Selective induction of apoptosis in mutant p53 premalignant and malignant cancer cells by PRIMA-1 through the c-Jun-NH_2-kinase pathway

Yin Li,[1] Yuehua Mao,[1] Paul W. Brandt-Rauf,[2] Ann C. Williams,[3] and Robert L. Fine[1]

[1]Experimental Therapeutics Program, Division of Medical Oncology, College of Physicians and Surgeons and [2]Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University Medical Center, New York, New York and [3]Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol, United Kingdom

Abstract

PRIMA-1 (p53 reactivation and induction of massive apoptosis) is a chemical compound that was originally identified as a selective mutant p53-dependent growth suppressor by screening a library of low-molecular-weight compounds. However, its mechanism of action is unknown. In this study, we examined toxicity of PRIMA-1 to three premalignant human colorectal adenoma cell lines (RG/C2, BR/C1, and AA/C1) and four colorectal carcinoma cell lines (DLD-1, SW480, LOVO, and HCT116) and its mechanism of action. It selectively induced apoptosis only in the mutant p53 premalignant and malignant colon cell lines, but was not toxic to the wild-type p53 premalignant and malignant colon cell lines. Using stable transfectants of temperature-sensitive p53 mutant Ala^{143} in null p53 H1299 lung cancer cells, we found that PRIMA-1 induced significantly more apoptosis in cells with mutant p53 conformation (37°C) than the wild-type p53 conformation (32.5°C). Cell cycle analysis indicated that its inhibition of cell growth was correlated with induction of G_2 arrest. Western blot analysis showed PRIMA-1 increased p21 and GADD45 expression selectively in the mutant p53 cells. However, Fas, Bcl-2 family proteins, and caspases were not involved in PRIMA-1–induced cell death. The c-Jun-NH_2-kinase (JNK) inhibitor SP 600125, but not p38 mitogen-activated protein kinase inhibitor SB 203580 or extracellular signal-regulated kinase inhibitor PD 98059, blocked PRIMA-1–induced apoptosis. Transfection with a dominant-negative phosphorylation mutant JNK, but not a dominant-negative p38 or wild-type JNK, inhibited PRIMA-1–induced cell death, suggesting that the JNK pathway plays an important role in PRIMA-1–induced apoptosis. PRIMA-1 is a highly selective small molecule toxic to p53 mutant cells and may serve as a prototype for the development of new p53-targeting agents for therapy of premalignant and malignant cells. [Mol Cancer Ther 2005;4(6):901–9]

Introduction

Under physiologic conditions in normal cells, p53 protein is expressed at low levels and has a short half-life due to rapid turnover mediated by ubiquitination and proteolysis (1). The p53 protein becomes stabilized and activated in response to a number of stimuli, including exposure of cells to DNA-damaging agents and oncogene activation. The activation of p53 allows it to carry out its function as a tumor suppressor through cell cycle arrest, apoptosis, DNA repair, differentiation, and antiangiogenesis (2–7). At least 50% of human tumors contain mutations or deletions of p53 (8). The fact that the specific DNA-binding function of p53 is disrupted in most tumors with a p53 mutation indicates that this function is critical for p53-mediated tumor suppression. p53-dependent cell death is a major pathway for the efficacy of cancer chemotherapy, and tumors carrying mutant p53 are often more resistant to chemotherapy than tumors with wild-type (WT) p53 (9). The wide range of the biological effects of p53 can be explained by its activation of expression of a number of target genes (>60), including p21; growth arrest; and DNA damage-inducible (GADD) family proteins, 14-3-3σ, MDM2, cyclins, transforming growth factor-β, Bax, and Fas/APO1 (1). p21 is not only a cyclin-dependent kinase inhibitor but also acts as a direct participant in regulating genes involved in growth arrest, senescence, and aging, thus providing an additional layer of control over the cell cycle (10). Induction of p21 caused a number of different cell lines to arrest in both G_1 and G_2 (11). GADD45 and 14-3-3σ are transcriptional targets of WT p53 and act as mediators of p53-dependent G_2 arrest. GADD45 is also regulated in a p53-independent manner (12).

The mitogen-activated protein kinase (MAPK) family comprises c-Jun-NH_2-kinase (JNK), p38 MAPK, and several extracellular signal-regulated kinases (ERK). This signal transduction pathway has been implicated in many physiologic processes, including growth, differentiation, survival, and cell death through a p53-independent mechanism (13). JNK and p38 MAPK signaling pathways are also activated by various and overlapping stimuli, such as heat shock, radiation, growth factors, and some chemotherapy drugs (14–16). In JNK family proteins, the

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Requests for reprints: Robert L. Fine, Division of Medical Oncology, College of Physicians and Surgeons, Columbia University Medical Center, Black Building 20-25, 650 West 168th Street, New York, NY 10032. Phone: 212-305-1168; Fax: 212-305-7348. E-mail: rlf20@columbia.edu

10 isoforms are encoded by three genes—*JNK1, JNK2*, and *JNK3* (17). JNK1 protein kinase activation requires phosphorylation on Thr^{183} and Tyr^{185} (18). Expression of the dominant-negative JNK (DN-JNK), which is the mutant JNK1 isoform where the sites of activating Thr^{183} and Tyr^{185} phosphorylation are replaced with Ala and Phe, respectively, markedly suppressed the ability of paclitaxel to induce apoptosis (19).

The p53 mutant Ala^{143} is a human temperature-sensitive mutant p53 with two conformational states. At 32.5°C, it possesses strong DNA-binding ability and functions like WT p53; but at 37°C, its ability to bind DNA and activate transcription is severely weakened or lost and acts like mutant p53 (20, 21). Using the human null p53 lung cancer cell line H1299 and prostate cancer cell line PC-3, we stably transfected the human temperature-sensitive p53 mutant Ala^{143} (Val-Ala) for use as our model in previous studies. With these cell lines, we previously reported that Fas-mediated apoptosis occurred only with the WT p53 phenotype at 32.5°C but not at the mutant p53 temperature (22).

