLASIA
www.neoplasia.com

Volume 15 Number 9 September 2013 pp. 1018–1027 1018

Chemopreventive Effects of the p53-Modulating Agents CP-31398 and Prima-1 in Tobacco Carcinogen–Induced Lung Tumorigenesis in A/J Mice[1]

Chinthalapally V. Rao*, Jagan Mohan R. Patlolla*, Li Qian*, Yuting Zhang*, Misty Brewer*, Altaf Mohammed*, Dhimant Desai†, Shantu Amin†, Stan Lightfoot‡ and Levy Kopelovich§

*Center for Cancer Prevention and Drug Development, Hematology-Oncology Section, Department of Medicine, Peggy and Charles Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, OK; †Department of Pharmacology, Penn State Hershey College of Medicine, Hershey, PA; ‡Department of Pathology and VA Hospital, Oklahoma City, OK; §Chemopreventive Agent Development Research Group, Division of Cancer Prevention, National Cancer Institute, Bethesda, MD

Abstract

Lung cancer is the leading cause of cancer deaths worldwide. Expression of the p53 tumor suppressor protein is frequently altered in tobacco-associated lung cancers. We studied chemopreventive effects of p53-modulating agents, namely, CP-31398 and Prima-1, on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)–induced lung adenoma and adenocarcinoma formation in female A/J mice. Seven-week-old mice were treated with a single dose of NNK (10 μmol/mouse) by intraperitoneal injection and, 3 weeks later, were randomized to mice fed a control diet or experimental diets containing 50 or 100 ppm CP-31398 or 150 or 300 ppm Prima-1 for either 17 weeks (10 mice/group) or 34 weeks (15 mice/group) to assess the efficacy against lung adenoma and adenocarcinoma. Dietary feeding of 50 or 100 ppm CP-31398 significantly suppressed ($P < .0001$) lung adenocarcinoma by 64% and 73%, respectively, after 17 weeks and by 47% and 56%, respectively, after 34 weeks. Similarly, 150 or 300 ppm Prima-1 significantly suppressed ($P < .0001$) lung adenocarcinoma formation by 56% and 62%, respectively, after 17 weeks and 39% and 56%, respectively, after 34 weeks. Importantly, these results suggest that both p53 modulators cause a delay in the progression of adenoma to adenocarcinoma. Immunohistochemical analysis of lung tumors from mice exposed to p53-modulating agents showed a significantly reduced tumor cell proliferation and increased accumulation of wild-type p53 in the nucleus. An increase in p21- and apoptotic-positive cells was also observed in lung tumors of mice exposed to p53-modulating agents. These results support a chemopreventive role of p53-modulating agents in tobacco carcinogen–induced lung adenocarcinoma formation.

Neoplasia (2013) 15, 1018–1027

Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; wt-p53, wild-type p53; mut-p53, mutant p53; AIN-76A, American Institute of Nutrition 76A; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling; PCNA, proliferating cell nuclear antigen; PARP, poly(ADP-ribose) polymerase; Rb, retinoblastoma; pRb, phospho-Rb; IHC, immunohistochemistry; H&E, hematoxylin and eosin; PBS, phosphate-buffered saline; TRITC, tetramethyl rhodamine isothiocyanate

Address all correspondence to: Chinthalapally V. Rao, PhD, Center for Cancer Prevention and Drug Development, University of Oklahoma Health Sciences Center, 975 NE 10th Street, BRC 1203, Oklahoma City, OK 73104. E-mail: cv-rao@ouhsc.edu

[1]This work was supported by NIH/NCI-NO2-CB-81013-74, NIH/NCI-CN-53300, and the Kerley-Cade Endowed Chair.

Received 28 June 2013; Revised 29 July 2013; Accepted 30 July 2013

Copyright © 2013 Neoplasia Press, Inc. All rights reserved 1522-8002/13/$25.00

DOI 10.1593/neo.131256

Introduction

Lung cancer is the leading cause of cancer mortality in both men and women in the United States [1]. *p53* is the most commonly mutated gene in human lung cancer [2] in which the mutation frequency is higher in lung cancers of smokers [3]. *p53* is a stress-induced tumor suppressor gene that plays a major role in multiple cellular functions such as cell cycle arrest and apoptosis, including DNA damage repair and cancer [4–6]. Nuclear localization is essential for the activity of p53 as a transcription factor [7], where it evokes cell cycle arrest or apoptosis through transcriptional activation of numerous effector genes [7–9]. p53 mutations result in loss of p53 tumor suppressor function and gain-of-function oncogenic mutations. About 50% of human cancers acquire somatic p53 mutations during the course of malignant conversion. Nonfunctional activity of p53 in cancer usually occurs in cancer by mutation, deletion, or inhibitory binding with viral oncoproteins [10–12]. Most p53 mutations occur in the DNA binding domain of the p53 protein, and about 80% are missense point mutations. Wild-type p53 (wt-p53) may also be inactivated in tumor tissues as a result of abnormal sequestration in the cytoplasm where it is functionally mutated [13,14]. Electrophilic prostaglandins produced by cyclooxygenase 2 have been shown to inhibit the wt-p53 activity by covalently binding to and impairing p53 in cytosol [15,16].

Over the past two decades, efforts have been made to restore or enhance p53 function in tumor cells. High-throughput screening has led to the identification of direct-acting inhibitors, such as a COOH-terminal peptide of p53, and small molecules, such as CP-31398, Prima-1, and MIRA-1 [17–21]. CP-31398, a styrylquinazoline that emerged from *in vitro* screening, can stabilize p53, protect against thermal denaturation, and maintain a monoclonal antibody 1620 epitope conformation in newly synthesized p53 [22] that restores a wt-p53 DNA binding conformation on mutant p53 (mut-p53) to suppress tumors *in vivo* [18]. CP-31398 was shown to promote stabilization of the DNA binding domain of p53 and to restore the transactivation function of certain mut-p53 proteins both *in vitro* and *in vivo* [18,20,23]. Studies from our laboratory demonstrated that CP-31398 inhibits intestinal polyp formation in $APC^{min/+}$ mice [24] and chemically induced colon cancers in a rat model [25] by reactivating p53. Prima-1 is another small molecule identified for its restoration of wild-type conformation and specific DNA binding to mut-p53 and for its ability to trigger apoptosis; it has mut-p53–dependent antitumor activity *in vivo* [26]. Previous reports have shown that Prima-1 inhibits the growth of cell lines derived from various human tumor types in a mut-p53–dependent manner [18,27].

Here, we have evaluated the chemopreventive efficacy of CP-31398 and Prima-1 on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)–induced lung adenoma and adenocarcinoma formation in female A/J mice to help elucidate whether direct-acting small molecules that rescue/activate p53 can prevent lung cancer in these mice. We chose the NNK-treated female A/J mouse model because these mice develop lung adenomas and adenocarcinomas in a manner that is similar to tobacco-induced lung cancer in humans and they are widely used in evaluating chemopreventive efficacy [28–32]. Furthermore, we investigated the effect of p53 modulators during initiation, including progression of early adenoma to adenocarcinoma by administrating these agents shortly after NNK treatment and well into the stage(s) in which adenoma occur. Together, these results support a chemopreventive role for direct-acting small molecules that rescue and activate mut-p53 in preventing tobacco carcinogen–induced lung adenocarcinoma formation.

