PRIMA-1 inhibits growth of breast cancer cells by re-activating mutant p53 protein

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Abstract. Mutation of the *p53* tumor suppressor gene is a common event in many types of tumors, including breast cancers. Mutant p53 (mtp53) protein is thought to promote tumor cell survival and resistance to chemotherapeutic drugs. Therefore, restoring p53 function by converting existing mtp53 to the wild-type p53 (wtp53) conformation is being pursued as one strategy to promote apoptosis of tumor cells. PRIMA-1 (p53 re-activation and induction of massive apoptosis) is a non-toxic small molecule that converts mtp53 to the active conformation and induces apoptosis in tumor cells. Here we examined whether PRIMA-1 activates mtp53 and induces cell death *in vitro* and *in vivo* in estrogen-responsive breast cancer cell lines that express mtp53 (BT-474, HCC-1428, and T47-D). Fluorescent staining with conformation-specific p53 antibodies demonstrated that PRIMA-1 converted mtp53 into the wtp53 conformation. *In vitro* treatment of tumor cells with PRIMA-1 (0-50 μM) led to a dose-dependent loss of cell viability and induced cell death markers. In contrast, PRIMA-1 had no effect on the viability of MCF-7 cells, normal breast cells, and endothelial cells, all of which express wtp53 protein. PRIMA-1 treatment of mice inhibited the growth of tumors from xenografts of BT-474, HCC-1428, and T47-D cells but did not influence xenografts obtained from MCF-7 cells. Mechanistic studies showed that PRIMA-1 induced the mitochondrial-dependent apoptotic pathway in mtp53-expressing breast cancer cells. Our findings suggest that PRIMA-1 renews the susceptibility of mtp53-expressing breast tumors to apoptosis and should be investigated for use in breast cancer therapy.

Introduction

Despite significant advances in breast cancer diagnosis and therapy, much remains to be done to prevent and treat breast disease in women (1). However, increasing knowledge of the molecular mechanisms of tumorigenesis and cancer progression is leading to novel approaches for preventing and treating cancer. The p53 protein is one of the best characterized of the tumor suppressor proteins, which protect against malignant transformation (2). Activation of p53 leads to apoptosis through either the death receptor pathway or the mitochondrial pathway (3). In the mitochondrial apoptotic pathway, p53 induces several genes, such as Bax, APAF-1, Puma, and Noxa (4-8). Bax expression can lead to increased mitochondrial membrane potential, which allows the release of cytochrome c into the cytoplasm (9). Cytochrome c release promotes apoptosis by interacting with caspase-9 and -3, resulting in initiation of the caspase cascade. Activation of effector caspases then trigger events that lead to apoptosis (10,11).

Mutation of the p53 tumor suppressor gene is the most common genetic alteration in human cancer, and ~50% of all breast cancers carry point mutations in the p53 gene (12). Whereas wild-type p53 (wtp53) is rapidly degraded in normal cells (2,13), the mutated p53 (mtp53) protein fails to undergo degradation in tumor cells and accumulates extensively (2,3,14,15). The majority of mtp53 alleles in breast cancer cells encode proteins that are defective in DNA binding, regulation at cell cycle check-points, and DNA damage-induced induction of apoptosis (12,16-18). Mutations in p53 or the p53 pathway are thought to play a key role in promoting tumor cell survival and tumor cell resistance to chemotherapeutic drugs (19,20). Therefore, restoring wtp53 function to the mtp53 protein present in tumor cells is a promising avenue of research for cancer therapy (3,21-23).

PRIMA-1 (p53 reactivation and induction of massive apoptosis) is a small molecule that has the ability to convert mtp53 to an active conformation, thereby restoring sequence-specific DNA binding and transcriptional activation of mtp53 (24). This in turns leads to p53-mediated apoptosis and cell-cycle arrest. PRIMA-1 is able to suppress the growth of tumor xenografts carrying mtp53 proteins (24,25), thus, by virtue of its ability to activate endogenous mtp53 protein, PRIMA-1 represents a unique model compound for the treatment of cancer.

A number of studies have examined the effects of PRIMA-1 on colon, lung, ovarian, lymphoma, and renal carcinomas (24,26,27). Studies of PRIMA-1 in breast cancer cells are limited, however (25,28,29). We have reported preliminary

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studies showing that PRIMA-1 restores wtp53 sequence-specific DNA-binding and increases the levels of p53 target gene products, such as p21, in BT-474, HCC-1428, and T47-D cells, all of which contain mtp53 (28). We also reported that PRIMA-1 inhibits hormone-induced expression of vascular endothelial growth factor in these breast cancer cells and inhibits T47-D breast tumor growth in nude mice (25,28). In the present study, we examined whether PRIMA-1 can restore the wtp53 conformation and induce apoptosis in a number of breast cancer cell lines that express mtp53, both *in vitro* and when growing as xenografts in a mouse model. We also sought to determine the mechanism responsible for PRIMA-1-induced tumor cell death. Our results demonstrate that PRIMA-1 is a non-toxic agent that suppresses the growth of breast cancer cells expressing mtp53, both *in vitro* and *in vivo*, by inducing apoptosis via the p53-dependent mitochondrial pathway.

Materials and methods

Cell culture. The following cell lines were used in this study: the mtp53-expressing breast cancer cell lines T47-D, BT-474, and HCC-1428 (28,30-32), and wtp53-expressing cells, including the breast cancer lines MCF-7, HCC-1500, and ZR-75; the normal breast-cell lines MCF-12A, MCF-12F, AG11132A, and 184A1; and human umbilical vein vascular endothelium cells (HUVECs). All cell lines were obtained from ATCC (Manassas, VA), except for AG11132A, and 184A1 that were obtained from Coriel Institute for Medical Research (Camden, NJ). BT-474, T47-D, MCF-7, and ZR-75 cells were grown in phenol red-free DME/F12 medium (Invitrogen Corp. and Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS). HCC-1428 and HCC-1500 were grown in RPMI-1640 medium (ATCC) supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g glucose/l, and 1.5 g sodium bicarbonate/l. HUVECs (passages 4-6) were cultured in F-12K medium supplemented with 0.1 mg/ml heparin, 0.05 mg/ml endothelial cell growth supplement, and 15% FBS. MCF-12A and MCF-12F cells were grown in DME/F12 medium supplemented with 20 ng/ml EGF, 100 ng/ml cholera toxin, 10 μg/ml insulin, 500 ng/ml hydrocortisone, and 5% horse serum (MCF-12A) or 5% Chelex-treated horse serum (MCF-12F) (Sigma, St. Louis, MO). AG11132A and 184A1 cells were grown in serum-free mammary epithelial growth medium (Coriel Institute for Medical Research, Camden, NJ), supplemented with 2 mM L-glutamine (AG11132A) or 5 μg/ml transferrin and 1 ng/ml cholera toxin (184A1). All cells were grown in 100x20-mm tissue culture dishes and harvested with 0.05% trypsin-EDTA (Invitrogen).