PRIMA-1 (p53 reactivation and induction of massive apoptosis) is a chemical compound originally identified as a selective mutant p53-dependent growth suppressor by screening a library of low-molecular-weight compounds. This small molecule (molecular weight: 185) restored sequence-specific DNA binding and functional p53 conformation to mutant p53 protein *in vitro* and *in situ* (23, 24). Results from screening PRIMA-1 in the National Cancer Institute cell line database showed a significant preference for growth inhibition of tumor cell lines expressing mutant p53 compared with lines expressing WT p53 (25). The mechanisms responsible for this preferential activity, its effect on premalignant cells, and the pathways of cell death are unknown.

In this study, we examined the toxicity of PRIMA-1 to human colon premalignant and malignant cells and lung cancer cells. We also investigated its mechanism of action in the intrinsic and extrinsic pathways of apoptosis, as well as its effects on cell cycle regulatory proteins and the MAPK pathways.

Materials and Methods

Reagents

PRIMA-1 was chemically synthesized by Karolinska Institute [Stockholm, Sweden; name: 2,2-*bis*(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one]. PRIMA-1 stocks were prepared in 100% DMSO and stored in aliquots at −80°C. MAPK inhibitors (SP 600125, PD 98059, and SB 203580) were purchased from Sigma (St. Louis, MO).

Cell Lines and Tissue Culture

The premalignant cell lines used in this study have been derived from human colorectal adenomas in the laboratory of Drs. Christos Paraskeva and Ann Williams (University of Bristol, Bristol, United Kingdom). RG/C2 (mutant p53/Arg^{282}Trp) and BR/C1 (mutant p53/deleted amino acids 262-266) are clonogenic, nontumorigenic adenoma-derived cell lines hemizygous for mutant p53 (26, 27). AA/C1 (WT p53) is a clonogenic, nontumorigenic adenoma cell line (28). These premalignant lines grow *in vitro* but cannot grow *in vivo* or in soft agar. H1299/ts p53-143#6 is the stably transfected temperature-sensitive p53 mutant Ala^{143} in null p53 H1299 lung cancer cells described in our previous study (22). The human colon cancer cell lines DLD-1 (mutant p53/Ser^{241}Phe), SW480 (mutant p53/Arg^{273}His), LOVO (WT p53), HCT116 (WT p53), and lung cancer cell line H1299 (null p53) were obtained from American Type Culture Collection (Rockville, MD). The premalignant colon cell lines RG/C2, BR/C1, and AA/C1 were maintained with a 1:1 mixture of DMEM/F12 supplemented with 5% horse serum, 4 mmol/L L-glutamine, 100 μg/mL penicillin/streptomycin, 20 mmol/L HEPES, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, and 20 ng/mL epidermal growth factor (26). DLD-1, SW480, LOVO, HCT116, and H1299 cells were maintained in RPMI 1640 supplemented with 10% FCS.

Antibodies

Anti-p21 polyclonal antibody (clone: H-164), anti-GADD45 polyclonal antibody (clone: H-165), anti-14-3-3σ polyclonal antibody (clone: C-18), anti-Bcl-XL polyclonal antibody (clone: H-5), anti-Bax polyclonal antibody (clone: N-20), and anti-JNK1 polyclonal antibody (clone: C-17) were obtained from Santa Cruz (San Diego, CA). Anti-human Fas polyclonal antibody was obtained from MIC (Watertown, MA). Anti–α-tubulin monoclonal antibody was obtained from Sigma.

DN-JNK Plasmid and Transfectants

Human WT JNK1 (p46 JNK, WT-JNK) cDNA was produced in our laboratory by reverse transcription-PCR and confirmed by DNA sequencing. DN-JNK cDNA was produced by amino acid substitution of threonine (ACG) 183 for alanine (GCG) and tyrosine (TAT) 185 for phenylalanine (TTT) in the dual-activating phosphorylation sites of WT-JNK.

The E1/E3-deleted adenovirus vectors pAd/CMV/WT-JNK (Ad-WT-JNK) and pAd/CMV/DN-JNK (Ad-DN-JNK) were constructed and propagated into 293A cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The virus particle titter was 3×10^8 pfu/mL as determined by plaque titration assays in 293A cells. pAd/CMV/DsRed (Invitrogen) was used as a control. For transient transfection, DLD-1 cells were infected with adenovirus containing either pAd/CMV/DsRed (Ad-vector), pAd/CMV/WT-JNK (Ad-WT-JNK), or pAd/CMV/DN-JNK (Ad-DN-JNK) for 36 hours, and their expression was assessed by Western blot.

4′,6-Diamidino-2-Phenylindole Staining

Cells were treated with various concentrations of PRIMA-1 for different periods of time. Cells were washed with PBS, fixed in 4% paraformaldehyde at room temperature for 1 hour, and stained with 50 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) at 4°C for 2 hours. Percentage of apoptotic cells was scored in at least 400 cells in each sample by fluorescence microscopy. The microscopy reader was blinded to the actual groups.

Annexin V Assay

Cells were treated with 100 μmol/L PRIMA-1 for 16 hours. To quantitate the incidence of apoptotic cells, cells were stained with phycoerythrin-labeled Annexin V using the apoptosis detection kit (MIC) according to the manufacturer's protocol. The percentage of Annexin V-phycoerythrin–positive cells was quantitated from the fluorescence intensity of 10,000 cells using FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

Terminal Deoxynucleotidyl Transferase Assay

Cells were preincubated for 16 hours at 37°C or 32.5°C and then treated with 50 or 100 μmol/L PRIMA-1 for 16 hours. Terminal deoxynucleotidyl transferase (TdT) assay was done with a MEBSTAIN Apoptosis Kit Direct (MIC) according to the manufacturer's instructions. The percentage of TdT-positive cells were quantitated from the fluorescence intensity of 5,000 cells using FACScan flow cytometry (Becton Dickinson).