Materials and Methods

Animals, Diets, Chemopreventive Agents, Chemicals, and Reagents

All animal experiments were performed in accordance with National Institutes of Health (NIH) guidelines and University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee–approved protocol (IACUC No. 09-067B). Female A/J mice were obtained at 6 weeks of age from Jackson Laboratory (Bar Harbor, ME). Ingredients for the semipurified diets were purchased from Bio-Serv (Frenchtown, NJ) and stored at 4°C before diet preparation. Diets were based on the modified American Institute of Nutrition 76A (AIN-76A) diet containing 20% casein, 52% cornstarch, 13% dextrose, 5% 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. CP-31398 and Prima-1 (Figure 1*A*) were kindly supplied by the National Cancer Institute, Division of Cancer Prevention Repository (Bethesda, MD). Test agents were premixed with a small quantity of casein and then blended into bulk diet using a Hobart Mixer. Both control and experimental diets were prepared weekly and stored in a cold room. The content of agent(s) in the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to ensure uniform distribution.

The cocktail of protease inhibitors was from Sigma (St Louis, MO). NNK was synthesized and provided by the laboratory of Dr Shantu Amin (Department of Pharmacology, Penn State Hershey, Hershey, PA). Anti–wt-p53 (Catalog No. OP33) and anti–mut-p53 (Catalog No. OP29) were purchased from Calbiochem (La Jolla, CA). Anti–poly(ADP-ribose) polymerase (PARP) and anti–caspase-3 were from Cell Signaling Technology (Beverly, MA). Anti-p21, anti–phospho-retinoblastoma (pRb), anti-total Rb, and anti–β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA).

Experimental Bioassay

The experimental design is summarized in Figure 1*B*. Female A/J mice at 6 weeks of age were purchased from Jackson Laboratory and were maintained in the pathogen-free Rodent Barrier Animal Facility at the University of Oklahoma Health Sciences Center. At 6 weeks of age, mice were fed control irradiated AIN-76A modified diet. At 7 weeks of age, the mice intended for carcinogen treatment received a single dose of 10 μmol (2.07 mg) NNK/mouse by intraperitoneal injection. All mice were weighed once every 2 weeks until termination of the study. Three weeks after NNK treatment, groups of mice (25 mice/group) were fed control AIN-76A or experimental diets containing 50 or 100 ppm CP-31398 or 150 or 300 ppm Prima-1 until termination. Mice were killed by CO_2 asphyxiation followed by cervical dislocation after 17 weeks (10 mice/group) or 34 weeks (15 mice/group) of exposure to test agents. At the time of sacrifice, lungs were lavaged, perfused, and fixed in phosphate-buffered formalin, transferred within 2 days to 70% alcohol, and evaluated under a dissecting microscope for the number of tumors and tumor size. Tumors on the lung surface were enumerated by at least two experienced readers, blinded to sample identifiers, using a dissecting microscope. Tumor diameters were measured using Fisher brand digital calipers.

Tumor Histology

Fixed lung samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). H&E-stained lung

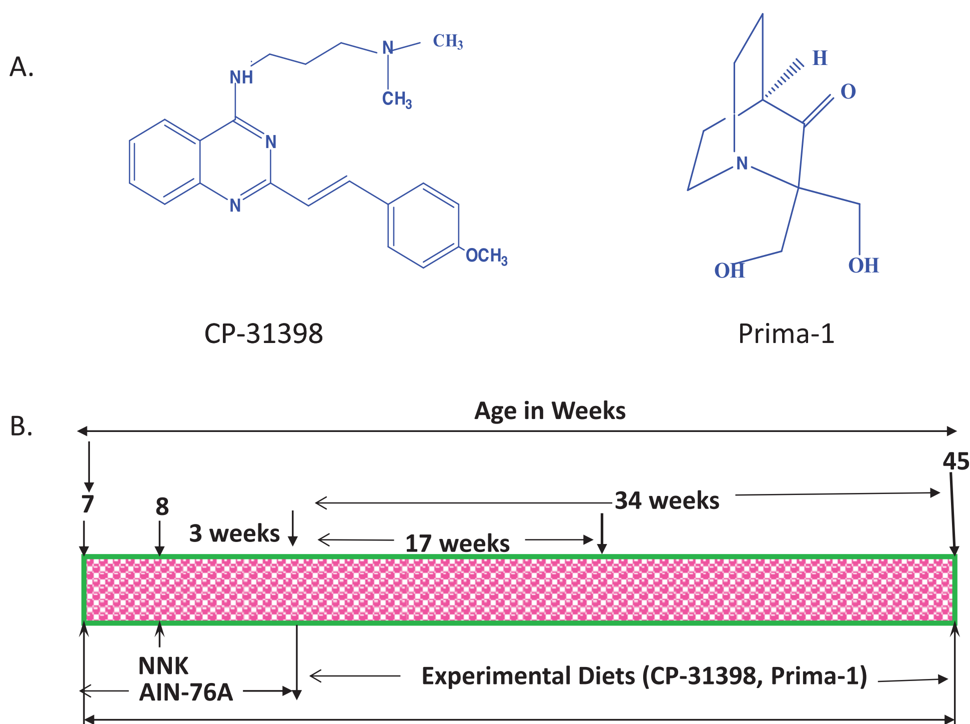

Figure 1. (A) Structure of CP-31398 and Prima-1. (B) Experimental protocol to assess the chemopreventive effects of CP-31398 and Prima-1 in NNK-induced lung tumorigenesis in A/J mice. Eight-week-old mice were injected intraperitoneally with NNK (10 μmol/mouse) and maintained on AIN-76A diet for 3 weeks. Three weeks after NNK injection, mice were fed control diet (AIN-76A) or diets containing 50 or 100 ppm of CP-31398 or 150 or 300 ppm Prima-1 and continued until the end of the study. At 20 weeks (10 mice/group) and at 37 weeks (15 mice/group) after NNK treatment, mice were killed and lungs were harvested for evaluation of lung tumors. Detailed information has been given in Materials and Methods section.

sections from three separate sections cut at predetermined depths were evaluated by a board-certified pathologist (Dr Stan Lightfoot, Department of Pathology and VA Hospital, Oklahoma City, OK) for the number of adenomas and adenocarcinomas. Tumors were categorized according to criteria of the Mouse Models of Human Cancers Consortium [33]. Adenomas were generally less than 5-mm-diameter, well-circumscribed areas of proliferative cuboidal to columnar cells lining an alveolus. Adenocarcinomas were typically >5 mm in diameter and showed invasion and loss in alveolar architecture, increased nuclear/cytoplasmic ratio, cellular atypia, a large mass of undifferentiated cells, and nuclear pleomorphism.

Immunohistochemistry

The effects of CP-31398 and Prima-1 on expression of proliferating cell nuclear antigen (PCNA), p21, wt-p53, and mut-p53 were evaluated in adenocarcinomas by immunohistochemistry (IHC). Briefly, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in phosphate-buffered saline (PBS). Antigen retrieval was carried out by treating sections in 0.01 M citrate buffer (pH 6.0) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in PBS for 5 minutes. Nonspecific binding sites were blocked by incubation with 2% BSA, and then sections were incubated overnight at 4°C with 1:100 dilutions of monoclonal antibodies against PCNA, p-21, wt-p53, or mut-p53. After several washes with PBS, the slides were incubated with appropriate secondary antibody for 2 hours and then washed and incubated with avidin-biotin complex reagent (Zymed Laboratories, Camarillo, CA). The slides were rinsed with PBS, incubated with the chromogen 3-diaminobenzidine for 3 minutes, and then rinsed and counterstained with hematoxylin. Nonimmune rabbit Igs were substituted for primary antibodies as negative controls. Slides were observed under an Olympus microscope IX701, and digital computer images were recorded with an Olympus DP70 camera.

