Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 352 (2007) 203–212
BBRC
www.elsevier.com/locate/ybbrc

PRIMA-1 induces apoptosis by inhibiting JNK signaling but promoting the activation of Bax

Tao Wang [a], Kyunghee Lee [a], Abdur Rehman [a], Sayed S. Daoud [a,b,c,*]

[a] *Department of Pharmaceutical Sciences, Washington State University, Pullman, WA, USA*
[b] *Cancer Prevention and Research Center, Washington State University, Pullman, WA, USA*
[c] *Center for Integrated Biotechnology, Washington State University, Pullman, WA, USA*

Received 1 November 2006
Available online 9 November 2006

Abstract

The p53 protein plays a major role in the maintenance of genome stability in mammalian cells. Mutations of p53 occur in over 40% of breast cancers and are indicative of tumor resistance to chemotherapeutic agents. Recently, there has been a high degree of interest in pharmacological approaches for restoring the normal function to mutant p53. The low molecular weight compound p53 reactivation and induction of massive apoptosis (PRIMA-1) was shown to induce cytotoxic effects and apoptosis in human tumor cells with mutant p53. Here, we studied the molecular mechanisms of PRIMA-1-induced apoptosis in human breast cancer cells with p53 mutations such as MDA-231 and GI-101A as compared to MCF-7 cells. We show that PRIMA-1 selectively induces apoptosis in human breast cancer cells MDA-231 and GI-101A compared to the MCF-7. This effect was paralleled by an increase in total p53 level in the nucleus and the induction of its phosphorylation at Ser-15 site. Using the chromatin immunoprecipitation (ChIP) assays, we show that PRIMA-1 restored p53 DNA binding activity to the promoters of the proapoptotic genes such as Bax and PUMA, but inhibited the binding activity to the promoters of the MAP4K4 gene. Knockdown of p53 protein in breast cancer cells using siRNA followed by PRIMA-1 treatment resulted in decline of Bax and PUMA proteins expression. Cell incubation with either PRIMA-1 or SP600125 (c-Jun NH_2-terminal kinase inhibitor) resulted in the abrogation of adriamycin-induced c-Jun NH_2-terminal kinase (JNK) activation, whereas Bax activation was not inhibited. We conclude that both Bax and PUMA but not JNK signaling are involved in PRIMA-1-induced apoptosis in breast cancer cells with p53 mutation.

Keywords: PRIMA-1; p53; Apoptosis; Breast cancer; Chromatin immunoprecipitation; ChIP; Bax; PUMA; c-Jun N-terminal kinase

The p53 protein plays a major role in the maintenance of genome stability in mammalian cells. It is a central transcription factor activated in response to a variety of cellular stresses, including DNA damage, mitotic spindle damage, heat shock, metabolic changes, hypoxia, viral infection, and oncogene activation [1]. p53 can induce growth arrest and apoptosis, events that prevent the survival of damaged cells. It can also promote early senescence in response to unregulated mitogenic signaling [2,3]. The transactivation function of p53 is mediated through sequence-specific binding of its central domain to *cis*-acting elements within the promoters or introns of responsive genes. It is also known that p53 is the most commonly mutated gene in human cancers and more than 40% of breast tumors are defective in p53 [4,5]. The frequent mutation of p53 in human tumors and the presumed dominant gain-of-function effect of p53 mutations make mutant p53 a prime target for pharmacological intervention in cancer therapeutics. Therefore, pharmacological reactivation of mutant p53

Abbreviations: PRIMA-1, p53 reactivation and induction of massive apoptosis; p53BS, p53 binding site; ADR, adriamycin; ChIP, chromatin immunoprecipitation; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PUMA, p53 upregulated modulator of apoptosis; MDM-2, murine double minute-2.

* Corresponding author. Fax: +1 509 335 5902.
E-mail address: daoud@wsu.edu (S.S. Daoud).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.006