Determination of p53 status by immunofluorescence staining. Cells were stained with conformation-specific antibodies as previously described (24,33). Briefly, BT-474, HCC-1428, T47-D, and MCF-7 cells ($1x10^4$-$2x10^4$/well), were seeded into 8-well chamber slides overnight. The cells were washed and treated with or without PRIMA-1 (Tocris Bioscience, Ellisville, MO) at doses of 10, 25, and 50 μM for 16 h at 37˚C, washed twice with Dulbecco's phosphate-buffered salt solution (D-PBS; Invitrogen) and fixed with 4% paraformaldehyde for 15 min. After 3 washes with D-PBS for 5 min each, the cells were permeabilized with 0.2% Triton X-100 for 3 min, then washed with D-PBS 3 times and blocked with 5% goat serum-PBS for 60 min at room temperature (RT). The cells were next washed and incubated overnight with or without antibody PAb240 (Calbiochem, San Diego, CA; 1:50 dilution in 5% goat serum-PBS) that recognizes mtp53, and antibody PAb1620 (Calbiochem; 1:40 dilution in 5% goat serum-PBS) that recognizes wtp53, washed 3 times with D-PBS, and incubated at RT with a rhodamine-conjugated goat anti-mouse IgG antibody (Chemicon International Inc., Temecula, CA; 1:100 dilution) for 1 h in the dark. Finally, the cells were washed 6 times for 5 min each with D-PBS, mounted with 90% glycerol in PBS, coverslipped, and sealed with nail polish. Images were captured using a Coolsnap digital camera mounted on the Olympus fluorescent microscope and processed with Image-Pro Express software provided by the supplier.

Cell viability assay. The sulforhodamine B (SRB) assay was used to measure cell viability, as previously described (34). This dye-binding assay measures the protein content in surviving cells as an index to determine cell growth and viability (35,36). Briefly, cells were seeded in each well of 96-well plates in 100 μl culture medium and incubated overnight at 37˚C in an atmosphere of 5% CO_2. The next day, the medium was removed and the cells were washed with DMEM/F12 medium and treated with different concentrations of PRIMA-1 for 24 or 48 h in DMEM/F12 + 5% FBS. Surviving or adherent cells were fixed *in situ* by withdrawing growth medium, adding 100 μl of PBS and 100 μl of 50% cold trichloroacetic acid, and incubating the cells at 4˚C for 1 h. Cells were washed with ice-cold water, dried, and stained in 50 μl of 4% SRB (Sigma) for 8 min at RT. Unbound dye was removed by 5 washes with cold 1% acetic acid, and the cells were dried at RT. Bound stain was solubilized with 150 μl 10 mM Tris. The absorbance of samples was measured at 520 nm with a SpecTRA MAX 190 microplate reader (Molecular Device, Sunnyvale, CA). Six wells were used for each concentration, and each experiment was performed at least twice.

Cell apoptosis and death assay. We used an Annexin V-FITC apoptosis detection kit (Biovision Research Products, Mountain View, CA) to detect the early stages of apoptosis and propidium iodide (PI) to detect DNA fragmentation or cell death. Cells were grown in 6-well plates overnight with 10% FBS DMEM/F12 medium. The next day, the medium was removed and the cells were washed and treated with PBS or with 50 or 100 μM PRIMA-1 in 5% FBS DMEM/F12 medium for 24 h. The percentage of Annexin V-FITC-positive and PI-positive cells were determined from the fluorescence of 10,000 cells by a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Experiments were performed twice.

The effect of PRIMA-1 on inhibition of breast tumor growth in vivo. Female athymic nude (nu/nu) mice, 5-6 weeks old and weighing 18-22 g, were purchased from Harlan Sprague Dawley, Inc (Indianapolis, IN). The mice were housed in a

laminar air-flow cabinet under specific pathogen-free conditions. All facilities were accredited by the American Association for Accreditation of Laboratory Animal Care, Inc. in accordance with the current regulations and standards of the United States Department of Agriculture and the Public Health Service.

Nude mice were inoculated with 17-ß-estradiol pellets (1.7 mg/pellet, 60-day release) or placebo pellets (both from Innovative Research of America, Sarasota, FL) on the back of mice 24-48 h before inoculation with tumor cells. Breast cancer cells in culture were harvested by trypsinization, washed twice with DMEM/F12 medium, and $5x10^6$ cells were resuspended in 0.15 ml of Matrigel (BD Biosciences, Bedford, MA)/DMEM/F12 medium [4:1 (v/v) for BT-474, T47-D, and HCC-1428 cells, and 1:4 (v/v) for MCF-7 cells]. Cells (0.15 ml) were then injected subcutaneously (s.c.) into both flanks of each mouse. Tumor size was measured every 2 or 3 days with a digital caliper and tumor volumes were calculated by the formula (L x W x H) x $\pi/6$ (37). Treatments were started when tumor volumes reached 100-150 mm^3. Animals were assigned to 2 groups of 6 mice each and administered PRIMA-1 (50 mg/kg per day) by intravenous (i.v., 0.2 ml) injection into the tail vein for 10 days and then additional 3 treatments of single injection every 3 days; the control group was injected with PBS. Animals were weighed twice weekly throughout the course of the experiments. At the end of the experiment, animals were sacrificed, and tumors were harvested and weighed. Fresh tumor tissues were immediately placed in 4% paraformaldehyde solution for immunohistochemical analysis or frozen in liquid nitrogen for future studies.