Immunohistofluorescence

For immunohistofluorescence, after overnight incubation with primary antibody of wt-p53 and mut-p53, the slides were washed thrice with PBS for 5 minutes and then incubated with secondary antibody tagged with tetramethyl rhodamine isothiocyanate (TRITC) in the dark for 1 hour. Slides were then washed with PBS for 5 minutes thrice in the dark room and incubated with 4′,6-diamidino-2-phenylindole (0.5 µg/ml) for 5 minutes. Slides were rinsed with PBS and observed for fluorescence under fluorescein isothiocyanate/TRITC filters using an Olympus microscope IX701, and digital computer images were recorded with an Olympus DP70 camera.

Apoptosis Assay

Paraffin sections of 5-µm thickness were mounted on slides, rehydrated, and stained using the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) method. TUNEL-positive cells were visualized by chromogenic staining with 3-diaminobenzidine, and slides were counterstained with malachite green. Stained apoptotic epithelial cells (a minimum

of 10 microscopic fields per section) were counted manually in a double-blind fashion.

Western Blot Analysis of Protein Expression

Lung tumors harvested from mice from different treatment groups were immediately frozen in liquid nitrogen and stored at −80°C for further analysis. During the time of marker analysis, large tumors were excised from the lungs, homogenized, and lysed in cold lysis buffer (Sigma). After a brief vortexing, the lysates were separated by centrifugation at 12,000*g* for 15 minutes at 4°C, and protein concentrations were measured with the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). An aliquot (50 μg protein/lane) of the total protein was separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking the membranes with 5% milk powder, adenocarcinomas were probed for expression of p21, total Rb, pRb–Ser-795, β-actin, PARP, caspase-3, wt-p53, and mut-p53 in hybridizing solution (1:500, in TBS–Tween 20) using the respective primary antibodies and then probed with their appropriate HRP-conjugated secondary antibodies. Detection was performed using the SuperSignal VWR West Pico Chemiluminescence procedure (Pierce, Rockford, IL). The bands were captured on Ewen Parker Blue sensitive X-ray films and quantified by densitometry. We reprobed every blot for β-actin, but we present only one β-actin blot from each of the two intervals of test agent exposure: 17 and 34 weeks.

Statistical Analysis

Differences in body weights among groups were analyzed by analysis of variance. Adenoma and adenocarcinoma multiplicities (number of tumors/mouse) are expressed as means ± SD. Protein expression, proliferative indices, and apoptotic indices were expressed as means ± SEM and were analyzed by unpaired *t* test with Welch correction. Dose-response effects were analyzed by linear regression analysis. Differences were considered statistically significant at $P < .05$.

Results

General Observations

Dose selection was based on our previous toxicity studies; CP-31398 doses of up to ∼400 ppm and Prima-1 doses of up to ∼600 ppm were tolerable in rodent models when administered chronically with AIN-76A diet for 9 weeks [25]. In the present study, we applied 12.5% and 25% of the maximally tolerated dose of CP-31398 and 25% and 50% of the maximally tolerated dose of Prima-1 to assess the chemopreventive efficacy. Unlike Prima-1, CP-31398 had been studied extensively for efficacy, pharmacokinetics, and pharmacodynamics in rodents [34]. Administration of 50 or 100 ppm CP-31398 or 150 or 300 ppm Prima-1 for either 17 or 34 weeks did not cause any body weight loss or any other histologic toxicity in major organ sites (data not shown). Overall, chronic dietary administration of CP-31398 and Prima-1 for either 17 or 34 weeks did not induce any overt toxicities in female A/J mice.

Dietary Administration of CP-31398 and Prima-1 Significantly Inhibited Lung Adenocarcinoma and Delayed the Progression of Adenoma to Adenocarcinoma Formation

NNK-treated mice fed the control diet developed 100% tumor incidence at 20 and 37 weeks after the carcinogen treatment (Figure 2, *A* and *B*). Control diet mice killed at 20 weeks after NNK treatment showed an average of 6.7 ± 1.4 lung adenomas and 9.22 ± 2.2 lung adenocarcinomas (means ± SEM, reflecting 41.4% of the tumors were adenomas and 58.6% were adenocarcinomas; Figure 2, *C* and *D*). Mice fed the control diet and killed at 37 weeks after NNK treatment showed 3.8 ± 1.1 adenomas and 19.4 ± 3.1 adenocarcinomas (16.4% of the tumors were adenomas and 83.6% were adenocarcinomas; Figure 2, *E* and *F*), reflecting an increased progression of adenomas to adenocarcinomas. The effects of CP-31398 and Prima-1 on lung adenoma and adenocarcinoma multiplicities are summarized in Figure 2, *C* to *F*. Mice administered 50 ppm CP-31398 for 17 weeks showed a slightly higher number of lung adenomas, whereas lung adenocarcinomas were significantly ($P < .0001$) suppressed by 64% or 73%, respectively, at the 50- or 100-ppm doses. A similar trend was observed in mice exposed to CP-31398 for 34 weeks; NNK-induced adenocarcinomas were inhibited by 47% to 56% at the two doses ($P < .0001$; Figure 2, *E* and *F*). However, we observed a slight increase in the lung adenomas, reflecting some delay in tumor progression from adenoma to adenocarcinoma in the CP-31398–treated mice.

Administration of 150 or 300 ppm Prima-1 significantly suppressed NNK-induced total lung adenocarcinoma formation by 57% or 62% ($P < .0001$), respectively, after 17 weeks of exposure and by 39% or 56% ($P < .0001$), respectively, after 34 weeks of exposure. As with administration of the lower (50 ppm) dose of CP-31398, administration of the lower (150 ppm) dose of Prima-1 also slightly increased the number of NNK-induced lung adenomas. We observed that there is no significant difference in lung spontaneous tumors in mice fed with control diet (adenomas, 0.4 ± 0.55/mouse; $N = 6$) compared to mice fed with CP-31398 and Prima-1 (adenomas, 0.2 ± 0.45; $P = .27$).

Effect of p53 Modulators on Apoptotic Index

Figure 3, *B* and *G*, summarizes the apoptotic effect of p53 modulators in lung adenocarcinomas of mice exposed for 34 weeks to 100 ppm CP-31398 or 300 ppm Prima-1. Apoptotic or TUNEL-positive cells are stained dark brown and nonapoptotic cells are counterstained with methyl green. Quantification of apoptotic staining revealed an apoptotic index of 10 ± 2.3 (mean ± SEM) TUNEL-positive cells in adenocarcinomas from mice fed the control diet as compared with an apoptotic index of 50 ± 6.1 or 35 ± 6 (mean ± SEM) TUNEL-positive cells in lung adenocarcinoma of mice fed 100 ppm CP-31398 or 300 ppm Prima-1, respectively, accounting for an increase in the apoptotic index of several fold by both agents.