Cell Cycle Analysis

For propidium iodide staining, cells were preincubated for 16 hours at 37°C or 32.5°C and then treated with 50 μmol/L PRIMA-1 for different periods of time. After collection by centrifugation at 1,200 rpm for 5 minutes, cell pellets were washed once in PBS and fixed in 70% ice-cold ethanol and kept at −20°C overnight. Fixed cells were centrifuged, washed once in PBS, and then resuspended in PBS containing 50 μg of propidium iodide per milliliter and 100 μg of DNase-free RNase A per milliliter. The cell suspension was incubated for 30 minutes at 37°C and protected from light and 10,000 cellswere analyzed by FACScan flow cytometry (Becton Dickinson).

Western Blot

Cell lysates were prepared in lysis buffer [20 mmol/L Tris·Cl (pH 7.6), 1 mmol/L EDTA (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, 10 μg/mL aprotinin, 5 mmol/L benzamidine, 50 μg/mL leupeptin, 10 μg/mL pepstatin A, and 1 mmol/L phenylmethylsulfonyl fluoride] for 15 minutes on ice and centrifuged at 10,000 × *g* for 30 minutes. Equal amounts of lysates (40 μg) were boiled in SDS sample buffer and loaded on SDS-PAGE. After transfer, immunoreactive products were detected by the ECL system (Amersham Pharmacia, Piscataway, NJ). Expression of α-tubulin was used as a control.

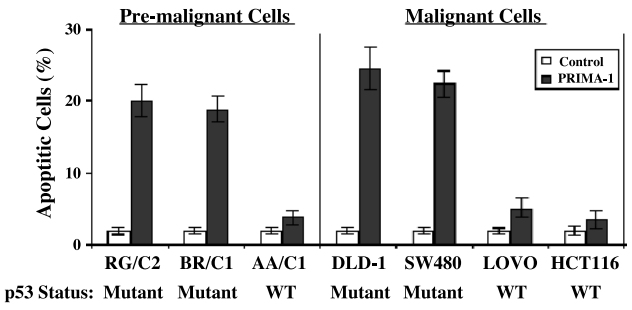

Figure 1. PRIMA-1–induced apoptosis in premalignant and malignant human colon cells by DAPI staining. Cells were treated with 100 μmol/L PRIMA-1 for 24 h, and changes in nuclear morphology were analyzed by DAPI staining. Percentage of apoptotic cells with nuclear morphology typical of apoptosis was scored in at least 400 cells in each sample by fluorescence microscopy. All microscopy experiments were done by a blinded reader to the actual groups. *Columns,* mean of three independent experiments.

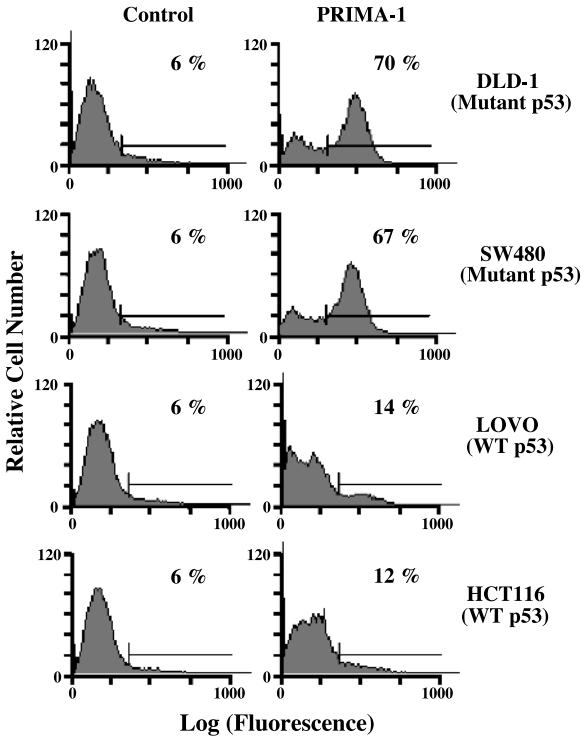

Figure 2. PRIMA-1 induced apoptosis in malignant human colon cells by Annexin V assay. Cells were treated with 100 μmol/L PRIMA-1 for 16 h. The Annexin V assays utilized the phycoerythrin-labeled apoptosis detection kit according to the manufacturer's protocol (MIC). The percentage of Annexin V-phycoerythrin–positive cells was quantitated by the fluorescence intensity of 10,000 cells using FACSCalibur. Experiments were done thrice and a representative experiment is shown.

Results

PRIMA-1–Induced Apoptosis in Mutant p53 Premalignant and Malignant Colon Cancer Cells

To investigate the selective toxicity of PRIMA-1 to premalignant and malignant colon cells, we studied three premalignant human lines (RG/C2, BR/C1, and AA/C1) and four malignant human lines (DLD-1, SW 480, LOVO, and HCT 116). In comparison to the WT p53 cell lines (AA/C1, LOVO, and HCT 116), a greater percentage of apoptotic cells were found in the mutant p53 lines (RG/C2, BR/C1, DLD-1, and SW480) after exposure to 100 μmol/L PRIMA-1 for 24 hours by DAPI staining (Fig. 1). In addition, Annexin V assays were done in human malignant DLD-1, SW480, LOVO, and HCT116 cell lines. As shown in Fig. 2, PRIMA-1 induced significantly more apoptosis in Annexin V assays in the mutant p53 lines DLD-1 and SW480 cells than the WT p53 lines (LOVO, HCT116) after exposure to 100 μmol/L PRIMA-1 for 16 hours (average 68% versus 13%, respectively).

The results showed that PRIMA-1 preferentially induced more cell death in the mutant p53 lines than in the WT p53 premalignant and malignant colon cells. Also, it showed for the first time that premalignant cells with mutant p53 were as sensitive to PRIMA-1 as malignant cells with mutant p53.