Western blots and the mitochondria-dependent apoptotic pathway. Whole-cell extracts were prepared with a nuclear extraction TransAm kit (Active Motif, Carlsbad, CA). Briefly, cells were grown and treated with PRIMA-1 in 100-mm culture dishes. Following treatment, the cells were washed with cold PBS containing phosphatase inhibitors (included with the kit) and harvested by gentle scraping with a cell lifter. The cells were centrifuged (200 x g, 5 min), and the pellets were re-suspended in complete lysis buffer provided in the nuclear extraction kit; 1 mM dithiothreitol, and 1% protease inhibitor cocktail was added prior to use) and samples were incubated on ice for 30 min with shaking. The samples were centrifuged at 15,000 rpm in an Eppindorf centrifuge (4˚C), for 20 min and the supernatant was transferred to a microcentrifuge tube, aliquoted, and stored at -80˚C. For Western blotting, samples containing 35-45 μg of protein were separated in a NuPAGE 10% Bis-Tris Gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at 120 V for 1.5 h using NuPAGE MES-SDS Running Buffer (Invitrogen). Separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) at 35 V for 1.5 h. Blots were blocked at RT for 1 h in TBS containing 0.1% Tween-20 and 5% non-fat dry milk (TBS-T) and incubated with primary antibodies against BAX, Bcl-2, and p21 (all from Santa Cruz Biotechnology, Santa Cruz, CA; used at a 1:200 dilution), caspase-3 (R&D, Minneapolis, MN; 1:300 dilution), caspase-9 (R&D; 1:200 dilution), or p53 (DO1, Santa Cruz Biotechnology; 1:1000 dilution) for 2 h at RT. The blots were washed 3 times with TBS-T, incubated with secondary antibody for 1 h at RT, and washed 7 times with TBS-T. Immunoreactive bands were visualized using an ECL Plus detection kit (Amersham, Pharmacia Biotech, Arlington Heights, IL). Membranes were stripped and re-blotted for ß-actin (Sigma), which was used as a control for protein loading.

Immunohistochemical analysis of xenografts. At the end of treatment animals were euthanized with an institutionally approved protocol and tumor tissue was removed, fixed overnight in 4% paraformaldehyde, followed by paraffin infiltration and embedding as described previously (25). Immunohistochemical analysis was carried out for p53, p21, Ki67, and cleaved caspase-3 also as described previously (25). p53 antibody DO-1 (Santa Cruz, Inc., CA) was used; it recognizes both the mt and wt form of protein of human origin. Five-micrometer sections were mounted onto ProbeOn Plus microscope slides (Fisher Scientific Inc., Pittsburgh, PA), stained with hematoxylin-eosin, and examined for cellularity by light microscopy. For immunohistochemical analysis, sections were de-waxed in xylene, rehydrated through graded concentrations of ethanol, rinsed in distilled water, and subjected to heat-induced epitope retrieval in 10 mM citrate buffer (pH 6.0) or proteinase K (20 μg/ml for factor VIII antigen). Slides were treated with 3% hydrogen peroxide in absolute methanol to inactivate endogenous peroxidase activity, washed 3 times with PBS, incubated in blocking buffer with 5% bovine serum albumin for 20 min, and treated with the primary antibodies for 60 min at RT. Sections were washed and subsequently incubated with a horseradish peroxidase labeled polymer conjugated with anti-rabbit antibodies (EnVision™ +, Dako, Carpenteria, CA) for 30 min at RT. Bound antibodies were visualized with 3, 3'-diaminobenzidine tetrahydrochloride (0.05% with 0.015% H_2O_2 in PBS). Sections were counter-stained with Mayer's hematoxylin, dehydrated, cleared, and coverslipped for microscopic examination.

Statistical analysis. Differences between groups or among groups were tested, respectively, using a t-test or One-way analysis of variance (ANOVA) with repeated measures over time. The assumption of the ANOVA was examined, and a non-parametric measure based on ranks was used if needed. Values are reported as mean ± SE. When ANOVA indicated a significant effect (F-ratio, $p<0.05$), the Student-Keuls multirange test was used to compare the means of the individual groups. All statistics was conducted using the SigmaStat software version 3.5.

Results

PRIMA-1 converts mtp53 to the wtp53 conformation. We used conformation-specific antibodies to test the ability of PRIMA-1 to convert mtp53 to the wtp53 conformation in various breast cancer cell lines, as described previously (24,33). The mtp53-specific antibody PAb240 stained p53 in BT-474, T47-D, and HCC-1428 cells but not in MCF-7 cells, which express wtp53 protein (Fig. 1A). We then examined the ability of PRIMA-1 to convert mtp53 into the wt conformation in T47-D and HCC-1428 cells, using