Modulation of PCNA, p21, mut-53, and wt-p53 Expression by CP-31398 and Prima-1 in NNK-Induced Lung Adenocarcinomas

Effects of 100 ppm CP-31398 and 300 ppm Prima-1 on lung adenocarcinoma cell proliferation were assessed through labeling of PCNA-positive cells (Figure 3*C*). PCNA labeling index was significantly lowered in tumors from CP-31398 or Prima-1–treated mice compared with tumors from mice fed a control diet. The quantification of PCNA staining showed a labeling index of 72 ± 5.8 (mean ± SEM) in adenocarcinomas from mice fed a control diet as compared with 30 ± 3.4 and 45 ± 3.5 (means ± SEM) in those from animals fed CP-31398 or Prima-1, accounting for a decrease in the proliferation

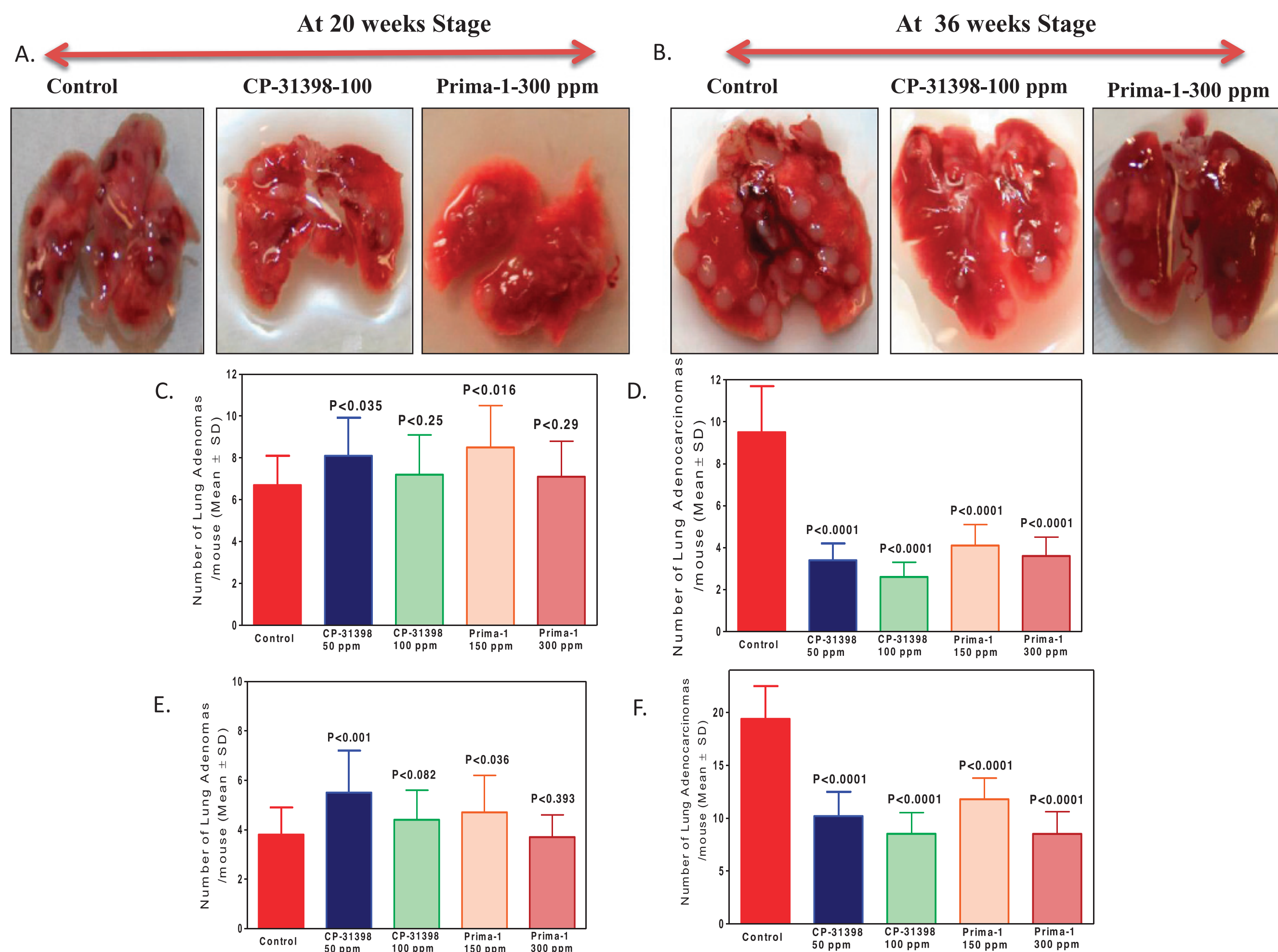

Figure 2. Effect of dietary feeding of p53 modulators on NNK-induced lung tumor growth in A/J mice. (A) Lungs with lesions from different groups at the 20-week stage [(i) control, (ii) 100 ppm CP-31398, and (iii) 300 ppm Prima-1]. (B) Lungs with lesions from different groups at the 37-week stage [(i) control, (ii) 100 ppm CP-31398, and (iii) 300 ppm Prima-1]. (C and D) Effect of CP-31398 and Prima-1 on lung adenoma and adenocarcinoma formation after NNK treatment at the 20-week stage. Values are means ± SD with $N = 10$. The significance of differences between control and treatment groups was analyzed by one-tailed *t* test with Welch correction and 95% confidence interval. (E and F) Effect of CP-31398 and Prima-1 on lung adenoma formation at the 37-week stage. Values are means ± SD with $N = 15$. The significance of differences between control and treatment groups was analyzed by one-tailed *t* test with Welch correction and 95% confidence interval.

index by 60% ($P < .0001$) or ∼38% ($P < .0002$), respectively (Figure 3*H*).

As shown in Figure 3*D*, expression of p21 was significantly reduced in lung adenocarcinomas of mice fed control diets, whereas dietary administration of CP-31398 and Prima-1 resulted in a significant increase in p21 protein expression levels in lung tumors. Figure 3, *E* and *F*, summarizes the expression of mut-p53 and wt-p53 in lung tumors of mice exposed to control diet or p53-modulating agents. We have not observed any significant differences in the expression of mut-p53 between the lung tumors from mice fed the control diet *versus* either of the p53-modulating agents. However, a significant increase in the expression of wt-p53 was observed in the lung tumors of mice treated with p53 modulators. From the immunofluorescence histochemistry analysis as shown in Figure 4, we observed an increased localization of wt-p53 in the nucleus of the lung tumors from mice fed 100 ppm CP-31398 when compared to those from mice fed the control diet. We also observed chromatin condensation and apoptosis of nuclei in tumor cells of mice fed the CP-31398 diet.

Western Blot Analysis of wt-p53, mut-p53, p21$^{WAF1/CIP1}$, Cleaved Caspase-3, and PARP Modulation by p53 Modulators

Expression of wt-p53, mut-p53, p21$^{WAF1/CIP1}$, cleaved caspase-3, and PARP was analyzed further by Western blot analysis. As shown in Figure 5, *A* and *B*, CP-31398 and Prima-1 caused significant induction of p21$^{WAF1/CIP1}$ expression in lung tumors harvested from mice after 17 or 34 weeks of exposure. CP-31398 caused a greater induction of p21$^{WAF1/CIP1}$ than did Prima-1. Figure 5*B* shows proteolytic cleavage of caspase-3 and decrease in PARP in mice fed CP-31398 compared with mice fed the control diet. The cleavage of caspase-3 was observed only in late-stage tumors and in mice fed the high dose of CP-31398. CP-31398 and Prima-1 induced wt-p53 in lung tumors, with CP-31398 showing greater wt-p53 induction as compared with Prima-1. No changes in expression of mut-p53

were detectable with either of the drugs, possibly because this mut-p53 antibody may recognize both wt-p53 and mut-p53 proteins under the denaturing conditions of the gel.

CP-31398 and Prima-1 Decrease the Phosphorylation of Rb

Members of Rb protein are phosphorylated by cyclin-dependent kinases (Cdks), leading to the activation of gene expression required for cell cycle progression and proliferation. Because p21 is an inhibitor of Cdks, we investigated whether p53 modulators modified the phosphorylation status of endogenous Rb protein. As shown in Figure 5*B*, CP-31398 and Prima-1 inhibited phosphorylation of Rb at Ser-795, but the total Rb protein levels were unaltered. CP-31398 caused greater inhibition of Rb phosphorylation than did Prima-1.