PRIMA-1 – Induced Apoptosis in the Temperature-Sensitive p53 Mutant 143 Model

To further substantiate whether PRIMA-1 was more toxic to mutant or WT p53 cancer cells for induction of apoptosis, we established stable transfectants of human temperature-sensitive p53 mutant ^{143}(Val-Ala) in the null p53 human lung adenocarcinoma cancer cell line H1299 (22). Cells were preincubated at 37°C or 32.5°C for 16 hours and then treated with 50 or 100 μmol/L PRIMA-1 for 24 hours. Apoptotic cells were determined by DAPI staining and TdT (TdT-mediated nick end labeling, TUNEL) assays. The results are shown in Fig. 3. PRIMA-1 was more toxic to H1299/ts p53-143#6 cells at the mutant p53 temperature (37°C) than at its WT p53 temperature (32.5°C). The degree of apoptosis was as follows: 58% at 37°C and 11% at 32.5°C by DAPI staining (Fig. 3A) and 65% at 37°C and 20% at 32.5 °C by TdT assay (Fig. 3B) after exposure to 100 μmol/L PRIMA-1. In addition, there was only 8% apoptosis in nontransfected null p53 H1299 cells exposed to 100 μmol/L PRIMA-1 at either temperature by both assays (Fig. 3A and B). These data indicated that PRIMA-1 was preferentially more toxic to cells expressing the mutant p53 form than WT p53 form in H1299 lung cancer cells and its induction of apoptosis was p53 dependent.

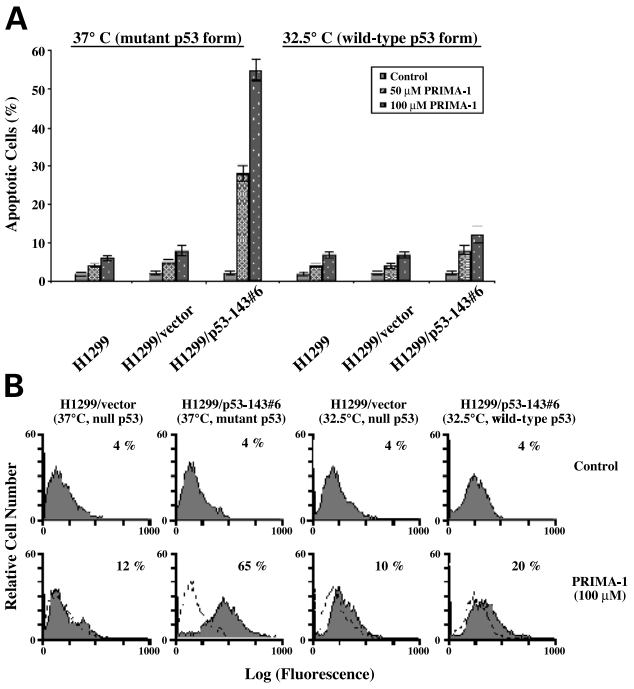

Figure 3. PRIMA-1 induced apoptosis in H1299 human lung cancer cells expressing a temperature-sensitive p53 mutant Ala^{143}. **A,** percentage of apoptotic cells by DAPI staining. Cells were preincubated for 16 h at 37°C or 32.5°C and then treated with 50 or 100 μmol/L PRIMA-1 for 24 h. Nuclear morphology was analyzed by DAPI staining from 400 cells. **B,** TdT (TUNEL) assay for apoptosis induced by PRIMA-1. Cells were preincubated for 16 h at 37°C or 32.5°C and then treated with 100 μmol/L PRIMA-1 for 16 h. DNA fragmentation was determined by TdT (TUNEL) assay from 5,000 cells according to the manufacturer's instructions. A representative histogram from an experiment shows relative apoptotic cell numbers from 5,000 cells studied in three independent experiments.

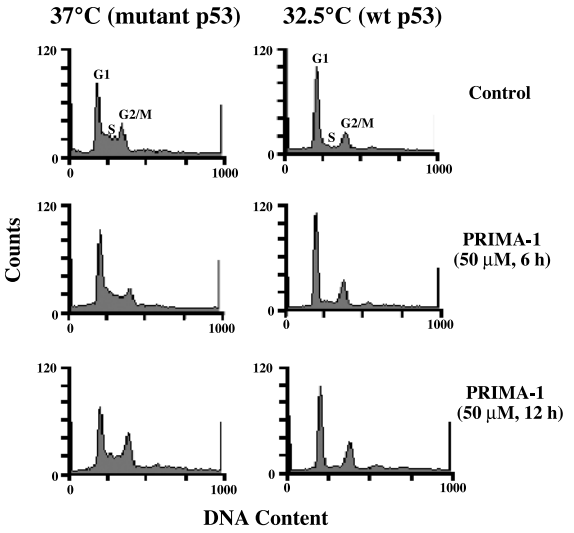

Figure 4. Time course effect of PRIMA-1 upon cell cycle in human lung cancer cells H1299/ts p53-143#6. Cells were preincubated for 16 h at 37°C or 32.5°C; treated with 50 μmol/L PRIMA-1 for 6, 12, 24, or 48 h; stained with propidium iodide; and 10,000 cells were analyzed by flow cytometry. The experiment was repeated thrice. A representative experiment is shown from three independent experiments. DNA content is presented as relative fluorescence.

The Effect of PRIMA-1 on Cell Cycle

To determine whether PRIMA-1 had an effect on the cell cycle, DNA content analyses for G_1 and G_2 peaks were done in temperature-sensitive p53 mutant H1299 stable transfectants. When cells were treated with 50 μmol/L PRIMA-1, the G_2 peak increased only at 37°C, but not at

Table 1. The effects of PRIMA-1 on the cell cycle of H1299 cells

Cell cycle phase	Control	6 h	12 h	24 h	48 h
37°C					
Sub-G_1	4	4	6	10	22
G_0-G_1	50	51	36	40	38
S	22	19	18	20	19
G_2-M	24	26	40	30	21
32.5°C					
Sub-G_1	4	4	4	6	6
G_0-G_1	52	54	50	46	48
S	20	19	21	23	23
G_2-M	24	23	25	25	23

NOTE: Data show cell cycle distribution of cells incubated at 37°C or 32.5°C for 16 hours and then treated with 50 μmol/L PRIMA-1. Values represent the results of flow cytometry experiments. Representative histograms for the 6- and 12-hour points are shown in Fig. 4.