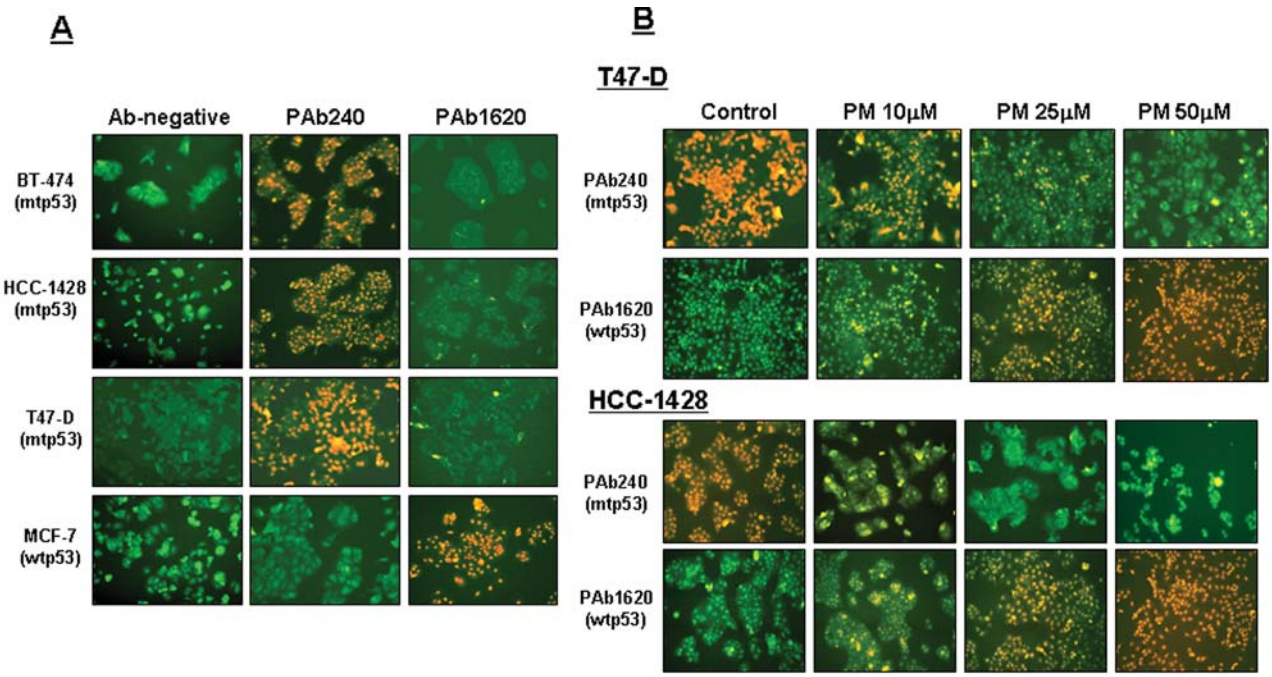

Figure 1. PRIMA-1 re-establishes the wt conformation of p53 in breast cancer cell lines expressing mtp53. (A) p53 status in BT-474, HCC-1428, T47-D, and MCF-7 cell lines as determined with the PAb 240 antibody, which recognizes only the mtp53 conformation and PAb 1620 which recognizes only wtp53 protein. Left panel represents controls where primary antibody was omitted, middle panel represents recognition of p53 with PAb240, and right panel represents recognition of p53 with PAb1620. (B) Cells were grown in 8-well chamber slides overnight and treated with PBS (control) or 10, 25, or 50 μM PRIMA-1 for 16 h. Treatment of breast cancer cells with PRIMA-1 converts the mtp53 protein (recognized by PAb240) to the wtp53 conformation, which is recognized by the conformation-specific antibody PAb1620. Results from T47-D and HCC-1428 cells are shown.

PAb1620 which specifically recognizes the restored wt protein. In a dose-dependent manner, PRIMA-1 reduced the conformation-specific staining of mtp53 by the PAb240 antibody in both T47-D and HCC-1428 cells, and increased staining with PAb1620 (Fig. 1B).

PRIMA-1 selectively reduces the cell viability of mtp53-expressing breast cancer cells but not in wtp53 containing normal mammary or endothelial cells. Treatment with PRIMA-1 reduced cell viability in a time- and dose-dependent manner in mtp53-expressing BT-474, HCC-1428, and T47-D cells, with half-maximal effects attained at concentrations between 10 and 25 μM (Fig. 2A). In contrast, PRIMA-1 did not affect the viability of breast cancer cell lines containing wtp53 protein (MCF-7, HCC-1500, and ZR-75), except for a minor effect on the viability of HCC-1500 and ZR-75 cells at the 50-μM concentration, indicating that high concentrations of PRIMA-1 may have a p53-independent effect on these cell types. Similarly, PRIMA-1 did not reduce the viability of normal breast cells (MCF-12A, MCF-12F, AG11132A) or endothelial cells (HUVECs) that express wtp53 (Fig. 2B), except for minor effects of 50 μM PRIMA-1 on AG11132A, 184A1, and MCF-12F cells.

PRIMA-1 selectively induces apoptosis and cell death in mtp53-expressing breast cancer cells. To determine whether PRIMA-1 causes apoptosis of breast cancer cells expressing mtp53, we performed a FACS analysis using the BT-474, HCC-1428, and T47-D (mtp53) cell lines and MCF-7 (wtp53) cell line. PRIMA-1 significantly induced apoptosis and cell death in the BT-474 (2.8- and 23.6-fold of control), HCC-1428 (1.6- and 4.5-fold of control), and T47-D (2.2- and 3.1-fold of control) cell lines, but no apoptosis was detected in MCF-7 cells (Fig. 2C and D).

PRIMA-1 selectively suppresses the growth of xenografts derived from mtp53-expressing breast cancer cells in nude mice. We next tested the ability of PRIMA-1 to inhibit the growth of tumor cells in xenografts in nude mice. We compared the growth of mtp53-expressing tumors grown from cells of the breast cancer lines BT-474, HCC-1428, and T47-D to the growth of wtp53-expressing tumors grown from MCF-7 cells. Nude mice were inoculated with 17-ß-estradiol pellets (1.7 mg/pellet) 24-48 h prior to inoculation of breast cancer cells, as described in Materials and methods. The protocol used for the *in vivo* PRIMA-1 study is shown in Fig 3; treatment was started when tumor volumes reached 100-150 mm^3 in size, which usually took 8-14 days, and treatment was continued for 20-22 days. PRIMA-1 treatment reduced tumor burden of xenografts obtained from mtp53-expressing cells, but not of those from wtp53-expressing MCF-7 cells (Fig 4). At the end of experiment, tumor volume and weight were inhibited by 82 and 85%, respectively, in the BT-474 tumor model, 79 and 55% in the HCC-1428 model, and 65 and 37% in the T47-D model.

PRIMA-1 induces apoptosis-related markers in xenografts obtained from mtp53-expressing breast cancer cells. To evaluate mechanism of PRMA-1 induced suppressive effects on tumor xenografts we performed immunohistochemical analyses of p53, p21, Ki67, VEGF, Factor VIII, and cleaved caspase-3 on tumor tissues treated or not treated with PRIMA-1.