Discussion

The NNK-induced mouse lung tumor model has been well established in studies of adenocarcinoma development, including chemoprevention [28–32]. High frequency of p53 mutations in lung cancer has led us to investigate the effects of direct-acting small molecules that rescue and activate mut-p53 for the prevention of lung cancer induced by the tobacco carcinogen NNK in female A/J mice. Here,

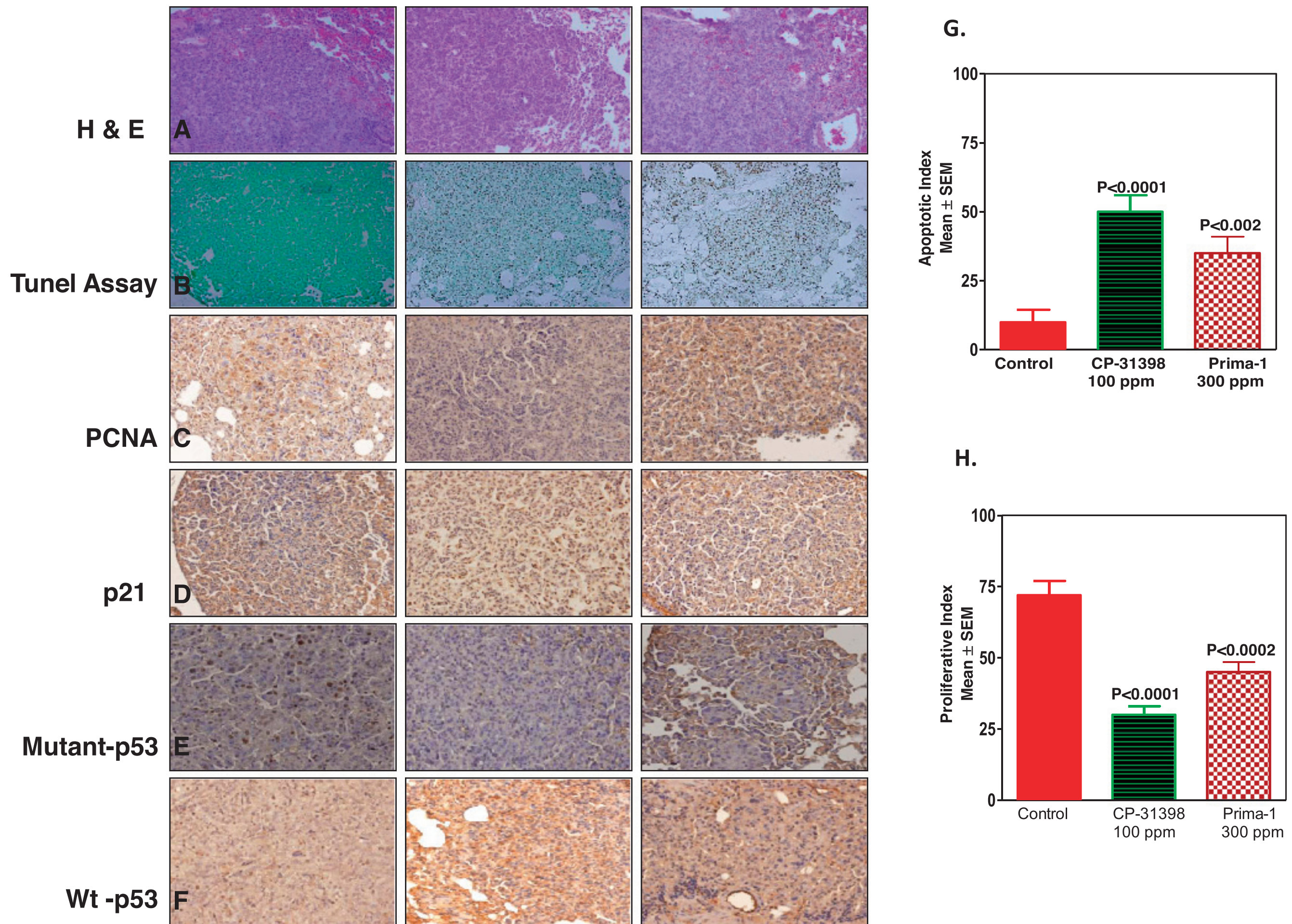

Figure 3. IHC staining of lung adenocarcinomas for different markers after 37 weeks with p53 modulators. Representative lung sections from the NNK-treated control group and from 100-ppm CP-31398 and 300-ppm Prima-1 treatment groups. Data are presented as means ± SEM; significance was analyzed by *t* test. (A) H&E: Images of H&E-stained lung sections from various groups. (B) TUNEL assay: *In situ* apoptotic cells were identified by brown staining for TUNEL-positive cells, with methyl green for counterstain. A significant induction of apoptosis was observed in adenocarcinomas of mice treated with CP-31398 or Prima-1 as compared with those from untreated control mice. (C) PCNA: Cell proliferation index was measured by staining with PCNA antibody and counterstaining with hematoxylin. A significant difference was observed in proliferative index with high PCNA expression in lung adenocarcinomas from NNK-treated groups and very low expression in those from CP-31398– and Prima-1–treated groups. Data are expressed as a percent of NNK control ($P < .05$). (D) p21: Paraffin sections of lung adenocarcinomas were subjected to IHC analysis using an anti-p21 antibody. There is intense staining of p21 in CP-31398– and Prima-1–treated groups compared with NNK control. (E) mut-p53: IHC staining of lung adenocarcinomas for mut-p53. Intense brown staining observed in controls is absent in the mice administered diets containing CP-31398 or Prima-1. (F) wt-p53: IHC staining of lung adenocarcinomas with wt-p53 monoclonal antibody. (G) Apoptotic index: Effect of CP-31398 and Prima-1 on NNK-induced lung tumor apoptotic index at the 36-week stage: Values are the means ± SEM, and significant difference between control and treatment groups was analyzed by *t* test. (H) Proliferative index: Effect of CP-31398 and Prima-1 on NNK-induced lung tumor proliferative index at the 36-week stage: Values are the means ± SEM, and significant difference between control and treatment groups was analyzed by *t* test.