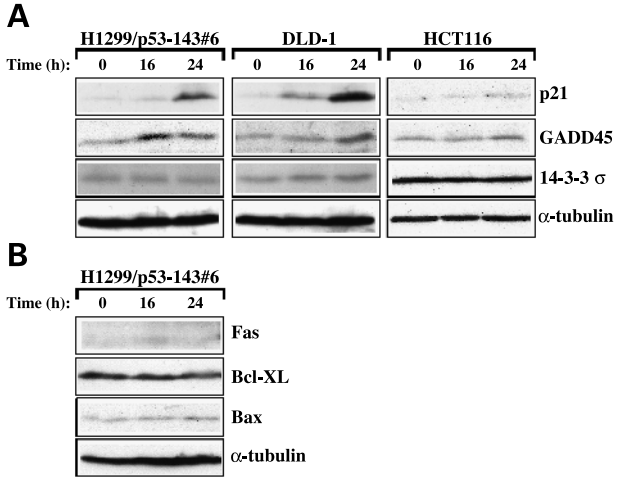

Figure 5. Effect of PRIMA-1 on *p53* target genes involved in cell cycle control (**A**) and apoptosis (**B**). **A,** cell lysates were prepared from H1299/ts p53-143#6 lung cancer cells at 37°C (mutant p53), DLD-1 colon cancer cells (mutant p53), and HCT116 colon cancer (WT p53) treated with 100 μmol/L PRIMA-1 for 0, 16, or 24 h. Expression of p21, GADD45, and 14-3-3σ was detected by using a polyclonal anti-p21 antibody (H-164), anti-GADD45 antibody (H-165), and anti-14-3-3σ antibody (C-18), respectively. Expression of α-tubulin was used as a control. **B,** Western blot time course for expression of Fas, Bcl-XL, and Bax in H1299/ts p53-143 #6 cells at 37°C (mutant p53) exposed to 100 μmol/L PRIMA-1. No changes were noted in these proteins in the H1299 cells (mutant p53) and similar results were obtained in DLD-1 and HCT116 cell lines (data not shown). Experiments were done thrice.

32.5°C, at 6- and 12-hour time points as shown in Fig. 4. The cell cycle distribution changes after treatment with 50 μmol/L PRIMA-1 for up to 48 hours is summarized in Table 1. In addition, these results were also replicated with BrdUrd staining/fluorescence-activated cell sorting analysis (data not shown). The data suggested that PRIMA-1 preferentially caused G_2 arrest in the H1299 cells expressing the mutant p53 form at 37°C, but not at the WT p53 temperature.

The Effect of PRIMA-1 on *p53* Target Genes Involved in Cell Cycle Control and Apoptosis

To investigate whether the G_2 arrest of PRIMA-1 on cell cycle was potentially related to cell cycle regulatory proteins directly transactivated by functional p53, we investigated the expression of p21, GADD45, and 14-3-3σ in H1299/ts p53-143 lung cancer cells, DLD-1, and HCT116 colon cancer cells. As shown in Fig. 5A, expression of p21 and GADD45, but not 14-3-3σ, was increased by PRIMA-1 exposure in a time course study in H1299/ts p53-143 cells with mutant p53 form at 37°C and the mutant p53 DLD-1 colon cancer cells. However, there was no change of expression of these proteins in the WT p53 HCT116 cells