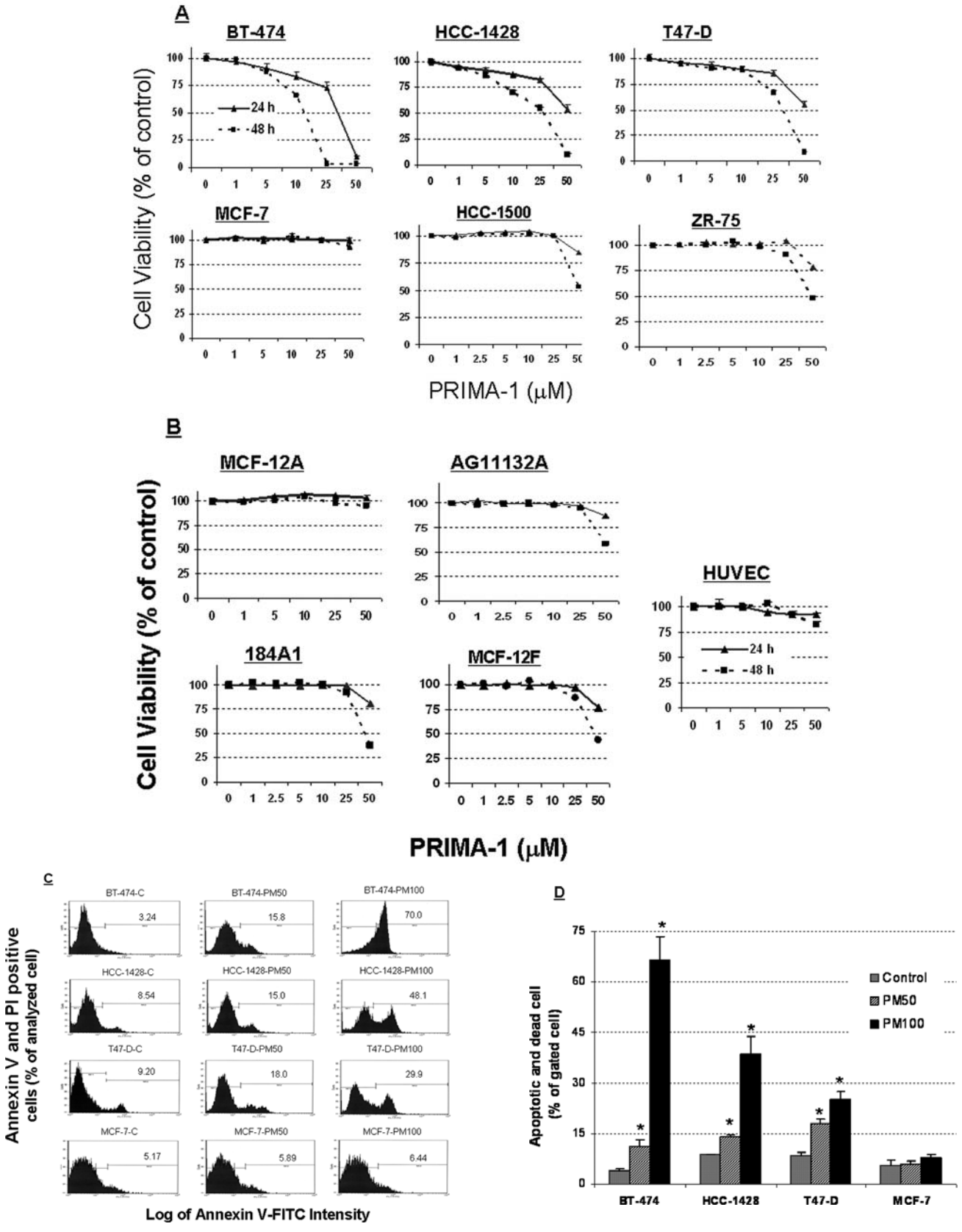

Figure 2. PRIMA-1 selectively inhibits proliferation of breast cancer cells expressing mtp53 protein. Cells were treated with various concentrations of PRIMA-1 for 24 (triangle) or 48 h (square) in MEM/F12 containing 5% FBS. Viable cells were identified with an SRB assay as described in Materials and methods. (A) BT-474, HCC-1428, and T47-D cells express mtp53, and MCF-7, HCC-1500, and ZR-75 cells contain wtp53. (B) PRIMA-1 does not inhibit growth of normal human mammary MCF-12A, MCF-12F, AG11132A, and 184A1 cells or HUVECs. (C) PRIMA-1 induces apoptosis and cell death in mtp53-expressing breast cancer cells. Representative images of the percentage of Annexin V-FITC-positive and PI-positive cells among BT-474, HCC-1428, and T47-D breast cancer cells, which express mtp53, and MCF-7 breast cancer cells, which express wtp53. Annexin V-FITC was used to detect the early stages of apoptosis and PI was used to detect DNA fragmentation or cell death. (D) Bar graph representing the proportion of Annexin V/PI-positive apoptotic cells described in (C). Experiments were performed in triplicate. *Significantly different compared with control group of each cell line. PM50, 50 μM PRIMA-1; PM-100, 100 μM PRIMA-1.

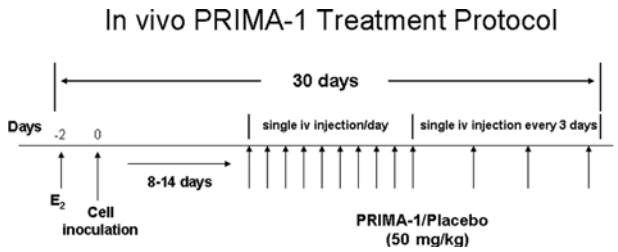

Figure 3. Protocol for treatment of xenografts with PRIMA-1.