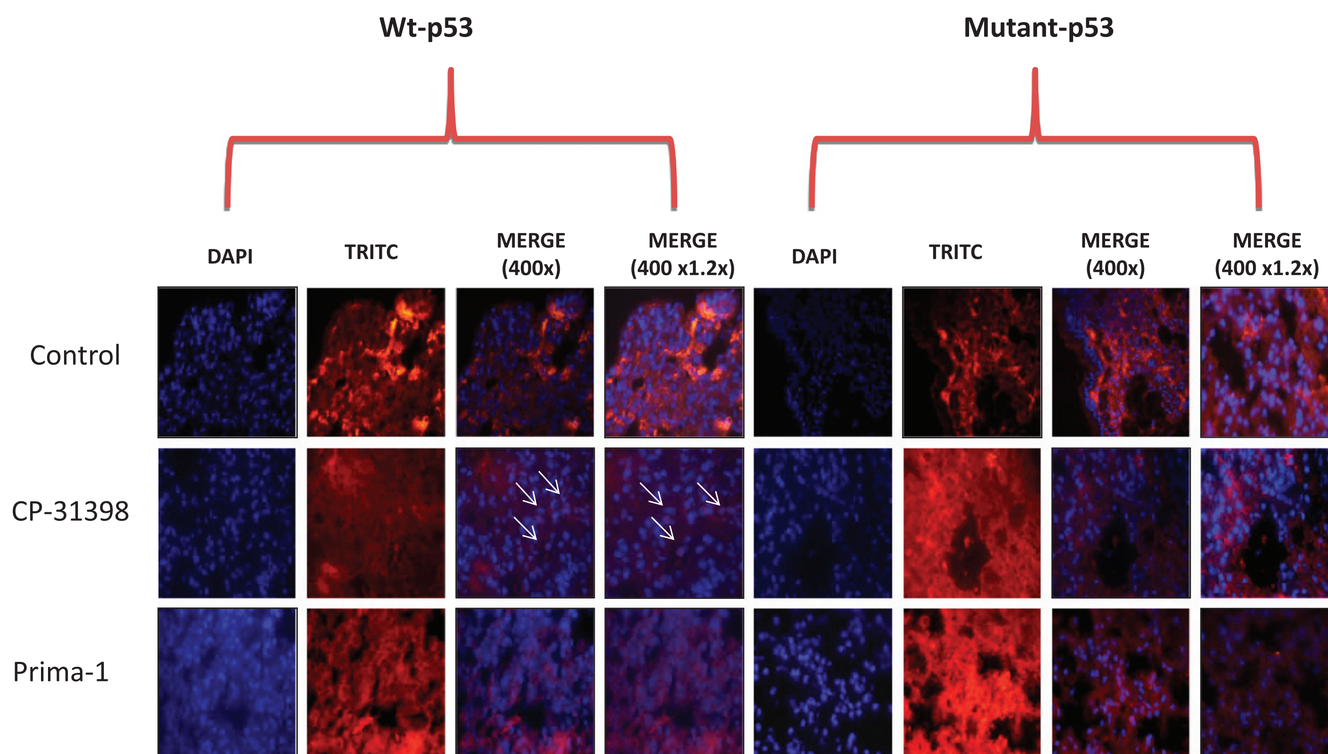

Figure 4. Modulation by p53 modulators of wt-p53 and mut-p53 expression and localization in lung adenocarcinomas. Monoclonal antibodies were used in connection with TRITC red–conjugated secondary antibody. 4′,6-Diamidino-2-phenylindole (blue signal) was used for counterstaining of the nucleus. The images were representative of three experiments (original magnification, ×400; a merged figure is shown at ×1.2 the original). The arrows in the merged figure indicate locations of increased wt-p53 and apoptosis in the nucleus of a tumor cell from a CP-31398 (100 ppm)–treated mouse compared to the nucleus from a control tumor. Even in the Prima-1 group, there is intense staining of wt-p53 and we could not find any apoptotic cells. We could not distinguish or localize the mut-p53 distribution.

we demonstrated that both CP-31398 and Prima-1 were well tolerated and lacked overt toxicities. Chronic exposure to nontoxic doses of each agent provided significant inhibition of lung adenocarcinoma (47% to 72%; $P < .0001$) and decreased tumor volume (Figure 2).

In the last few decades, extensive efforts have been made to develop direct targeting approaches, including gene therapy using viral vectors and peptide molecules, for rescue of endogenous mutated tumor suppressors [18,35–37]. The use of small molecules that rescue mut-p53 and enhance the wt-p53 activity would lend itself to effective intervention, including prevention and/or delay of onset of cancers in which p53 is likely to play a causative role. Re-expression of functional p53 and/or activation of p53 has been shown to induce apoptosis and growth arrest of cells undergoing neoplastic transformation [38,39]. Our previous observations on chemically induced and spontaneous models of colon cancer [24,25], including skin tumorigenesis [40], demonstrated significant efficacy of p53 modulators.

We found increased expression of wt-p53 and its nuclear localization on treatment with direct-acting p53-modulating agents shown by histologic and Western blot analyses, although we failed to observe significant changes in mut-p53 expression in NNK-induced lung adenocarcinomas and/or with p53 modulatory agent treatments (Figures 3 4 5). It is possible that the mut-p53 antibody may not be able to recognize NNK-induced p53 mutant forms or very low levels of p53 mutations. Our results suggest that CP-31398 and Prima-1 may induce wt-p53 expression, leading to suppression of lung tumor inhibition that is supported by our study and other studies in *in vitro* and *in vivo* models of other cancers [18,24–27].

To identify specific molecular alterations that may contribute to the protective effect of p53 modulators in NNK-induced lung cancer of A/J mice, we examined the expression of downstream effector molecules that modulate cell proliferation. Here, we observe that $p21^{WAF1/CIP1}$ was expressed at very low levels in tumors of mice fed a control diet, whereas its expression was upregulated by two- to three-fold in the CP-31398– and Prima-1–fed mice. We also found decreased levels of pRb without influencing total Rb protein expression levels in lung tumors in mice fed CP-31398–containing diet. The induction of $p21^{WAF1/CIP1}$ by CP-31398 treatment can inhibit the formation of one Cdk complex, and this, in turn, could be responsible for reduced phosphorylation of Rb. Our results are in agreement with studies carried out by Takimito et al. [20], where they show that CP-31398 inhibits pRb in a panel of human cancer cell lines.

We observed a significant up-regulation of TUNEL-positive cells, as well as cleavage of caspase-3, in lung tumors from the mice fed CP-31398 and Prima-1 (Figures 3 and 5), suggesting that p53-induced apoptotic effects are mediated, in part, through the induction of caspase-3 and PARP cleavage. In support of this conclusion, we have previously shown that CP-31398 inhibition of intestinal tumors in $APC^{min/+}$ mice is associated with induction of wt-p53 and caspase-3 and PARP cleavage [24]. However, Prima-1 exposure led to induction of apoptosis in lung tumors but did not show any cleavage of caspase-3, suggesting involvement of different mechanisms. Previous reports suggest that Prima-1 potentiated the effect of adriamycin and triggers an apoptotic response in non–small cell lung cancer cells [26]. Prima-1 also inhibited tumor cell growth *in vivo* in mice carrying

endogenous missense mutations in p53 [27]. Previous studies have shown that the PCNA labeling index is high in human lung tumors and the bronchial epithelial cells of smokers [41,42]. Dietary administration of both p53 modulators decreased the size of the lung tumors as well as the PCNA labeling index as compared with those in mice fed the control diet (Figures 2 and 3).

In summary, this is the first report on the chemopreventive effects of CP-31398 and Prima-1 on NNK-induced lung carcinogenesis in the A/J mouse strain. Both chemopreventive agents were able to inhibit adenocarcinoma multiplicity and tumor size whether mice were exposed to either short term or long term. Our observations suggest that p53-modulating agents delay the adenoma to adenocarcinoma progression, and there is ~30% to 50% inhibition (adenoma + adenocarcinoma) in the treatment groups when compared to control diet–fed mice. These results clearly suggest that some of the NNK-induced lung lesions may not even progress to adenomas or that in turn to adenocarcinomas. There is relatively less adenocarcinoma inhibition at the 34-week stage compared to the 17-week stage, suggesting that even in treatment groups several adenomas had progressed to adenocarcinomas, indicating that these adenocarcinomas might escape the tumor inhibitory effect of p53 modulators. Our observations suggest a substantial delay of adenoma progression to adenocarcinomas and relatively lesser tumor cell growth in adenocarcinomas in mice exposed to p53 modulators at 34 weeks. The biomarkers we have analyzed on lung tumor proliferation and apoptosis at 34 weeks support tumor growth inhibitory effects of p53-modulating agents. However, in-depth molecular pathway analysis is required to address why some adenomas escape and progress to adenocarcinomas.