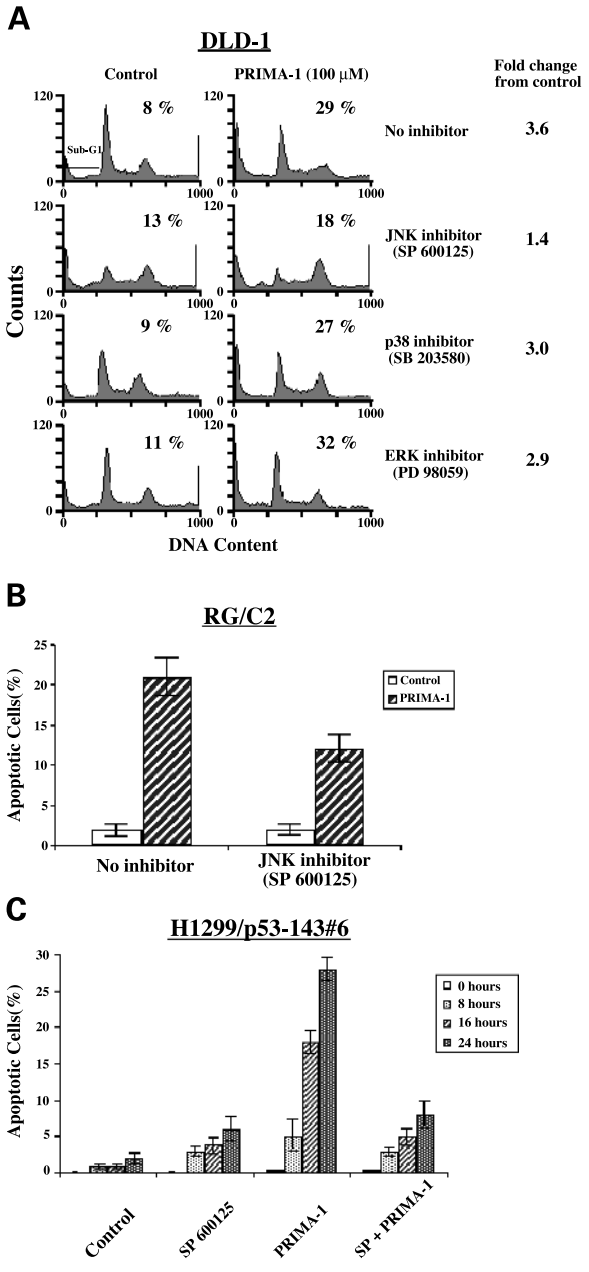

Figure 6. Effects of MAPK inhibitors on PRIMA-1 – induced apoptosis. **A,** propidium iodide staining in mutant p53 human colon cancer cell line DLD-1. Cells were preincubated with 50 μmol/L MAPK inhibitor SP 600125 (JNK inhibitor), SB 203580 (p38 MAPK inhibitor), or PD 98059 (ERK inhibitor) for 2 h and then treated with 100 μmol/L PRIMA-1 for 24 h, stained with propidium iodide, and analyzed by flow cytometry. DNA content is presented as relative fluorescence. Data represents the mean of three experiments. The fold increase above control is shown at the right of each figure. **B,** DAPI staining in mutant p53 human colon premalignant cell line RG/C2. Cells were preincubated with 50 μmol/L JNK inhibitor SP 600125 for 2 h and then treated with 100 μmol/L PRIMA-1 for 24 h. Nuclear morphology was analyzed by DAPI staining. Percentage of cells with nuclear morphology typical of apoptosis was scored in at least 400 cells in each sample by fluorescence microscopy. All microscopy experiments were done by a reader blinded to the actual groups. *Columns,* mean of three experiments. **C,** time course study by DAPI staining in human lung cancer cell line H1299/ts p53-143#6 at 37°C (mutant p53 form). Cells were pretreated with 50 μmol/L JNK inhibitor SP 600125 for 2 h and then treated with 50 μmol/L PRIMA-1 for 0, 8, 16, or 24 h. Nuclear morphology was analyzed by DAPI staining as above. *Columns,* mean of three experiments.

from the same exposure to PRIMA-1 (Fig. 5A). These data suggested that PRIMA-1 induced G_2 arrest and it was preceded or occurred concomitantly with increased levels of p21 and GADD45. Levels of Bax, Bcl-XL, and FAS were examined from the above experimental groups to investigate the involvement of the intrinsic and extrinsic pathways in the mechanism of apoptosis, respectively. As shown in Fig. 5B, the H1299 stably transfected temperature-sensitive p53 line showed no change in the levels of these proteins. The same findings were also detected in the DLD-1 and HCT116 cell lines (data not shown), implying that the intrinsic and extrinsic pathways of apoptosis were not involved in the mechanism of cell death by PRIMA-1 in these different cell lines.

The Effect of MAPK Inhibitors on PRIMA-1–Mediated Apoptosis

To investigate other possible mechanisms of PRIMA-1–induced apoptosis in mutant p53 cells, MAPK inhibitors were used to determine whether segments of the MAPK pathway were involved. Fig. 6A showed the results of the effect of three inhibitors on PRIMA-1–mediated apoptosis by propidium iodide staining. The JNK inhibitor SP 600125 at 50 μmol/L reduced the increase in apoptosis from 3.6-fold to 1.4-fold in the mutant p53 colon cancer cell line DLD-1 after exposure to 100 μmol/L PRIMA for 24 hours. Similar results were also obtained in the pre-malignant RG/C2 mutant p53 cells by DAPI staining as shown in Fig. 6B. In addition, similar results were detected in a time course study of 50 μmol/L JNK inhibitor SP 600125 in H1299/ts p53-143 cells at 37°C with DAPI staining as shown in Fig. 6C. However, the p38 MAPK inhibitor SB 203580 and the ERK inhibitor PD 98059 at 50 μmol/L did not significantly inhibit PRIMA-1–induced cell death (Fig 6A). All three MAPK inhibitors at 50 μmol/L did not show significant toxicity in control experiments without PRIMA-1 in the DLD-1, RG/C2, and H1299/ts p53-143 cell lines (Fig. 6A-C). These results indicated that the JNK pathway played an important role in PRIMA-1–induced apoptosis in the three different cell lines with mutant p53.

Expression of DN-JNK and Its Effect on PRIMA-1–Induced Cell Death

To further show that the JNK pathway was essential to the mechanism of cell death induced by PRIMA-1, we modulated JNK expression via an Ad5 adenovirus system. DLD-1 cells were transiently transfected with adenovirus containing either control Ad-vector, Ad-WT-JNK, or Ad-DN-JNK with three different multiplicities of infection (MOI) for 36 hours. The expression of WT-JNK and DN-JNK was detected by Western blot as shown in Fig. 7A. Approximately equal amounts of WT-JNK and DN-JNK were expressed from transfection of 50 or 100 MOI adenovirus in a dose-dependent manner. Cells were treated with 50 or 100 μmol/L PRIMA-1 for an additional 24 hours after being transfected with 50 MOI adenovirus of either Ad-vector, Ad-WT-JNK, or Ad-DN-JNK for 36 hours. The TdT assay was done for detection of apoptosis. DN-JNK expression decreased induction of apoptosis by 100 μmol/L PRIMA-1 from 28% to 11% in DLD-1 cells (Fig. 7B). In experiments with H1299/ts p53-143#6 cells treated with 50 μmol/L PRIMA-1, DN-JNK expression reduced apoptosis from 21% to 10% (Fig. 7C). However, transfection with Ad-WT-JNK did not produce a significant reduction in apoptosis in these cells (Fig. 7B and C). These results with JNK chemical inhibitor and DN-JNK showed that the JNK MAPK pathway played a critical role for PRIMA-1–induced apoptosis in these human premalignant and malignant cell lines.