To determine whether PRIMA-1 induces apoptotic markers at an early stage following treatment, we collected tumor samples from BT-474, HCC-1428, and MCF-7 xenografts 16 h after injection of 50 mg/kg PRIMA-1 or PBS into the tail vein. The tumor samples were immediately fixed and embedded in paraffin as described in Materials and methods. PRIMA-1 increased the expression of p53 and p53-dependent proteins such as p21 in both BT-474 and HCC-1428 tumor tissue, but not in MCF-7 xenografts (Fig. 5A). Ki67 remained

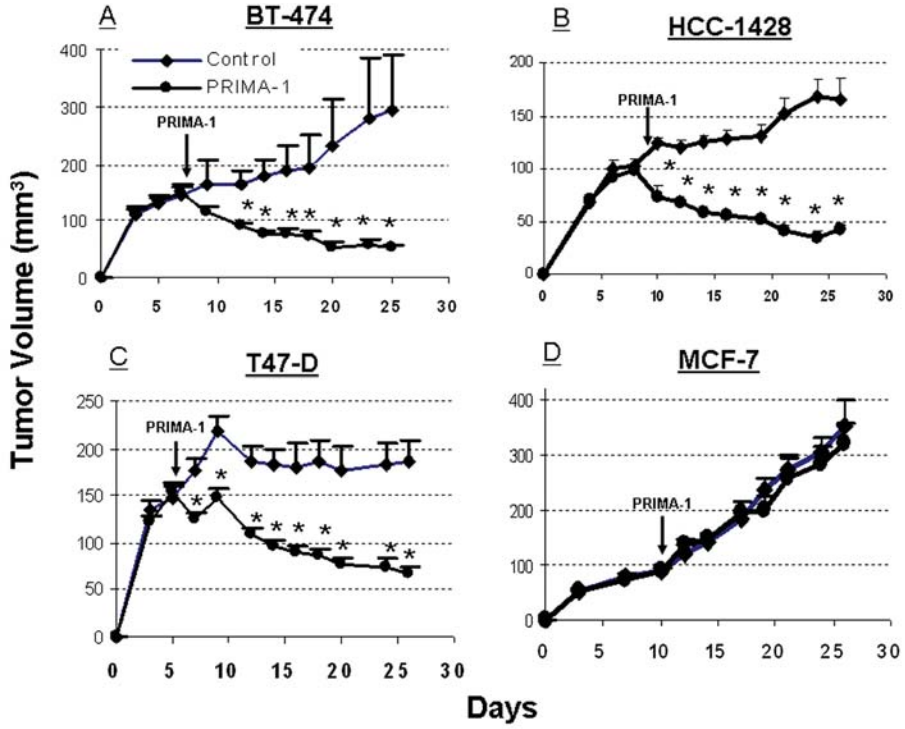

Figure 4. PRIMA-1 selectively suppresses xenografts derived from human breast cancer cells expressing mtp53 protein. Tumor cells were inoculated subcutaneously into both flanks of 5- to 6-week-old nude mice (nu/nu) as described in Materials and methods (n=5-9 animals/group). PRIMA-1 treatment was started when tumor volumes reached 100-150 mm^3 as described in Fig. 3. PRIMA-1 significantly inhibited tumor growth of xenografts obtained from mtp53-expressing breast cancer cells (A-C) but not of xenografts from wtp53-expressing cells (D). $^*P<0.05$ vs. control (t-test).

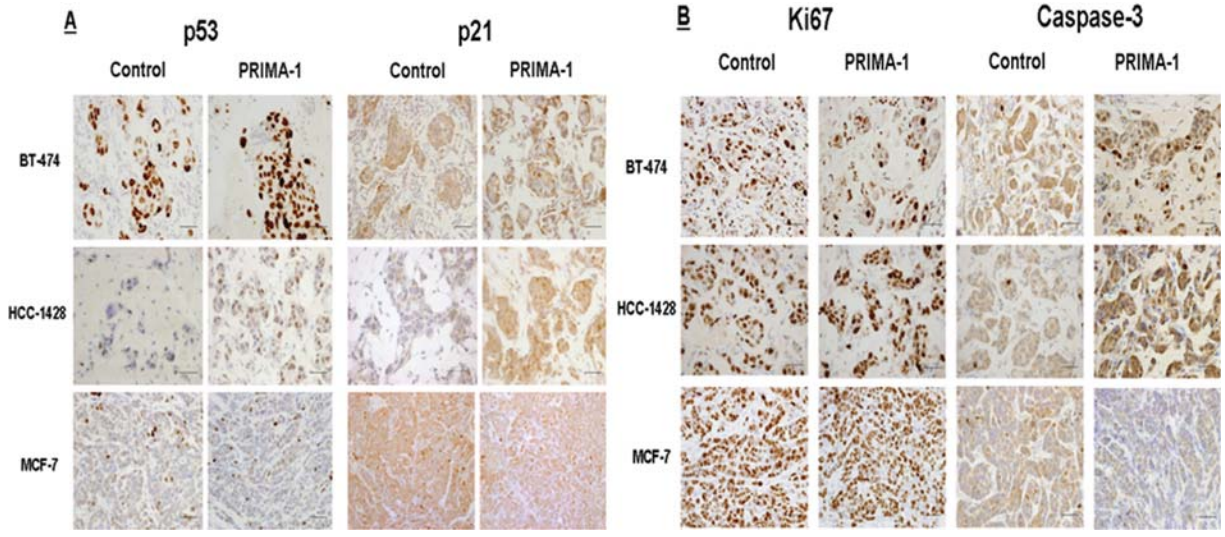

Figure 5. Representative pictures of immunohistochemical staining for p53, p21, Ki67, and caspase-3. Tumor-bearing animals were treated with PBS or PRIMA-1 (50 mg/kg) by i.v. injection 16 h before the end of experiment and then again at 2 h prior to tumor collection. (A) PRIMA-1 increased p53 and p21 expression in BT-474 and HCC-1428 xenografts, but not in MCF-7-derived tumor tissue. (B) PRIMA-1 did not influence tumor cell proliferation (as reflected by Ki67 expression) but did induce expression of the apoptosis marker caspase-3 in xenografts obtained from BT-474 and HCC-1428 cells, but not in MCF-7 tumor tissue.