Our results suggest that some of the NNK-induced lung lesions may not even progress to adenomas in the treatment groups, in addition to progression delay as discussed above. Thus, p53-modulating agents favor tumor prevention. In support of this view, our previous studies suggest that early and continuous intervention with CP-31398 provides better efficacy and that combining with other preventive agents also improve the colon adenocarcinoma inhibitory effects [24,25]. Furthermore, our unpublished data suggest that the administration of CP-31398 at late adenoma and adenocarcinoma stage had modest efficacy effects in rat colon cancer model. More recently, we have shown that CP-31398 administration inhibits the progression of low-grade papillary transitional cell carcinomas to invasive transitional cell carcinomas [43]. Thus, p53 modulators may block the NNK-induced adenoma formation and also progression of adenoma/adenocarcinoma growth.

In the present study, we have observed a nonstatistically significant reduction of spontaneous lung tumors in mice exposed to p53-modulating agents. Previous studies by the group of Dr Ming You

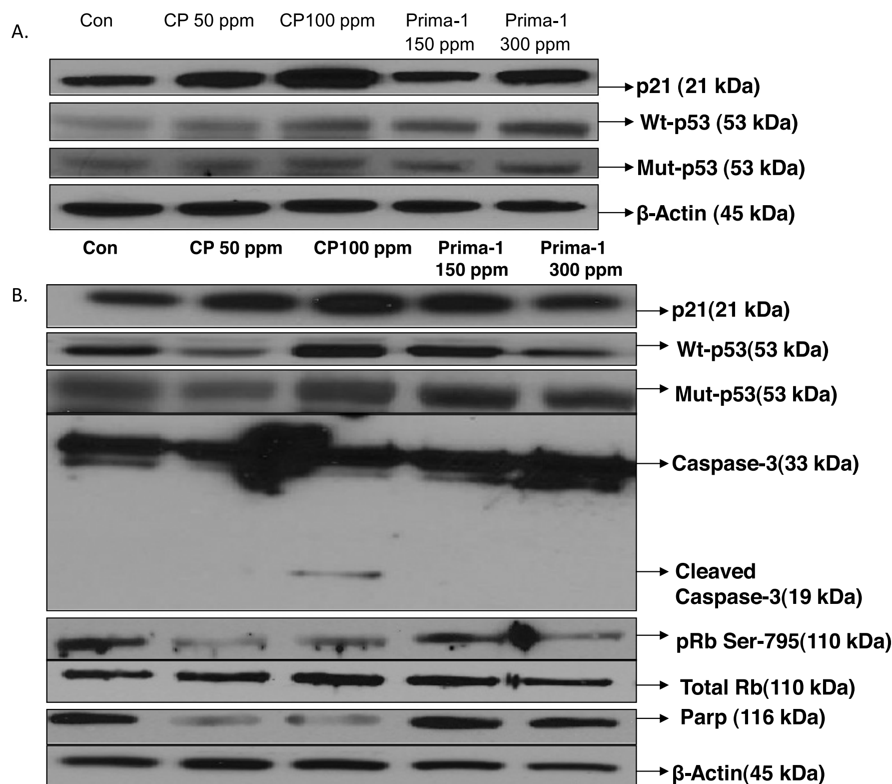

Figure 5. Immunoblot analyses of lung tumor lysates derived from A/J mice treated with NNK control (C), 50 or 100 ppm CP-31398 (low-dose and high-dose CP-31398, respectively), or 150 or 300 ppm Prima-1 (low-dose and high-dose Prima-1, respectively) and sacrificed after 20 or 37 weeks of treatment. (A) Expression of p21, wt-p53, mut-p53, and β-actin after 20 weeks with p53 modulators. (B) Expression of p21, wt-p53, mut-p53, pRb–Ser-795, total Rb, PARP, and cleaved caspase-3 protein from lung tumor samples of A/J mice administered 50 or 100 ppm CP-31398 or 150 or 300 ppm Prima-1 for 37 weeks. Lung tumor tissue lysates were homogenized in lysis buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blot analysis as described in Materials and Methods section. Nitrocellulose was probed with primary antibodies specific for p53, $p21^{CIP1}$, cleaved caspase-3, and cleaved PARP and then incubated with appropriate peroxidase-conjugated secondary antibodies. Proteins were visualized with enhanced chemiluminescence detection system.

[44–46] have reported the role of p53 in A/J mice lung tumorigenesis with spontaneous and NNK-treated mice. These studies suggest that there is no significant difference (modest risk) in lung spontaneous tumors in dominant mutant A/Jp53Val^{135} mice compared to wild-type mice, whereas there is a two- to three-fold increase risk of NNK-induced lung tumors in A/Jp53Val^{135} mice compared to wild-type NNK-treated mice, thus suggesting the limited role of p53 in spontaneous lung tumors of A/J mice compared to the highly significant role of p53 in tobacco carcinogen–induced lung tumor development. In our study, we observed that there is no significant difference in lung spontaneous tumors in mice fed with control diet (adenomas, 0.4 ± 0.55/mouse; N = 6) compared to mice fed with CP-31398 and Prima-1 (adenomas, 0.2 ± 0.45; P = .27). Overall, results from the present study suggest that CP-31398 and Prima-1 can delay progression of NNK-induced lung adenoma formation and delay the adenoma progression to adenocarcinomas. Oral administration of nontoxic p53-modulating small molecules may provide a promising approach for the prevention of lung cancer in individuals at high risk, both ex-smokers and current smokers.

Acknowledgments

The authors thank Julie Sando for valuable suggestions and editorial help with the manuscript and the University of Oklahoma Health Sciences Center Rodent Barrier Facility Staff.

References

[1] American Cancer Society (ACS) (2013). *Cancer Facts & Figures 2013*. American Cancer Society, Atlanta, GA.

[2] Reenblatt MS, Bennett WP, Hollstein M, and Harris CC (1994). Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* **54**, 4855–4878.

[3] Husgafvel-Pursiainen K, Boffetta P, Kannio A, Nyberg F, Pershagen G, Mukeria A, Constantinescu V, Fortes C, and Benhamou S (2000). *p53* mutations and exposure to environmental tobacco smoke in a multicenter study on lung cancer. *Cancer Res* **60**, 2906–2911.

[4] Ko LJ and Prives C (1996). p53: puzzle and paradigm. *Genes Dev* **10**, 1054–1072.

[5] Giaccia AJ and Kastan MB (1998). The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev* **12**, 2973–2983.

[6] Lakin ND and Jackson SP (1999). Regulation of p53 in response to DNA damage. *Oncogene* **18**, 7644–7655.

[7] Micheal BK and Gerad PZ (2003). Parcing p53 in the cytoplasm. *Cell* **112**, 1–5.

[8] El-Deiry WS (1998). Regulation of *p53* downstream genes. *Semin Cancer Biol* **8**, 345–357.

[9] Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, and Beach D (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* **366**, 701–704.

[10] Lane DP (1992). p53, guardian of the genome. *Nature* **358**, 15–16.

[11] Vogelstein B and Kinzler KW (1992). p53 function and dysfunction. *Cell* **70**, 523–526.

[12] Agarwal ML, Taylor WR, Chernov MV, Chernova OB, and Stark GR (1998). The p53 network. *J Biol Chem* **273**, 1–4.

[13] Moll UM, Laquaglia M, Bénard J, and Riou G (1995). Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc Natl Acad Sci USA* **92**, 4407–4411.

[14] Issacs JS, Hardman R, Carman TA, Barrnet JC, and Weissman BE (1998). Differential subcellular p53 localization and function in N- and S-type neuroblastoma cell lines. *Cell Growth Differ* **9**, 545–555.