Discussion

The low-molecular-weight compound PRIMA-1 was identified in a cellular screen of a chemical library from the National Cancer Institute using Saos-2-His^{273} osteosarcoma cell line that expresses tetracycline-regulated mutant p53 (23). Li et al. (24) and Baker et al. (25) had reported that PRIMA-1 restored sequence-specific DNA binding and the active conformation to mutant p53 proteins *in vitro* and *in situ*. PRIMA-1 induced apoptosis in a mutant p53–dependent manner using a panel of cell lines with expression of most common p53 mutant proteins. In this study, we showed, for the first time, that PRIMA-1 was not only toxic to malignant mutant p53 cells but was also toxic to premalignant mutant p53 human colon cells using DAPI and Annexin V assays (Figs. 1 and 2). In addition, PRIMA-1 was significantly more toxic to the mutant p53 conformation than the WT p53 form in lung cancer H1299 cells stably transfected with a human temperature-sensitive p53 mutant Ala^{143} (Fig. 3). We have also found the selective induction of apoptosis by PRIMA-1 in mutant p53 human cancer cells from breast, mesothelioma, and pancreatic cell lines compared with WT p53 lines.[4]

PRIMA-1 induced G_2 arrest in H1299 cells expressing human temperature-sensitive p53 mutant Ala^{143} at 37°C but not at 32.5°C (Fig. 4; Table 1). This was associated temporally with an induction of p21 and GADD45 in these cells, as well as in the mutant p53 DLD-1 cells, which is likely responsible for the PRIMA-1–induced G_2 arrest. However, p21 and GADD45 were not induced in the HCT116 cells with WT p53 (Fig. 5A). Induction of p21 by PRIMA-1 has been observed in human lung cancer cell line H1299 expressing mutant p53 His^{175}, as well as in the human colon mutant p53 cancer cell line SW 480 (23). Induction of p21 mainly occurs in p53-dependent and p53-independent G_1 arrest and apoptosis (29–32). p21 also participates in the G_2 checkpoint, as shown in altered HCT116 colon cancer cells lacking either p21 or p53, which did not arrest in G_2 after exposure to ionizing radiation (33). Cisplatin and BBR3464, a trinuclear platinum complex that exhibits potent cytotoxicity and efficacy against cisplatin-resistant tumors, induced p21 expression and caused a selective G_2-M arrest in ovarian cancer cells (34). T-cadherin–mediated cell growth regulation involves G_2, but not G_1, phase arrest and requires p21 expression (35).

[4] Y. Li and R.L. Fire, unpublished data.

These observations suggested a novel mechanism of growth regulation for drugs, inducing p21 expression and subsequent G_2 arrest. Our results support the concept that p21 induction by PRIMA-1 in mutant p53 cells can mediate a p53-dependent G_2 arrest mechanism. GADD45 is another transcriptional target of p53 implicated in regulating the G_2-M transition, but whether GADD45 plays a direct role in apoptosis remains unclear (12). GADD genes are stress response genes and classified as *GADD34*, *GADD45*, and *GADD153*, and they are induced by stressors, such as UV irradiation, chemical carcinogens, and starvation, respectively (36, 37). The GADD family of genes, by means of the p38 MAPK pathway, has been shown to play an important role in melanoma differentiation-associated gene-7 (*mda-7*)–induced apoptosis in human melanoma cells (38). Peroxynitrite-mediated oxidative stress activated p38 MAPK to induce three GADD genes in human neuroblastoma SH-SY5Y cells (39). We found the induction of *GADD45* by PRIMA-1 in cell lines with only mutant p53 including the human lung cancer H1299 temperature-sensitive p53 mutant Ala^{143} cells and human colon cancer DLD-1 cells, but not in the HCT116 line with WT p53 (Fig. 5A). The p38 MAPK inhibitor SB 203580 did not block PRIMA-1–induced apoptosis in DLD-1 cells (Fig. 6A), suggesting that the *GADD45* gene was involved in the G_2 arrest of the cell cycle in PRIMA-1, but the p38 MAPK pathway may not be required for PRIMA-1 to induce cell death in these colon cancer cells. Induction of apoptosis by PRIMA-1 was potently inhibited in DLD-1, RG/C2, H1299/p53-143#6 cell lines by specific JNK inhibitor SP 600125 and DN-JNK (Figs. 6 and 7B, C). Generally, activation of ERK forms of MAPK elicits survival responses, whereas activation of the stress-activated protein kinases, JNK, and p38 generally promote cell death (40, 41). The effects of these MAPKs seem to be cell type specific. Paclitaxel-induced apoptosis in MCF-7 cells (WT p53) can be inhibited in a p53-dependent manner by the JNK inhibitor SP 600125 (42) and can also be blocked with inhibitors of the ERK and p38 MAPK pathways in a p53-independent manner (43). Cantharidin-induced apoptosis via activation of p38 MAPK and JNK pathways were associated with mutant p53 and activation of caspase-3 (44). These data suggest that agent-induced G_2 arrest and apoptosis can be through p53-dependent and p53-independent MAPK pathways in a cell type–specific manner.

Specific caspase-8, caspase-9, and caspase-3 inhibitors, each at 4 μmol/L, either alone or all together did not block PRIMA-1–induced cell death under the conditions in our study in the lung cancer cell line H1299 temperature-sensitive p53-143 and colon cancer cell line DLD-1 (data not shown). However, the pan-caspase