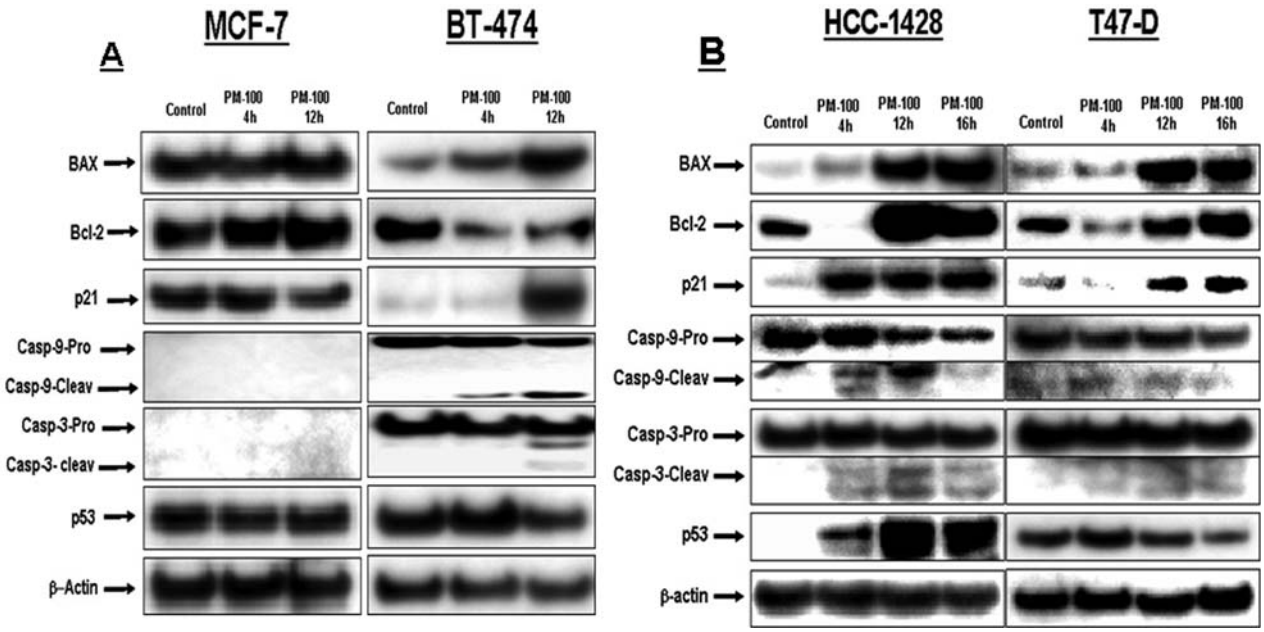

Figure 6. PRIMA-1 activates the mitochondria-dependent apoptotic pathway in mtp53-expressing breast cancer cells. Cells were treated with 100 μM PRIMA-1 for 4, 12, or 16 h, and whole-cell extracts were analyzed by Western blotting for the expression of apoptosis markers. Membranes were stripped and reblotted for ß-actin as a control for loading. PM-100, 100 μM PRIMA-1; Casp-9-Pro, procaspase-9; Casp-9-Cleav, cleaved caspase-9; Casp-3-Pro, procaspase-3; Casp-3-Cleav, cleaved caspase-3.

unchanged indicating that PRIMA-1 did not inhibit cell proliferation at this time, but PRIMA-1 did induce apoptosis, as judged by the upregulation of caspase-3 expression in BT-474 and HCC-1428 tumor tissues (Fig. 5B). These effects were not observed in MCF-7 tumor tissues (Fig. 5).

PRIMA-1 induces cell death by activating the mitochondria-dependent apoptotic pathway. Lastly, we sought to determine *in vitro* the signaling mechanism by which PRIMA-1 decreases cell viability *in vitro* and reduces tumor burden *in vivo* (Fig. 6). We treated cells for 4-12 h (BT-474 and MCF-7) or 4-16 h (HCC-1428 and T47-D) with 100 μM PRIMA-1 as described previously (28), then collected them and performed a Western blot analysis of whole-cell extracts. We found that in BT-474, HCC-1428, and T47-D cells, PRIMA-1 induced the pro-apoptotic proteins Bax and p21, increased cleavage of caspase -3 and -9, and inhibited expression of the survival protein Bcl-2 (Fig. 6). Such effects were not observed in MCF-7 cells (Fig. 6). The kinetics of these responses were cell-type specific. For example, although p21 was induced within 4 h in HCC-1428 cells, it was not upregulated until 12 h in BT-474 cells. Similarly, the survival of Bcl-2 was downregulated in BT-474 cells and remained low at 12 h, although in HCC-1428 and T47-D cells its protein levels were reduced only at 4 h and returned to control levels by 12 h after PRIMA-1 treatment. p53 levels were also induced to some extent in BT-474 and T47-D cells but not in MCF-7 cells. As previously noted (28) PRIMA-1 massively increased p53 expression in HCC-1428 cells. These results, combined with our FACS analysis data, show that PRIMA-1 induces apoptosis in BT-474, HCC-1428, and T47-D cells by activating a mitochondria-dependent signaling pathway. PRIMA-1 is, however, ineffective against MCF-7 cells, which express the wtp53 protein.

Discussion

Bykov *et al* (24) reported that the new investigational drug PRIMA-1 can restore the wtp53 conformation to various mtp53 proteins expressed in cancer cell lines originating from osteosarcomas, lung adenocarcinomas, and ovarian carcinomas. In this study, we have shown that PRIMA-1 also restores mtp53 to the wtp53 conformation in breast cancer cells harboring a variety of p53 mutations, and promotes tumor cell apoptosis via the mitochondria-dependent apoptotic pathway. Importantly, PRIMA-1 did not affect the survival of tumor cells that retain the wtp53 protein, such as MCF-7 cells, normal breast cancer cells, or human endothelial cells. Thus, PRIMA-1 appears to be an agent that can effectively target tumor cells which express mtp53 while sparing normal cells that express low levels of wtp53 protein. As previously observed (24,25), in this study PRIMA-1 was not toxic to nude mice treated *in vivo* and no loss of animal weight was observed over the duration of treatment (data not shown).