[15] Moos PJ, Edes K, and Fitzpatrick FA (2000). Inactivation of wild-type p53 tumor suppressor by electrophilic prostaglandins. *Proc Natl Acad Sci USA* **97**, 9215–9220.

[16] Swamy MV, Herzog CR, and Rao CV (2003). Inhibition of COX-2 in colon cancer cell lines by celecoxib increases the nuclear localization of active p53. *Cancer Res* **63**, 5239–5242.

[17] Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, Bergman J, Wiman KG, and Selivanova G (2002). Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* **8**, 282–288.

[18] Foster BA, Coffey HA, Morin MJ, and Rastinejad F (1999). Pharmacological rescue of mutant p53 conformation and function. *Science* **286**, 2507–2510.

[19] Hietanen S, Lain S, Krausz E, Blattner C, and Lane DP (2000). Activation of p53 in cervical carcinoma cells by small molecules. *Proc Natl Acad Sci USA* **97**, 8501–8506.

[20] Takimoto R, Wang W, Dicker DT, Rastinejad F, Lyssikatos J, and El-Deiry WS (2002). The mutant p53-conformation modifying drug, CP-31398, can induce apoptosis of human cancer cells and can stabilize wild-type p53 protein. *Cancer Biol Ther* **1**, 47–55.

[21] Bykov VJ, Issaeva N, Zache N, Shilov A, Hultcrantz M, Bergman J, Selivanova G, and Wiman KG (2005). Reactivation of mutant p53 and induction of apoptosis in human tumor cells by maleimide analogs. *J Biol Chem* **280**, 30384–30391.

[22] Bullock AN, Henckel J, and Fersht AR (2000). Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy. *Oncogene* **19**, 1245–1256.

[23] Wang W, Takimoto R, Rastinejad F, and El-Deiry WS (2003). Stabilization of p53 by CP-31398 inhibits ubiquitination without altering phosphorylation at serine 15 or 20 or MDM2 binding. *Mol Cell Biol* **23**, 2171–2181.

[24] Rao CV, Swamy MV, Patlolla JM, and Kopelovich L (2008). Suppression of familial adenomatous polyposis by CP-31398, a TP53 modulator, in APC$^{min/+}$ mice. *Cancer Res* **68**, 7670–7675.

[25] Rao CV, Steele VE, Swamy MV, Patlolla JM, Guruswamy S, and Kopelovich L (2009). 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. *Cancer Res* **69**, 8175–8182.

[26] Bykov VJN, Issaeva N, Selivanova G, and Wiman KG (2002). Mutant p53-dependent growth suppression distinguishes Prima-1 from known anticancer drugs: a statistical analysis of information in the National Cancer Institute database. *Carcinogenesis* **23**, 2011–2018.

[27] Li Y, Mao Y, Brandt-Rauf PW, Williams AC, and Fine RL (2005). Selective induction of apoptosis in mutant p53 premalignant and malignant cancer cells by Prima-1 through the c-Jun-NH_2-kinase pathway. *Mol Cancer Ther* **4**, 901–909.

[28] Stoner GD (1998). Introduction to mouse lung tumorigenesis. *Exp Lung Res* **24**, 375–383.

[29] Hecht SS (2005). Carcinogenicity studies of inhaled cigarette smoke in laboratory animals: old and new. *Carcinogenesis* **26**, 1488–1492.

[30] Hecht SS, Isaacs S, and Trushin N (1994). Lung tumor induction in A/J mice by the tobacco smoke carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[a]pyrene: a potentially useful model for evaluation of chemopreventive agents. *Carcinogenesis* **15**, 2721–2725.

[31] Malkinson AM (1998). Molecular comparison of human and mouse pulmonary adenocarcinomas. *Exp Lung Res* **24**, 541–555.

[32] Malkinson AM (2001). Primary lung tumors in mice as an aid for understanding, preventing, and treating human adenocarcinoma of the lung. *Lung Cancer* **32**, 265–279.

[33] Nikitin AY, Alcaraz A, Anver MR, Bronson RT, Cardiff RD, Dixon D, Fraire AE, Gabrielson EW, Gunning WT, Haines DC, et al. (2004). Classification of proliferative pulmonary lesions of the mouse: recommendations of the mouse models of human cancers consortium. *Cancer Res* **64**, 2307–2316.

[34] Kapetanovic IM, Muzzio M, McCormick DL, Thompson TN, Johnson WD, Horn TL, Mohammed A, Rao CV, and Kopelovich L (2012). Pharmacokinetics and tissue and tumor exposure of CP-31398, a p53-stabilizing agent, in rats. *Cancer Chemother Pharmacol* **69**, 1301–1306.

[35] Roth JA (2006). Adenovirus p53 gene therapy. *Expert Opin Biol Ther* **6**, 55–61.

[36] Peng Z (2005). Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. *Hum Gene Ther* **16**, 1016–1027.

[37] Selivanova G (2010). Therapeutic targeting of p53 by small molecules. *Semin Cancer Biol* **20**, 46–56.

[38] Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, and Jacks T (1994). p53 status and the efficacy of cancer therapy *in vivo*. *Science* **266**, 807–810.

[39] Takahashi T, Carbone D, Nau MM, Hida T, Linnoila I, Ueda R, and Minna JD (1992). Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. *Cancer Res* **52**, 2340–2343.

[40] Tang X, Zhu Y, Han L, Kim AL, Kopelovich L, Bickers DR, and Athar M (2007). CP-31398 restores mutant p53 tumor suppressor function and inhibits UVB-induced skin carcinogenesis in mice. *J Clin Invest* **117**, 3753–3764.

[41] Sunday ME, Willett CG, Graham SA, Oreffo VI, Linnoila RI, and Witschi H (1995). Histochemical characterization of non-neuroendocrine tumors and neuroendocrine cell hyperplasia induced in hamster lung by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone with or without hyperoxia. *Am J Pathol* **147**, 740–752.

[42] Khuri FR, Lee JS, Lippman SM, Lee JJ, Kalapurakal S, Yu R, Ro J, Morice RC, Hong WK, and Hittelman WN (2001). Modulation of proliferating cell nuclear antigen in the bronchial epithelium of smokers. *Cancer Epidemiol Biomarkers Prev* **10**, 311–318.

[43] Madka V, Zhang Y, Li Q, Mohammed A, Sindhwani P, Lightfoot S, Wu XR, Kopelovich L, and Rao CV (2013). p53-stabilizing agent CP-31398 prevents growth and invasion of urothelial cancer of the bladder in transgenic UPII-SV40T mice. *Neoplasia* **15**, 966–974.

[44] Matzinger S, Crist KA, Stoner GD, Anderson MW, Pereira MA, Steele VE, Kelloff GJ, Lubet RA, and You M (1995). K-*ras* mutations in lung tumors from A/J and A/J×TSG-*p53* F_1 mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and phenethyl isothiocyanate. *Carcinogenesis* **16**, 2487–2492.

[45] De Flora S, Balansky RM, D'Agostini F, Izzotti A, Camoirano A, Bennicelli C, Zhang Z, Wang Y, Lubet RA, and You M (2003). Molecular alterations and lung tumors in p53 mutant mice exposed to cigarette smoke. *Cancer Res* **63**, 793–800.

[46] Wang Y, Zhang Z, Lubet R, and You M (2005). Tobacco smoke-induced lung tumorigenesis in mutant A/J mice with alterations in K-ras, p53, or Ink4a/Arf. *Oncogene* **24**, 3042–3049.