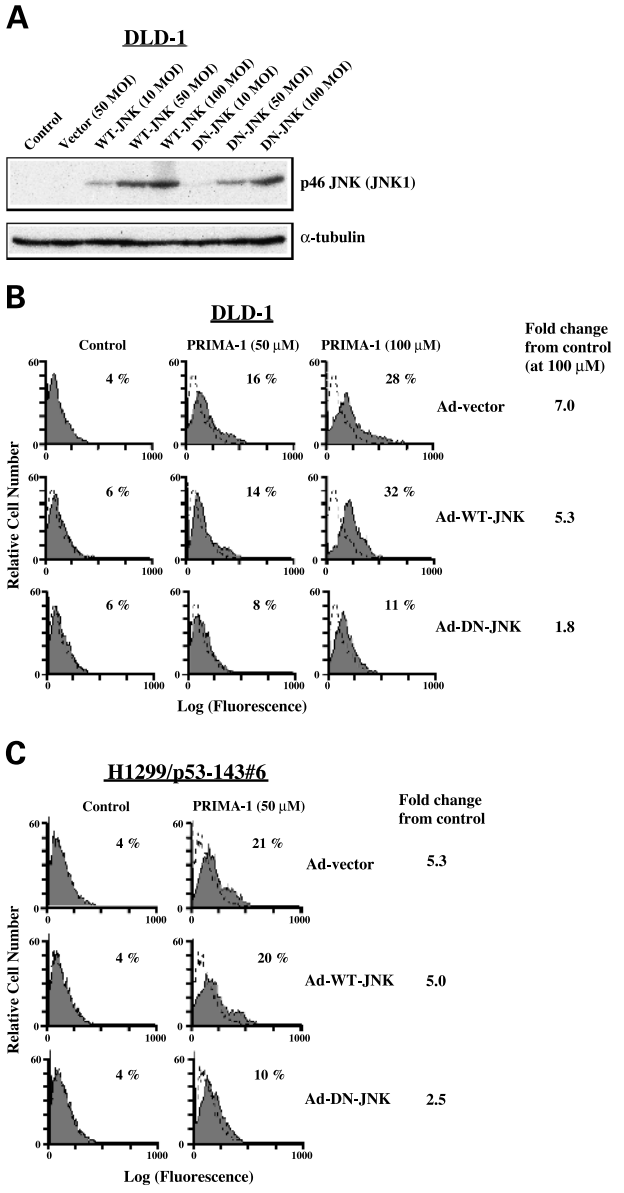

Figure 7. The effect of DN-JNK on PRIMA-1–induced apoptosis. **A,** expression of WT-JNK and DN-JNK in mutant p53 colon cancer DLD-1 cell line. Cells were infected with different MOIs of adenovirus containing either pAd/CMV/DsRed (*Ad-vector*), pAd/CMV/WT-JNK (*Ad-WT-JNK*), or pAd/CMV/DN-JNK (*Ad-DN-JNK*) for 36 h. Expression of JNK and DN-JNK proteins was detected by Western blot using anti-JNK1 polyclonal antibody (clone: C-17). Expression of α-tubulin was used as a control. **B,** DN-JNK blocked PRIMA-1–induced cell death in the DLD-1 cell line. Cells were infected with 50 MOI of adenovirus containing either Ad-vector, Ad-WT-JNK, or Ad-DN-JNK for 36 h. DLD-1 cell transient transfectants were treated with 50 or 100 μmol/L PRIMA-1 for an additional 24 h. Apoptotic cells were determined by TdT (TUNEL) assay and quantitated by flow cytometry from 5,000 cells tested according to the manufacturer's instructions. The histograms show relative apoptotic cell numbers of a representative experiment from three experiments. The fold increase above control is shown at the right of each figure. **C,** DN-JNK blocked PRIMA-1–induced cell death in H1299/ts p53-143#6 cells at 37°C (mutant p53 form). Cells were infected with 50 MOI of adenovirus containing either Ad-vector, Ad-WT-JNK, or Ad-DN-JNK for 24 h. H1299 transient transfectants were treated with 50 μmol/L PRIMA-1 for an additional 24 h. Apoptotic cells were determined by TdT (TUNEL) assay from 5,000 cells tested as above. The histogram shows relative apoptotic cell numbers of a representative experiment from three studies. The fold increase above control is shown at the right of each figure.

inhibitor VAD-FMK, at 100 μmol/L, completely blocked PRIMA-1–mediated cell death in human non–small cell lung carcinoma cell lines H460 and H23 (45). It is possible that the extraordinarily high concentration of the pan-caspase inhibitor (100 μmol/L) in this previous study may have been nonselective. In our experiments, the pan-caspase inhibitor VAD-FMK at 40 μmol/L also reduced PRIMA-1–induced cell death by 40% in mutant p53 colon cancer DLD-1 cells. Thus, PRIMA-1 did not induce any activities of the caspases tested (caspase-8, caspase-9, or caspase-3), but high concentrations of the pan-caspase inhibitor VAD-FMK reduced apoptosis induced by PRIMA-1, suggesting either a nonspecific toxic effect or an unknown caspase may be involved in this mechanism. Collectively, these results and prior studies suggest that PRIMA-1–induced apoptosis can be through a caspase-dependent or caspase-independent pathway possibly dependent upon cell line type.

Tumors carrying mutant p53 are often more resistant to conventional cancer therapy (9, 46, 47). Therefore, it is important to develop novel therapeutic strategies that target mutant p53 in tumors. A number of small molecules, such as CP-31398, WR1065, and PRIMA-1, have been identified, which restore partial degree of native conformation and WT function to mutant p53 (48). The study of the structure-activity relationship of these molecules could be helpful in the future for the discovery of more potent analogues for restoration of p53 function in mutant p53 tumors.

In conclusion, PRIMA-1 selectively induced apoptosis only in mutant p53, but not in WT p53, premalignant colon cells, and in malignant colon cells. It also induced significantly more apoptosis in human lung cancer H1299 cells with mutant p53 conformation than the WT p53 conformation. Induction of G_2 arrest by PRIMA-1 is probably conferred by up-regulation of p21 and GADD45. Induction of apoptosis occurred through the JNK pathway but did not require the p38 MAPK or ERK pathways as shown in the dominant-negative and pharmacologic inhibitor experiments. There was no evidence for involvement of Fas, the Bcl-2 family proteins, and caspase-8, caspase-9, and caspase-3 in PRIMA-1–induced apoptosis in premalignant and malignant colon cells and malignant lung cells with mutant p53. Our new findings suggest that PRIMA-1–induced cell death is through the JNK pathway in a p53-dependent manner and this may have important implications for cancer treatment, especially for colon cancer in different phases of transformation, including premalignant and malignant states. This work shows the selective efficacy of PRIMA-1 for inducing apoptosis in premalignant mutant p53 colon cells, which could possibly eliminate cells in evolution to frank malignancy. There are currently very few therapeutics or clinical modalities outside of surgery that have this potential. PRIMA-1 may serve as a leading prototypic compound for the development of new p53-targeting agents for more potent small molecules that work at clinically achievable concentrations.

Acknowledgments

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Yin Li, Yuehua Mao, Paul W. Brandt-Rauf, et al.

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