Although tumor cells that expressed wtp53 (MCF-7, HCC-1500, ZR-75) were not responsive to PRIMA-1 at lower concentrations, the viability of HCC-1500 and ZR-75 cells did decrease somewhat after exposure to high concentrations of PRIMA-1 for 48 h. This likely reflects a recently reported general toxic effect of PRIMA-1, which may become apparent after long-term *in vitro* exposure (38). Interestingly, MCF-7 cells appeared most resistant to PRIMA-1 and showed no signs of cytotoxicity in the concentration range tested for all cells. The response of some normal breast cells (184A1, MCF-12F) was similarly influenced by higher concentrations of PRIMA-1, although MCF-12A and AG11132A remained resistant to PRIMA-1 treatment. Importantly, HUVECs did not respond to PRIMA-1, indicating that the *in vivo* effects

of PRIMA-1 are not likely to result from a direct apoptotic effect on tumor endothelial cells; rather, the effects are most likely due to the induction of apoptosis in tumor epithelial cells expressing mtp53. However, further experiments will be needed to determine this conclusively.

To test whether PRIMA-1 induces tumor cell apoptosis, we used FACS to measure the expression of the apoptosis marker Annexin V and cell death with PI in PRIMA-1-treated tumor cells. Consistent with the viability results, PRIMA-1 induced apoptosis and cell-death in mtp53-expressing tumor cells in a dose-dependent manner, with BT-474 cells being the most sensitive to PRIMA-1 and T47-D cells the least sensitive. However, PRIMA-1 was ineffective against wtp53-expressing MCF-7 cells, which did not express Annexin V or stained for cell death with PI even at concentrations of PRIMA-1 as high as 100 μM.

To determine whether *in vivo* administration of PRIMA-1 induced tumor cell apoptosis prior to tumor regression, we injected it into the tail veins of nude mice bearing BT-474, HCC-1428, and MCF-7 xenografts at 16 h and again at 2 h before collecting tumors. PRIMA-1 treatment led to elevated levels of p21 and caspase-3 in BT-474 and HCC-1428 cells, but not in MCF-7 cells, and did not seem to influence expression of the proliferative marker Ki67 at early treatment times. It therefore appears that the primary mode of action of PRIMA-1 in mice harboring xenografts from mtp53-expressing cells is to induce tumor cell apoptosis, thereby leading to a reduced tumor burden. Since the breast cancer cells used in this experiment grow in response to estrogen, we also examined whether there was an effect on the expression of estrogen or progesterone receptors, since this might also account for lack of tumor progression. Because such changes can only be observed after a relatively prolonged period, we examined tissue isolated from tumors after 3 days of PRIMA-1 treatment, when the tumors were regressing. No loss of estrogen receptor α, estrogen receptor ß, or progesterone receptor was detected in the PRIMA-1-treated groups compared with the controls (data not shown). Thus it appears that the effects of PRIMA-1 are independent of any effect on estrogen or progesterone receptor expression. Although we cannot rule out effects on steroid receptor activity altogether, our earlier studies suggest that PRIMA-1 does not block the transcriptional activity of steroid receptors (28). In addition, PRIMA-1 treatment did not influence basal VEGF levels in tumor samples following treatment for either 16 h, 3 days or 10 days. This indicates that suppression of VEGF was not the major cause of tumor loss in the current model tested (not shown) suggesting that angiogenesis is not the target for PRIMA-1 mediated effects in the current model system. This is in contrast to the reduction of progestin-induced VEGF levels and number of blood vessels by PRIMA-1 in progestin-dependent mammary tumor model that we recently published (39).

p53-induced apoptosis may occur either through the death receptor pathway or the mitochondrial pathway (3,4,40). In the mitochondrial apoptotic pathway, p53 induces several genes, including Bax, APAF-1, Puma, and Noxa. Bax expression can lead to an increased mitochondrial membrane potential, which allows the release of cytochrome c into the cytoplasm (9). Cytochrome c release promotes apoptosis by activating caspase-9 and -3, resulting in initiation of the caspase cascade. Caspase-9 then activates effector caspases, which trigger events that lead to apoptosis (10,11). To further understand the molecular mechanism for PRIMA-1-induced apoptosis, we examined the effect of PRIMA-1 on the expression levels of several proteins involved in the mitochondria-dependent apoptotic pathway (Fig. 6). PRIMA-1 treatment increased the levels of Bax and p21 in mtp53-expressing cells but not in wtp53-expressing MCF-7 cells. Bcl-2 protein was initially downregulated in all mtp53-expressing cells, but the levels rebounded in HCC-1428 and T47-D cells, suggesting that these cells may undergo some form of survival response e.g., production of excess growth or survival factors that prevents apoptosis. All mtp53-expressing cells, but not MCF-7 cells, also showed caspase-3 and -9 cleavage products, indicating that apoptosis had occurred. The expression of procaspase-3 and -9 is under the control of p53, and MCF-7 cells also lacked bands for these caspase precursors, as previously reported by Janicke *et al* (41). Thus PRIMA-1 induces apoptosis of mtp53-containing breast cancer cells via the mitochondria-dependent intrinsic pathway by regulating proteins that are normally under the control of the wtp53 protein. We should point out, however, that these observations do not rule out any direct effects of re-activated p53 on mitochondrial functions independent of transcriptional effects, as reported recently (42), or long-term effects on extrinsic events, such as regulation of the TRAIL pathway and involvement of these pathways remain to be tested.

In conclusion, we have shown that PRIMA-1 converts mtp53 into a protein with wtp53 functions in breast cancer cells and re-initiates the intrinsic apoptotic pathway. We therefore suggest that because most breast tumor cells with mtp53 fail to respond to first-line chemotherapeutic treatment (43), PRIMA-1 should be investigated as a potential alternative treatment strategy, either alone for tumors expressing high levels of p53, or in combination with anti-hormones, anti-angiogenesis based antibodies, chemotherapeutic compounds, or vascular disrupting agents. We believe that such novel therapies may lead to a higher rate of success in the treatment of breast cancer. Our recent studies have demonstrated that PRIMA-1 as well as VEGF-specific antibodies are effective non-toxic suppressors of progestin-induced progression of mtp53 expressing breast cancer cells (39). Thus clinical studies involving PRIMA-1 and other chemotherapeutic alternatives are warranted in treatment of breast cancer.

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