should efficiently eliminate tumor cells through the induction of apoptosis with minor unwanted side effects on normal tissues. Hence, the reactivation of mutant p53 in tumors has emerged as an attractive strategy for novel tumor therapies. Various strategies have been designed to restore normal function to mutant p53 [6]. The low molecular weight compound PRIMA-1 (p53 reactivation and induction of massive apoptosis) (2,2-bis(hydroxymethyl)-1-azabicyclo(2,2,2)octan-3-one) was identified in cellular screen of chemical library as a compound that restores tumor suppressor function to mutant p53 proteins with subsequent noticeable *in vitro* and *in vivo* anti-tumor activity [7]. However, the precise mechanism(s) of apoptosis elicited by PRIMA-1 remained under investigation by various laboratories. For example, recent study by Schuler and Wiman groups [8,9] showed the involvement of the proapoptotic Bax and PUMA in PRIMA-1-mediated apoptosis. In contrast, Li et al. [10] reported the involvement of the c-Jun N-terminal kinase (JNK) pathway and not the Bcl-2 proteins. Hence the aim of this study was to further elucidate the apoptotic signal transduction pathway that is activated upon restoration of the transcriptional transactivation function with PRIMA-1 in breast cancer cells. Direct evidence is provided for the requirement of Bax and PUMA activation as direct targets for PRIMA-1-induced apoptosis. In contrast, PRIMA-1 inhibited the binding of p53 to the promoters of the MAP4K4 gene and abrogated adriamycin-mediated activation of JNK signaling. Taken together, these results suggest a critical role of Bcl-2 proapoptotic proteins and p53 in PRIMA-1-induced apoptosis of human breast cancer cells. Parts of the current study were published previously as an abstract [11].

Materials and methods

Chemicals, kits, and antibodies. PRIMA-1 (NSC-281668) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD); Annexin V-PI kit was purchased from BD Biosciences (San Diego, CA). Focus™-Cytoplasmic & Nuclear Protein Extraction and Focus™ Mitochondria kits were from Geno Technology, Inc. (St. Louis, MO). ChIP-IT™ kit was from Active Motif (Carlsbad, CA). CB-XTM Protein Assay kit was from Biosciences (St. Louis, MO). Primary antibodies against p53 (DO-1), phos-p53(ser20), c-Jun(H-79), and p-c-Jun(Ser 63/73) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); phosp53(ser15) and phos-p53(ser46) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-PUMA(CT)bbc3 antibody was from ProSci Inc. (Poway, CA), anti-Bax(Ab-3) antibody was from Calbiochem (EMD Biosciences Inc., San Diego, CA). The goat anti-mouse, anti-rat, and anti-rabbit secondary antibodies labeled with IRDye™ 38 were purchased from LI-COR, Inc. Biosciences (Lincoln, NE). PageRuler™ Prestained Protein Ladder was the product of Fermentas Life Sciences (Hanover, MD). Adriamycin was from SIGMA (St. Louis, MO). SP600125 (JNK inhibitor II) was from Calbiochem (EMD Biosciences Inc., San Diego, CA). siGENOME SMARTpool tumor protein p53 (Li-Fraumeni syndrome) siRNA, si*CONTROL* Non-Targeting siRNA #2, and DharmaFECT 2 Transfection Reagent were purchased from Dharmacon Research Inc. (Lafayette, CO). All other chemicals were of reagent grade. All ChIP primers used in this study were synthesized by Invitrogen (Carlsbad, CA) and the sequences are shown in Table 1.

Cell lines and flow cytometry. The human breast MDA-231 and GI-101A carcinoma cells were used in this study. MDA-231 expresses endogenous A278P, R280K, and M385T p53 mutations and GI-101A has Y236C, A278P, and *R72P (polymorphism) mutant p53. In addition, we used the human breast MCF-7 carcinoma cells with wild-type p53. These cells were routinely maintained in monolayer cultures in RPMI-1640 medium (Invitrogen, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cells were grown in a humidified atmosphere of air containing 5% CO_2 at 37 °C. Exponentially growing cultures at 80% confluence were used in all experiments, as previously reported [12]. For flow cytometry, cells were treated with 100 μM PRIMA-1 for 24 h. After drug treatment, the cultures were rinsed with PBS and re-incubated with fresh medium for 24, 48, and 72 h, then trypsinized, centrifuged for 10 min at 600–700 rpm, and resuspended in binding buffer. A mixture of 0.1 ml of cell suspensions, 5 μl of Annexin V-FITC, and 10 μl of PI was incubated for 15 min at room temperature in the dark. A flow-cytometric analysis proceeded within 1 h after the addition of 400 μl of binding buffer. These experiments were carried out using the FACSort™ flow cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA) and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Each experiment was repeated three times.

Immunoblotting. Cells were incubated with 0 or 100 μM PRIMA-1 for 0, 2, 4, 8, 12 or 24 h, washed twice with PBS (pH 7.4), and harvested. Harvested cells were either used to prepare the whole cell lysates or for subcellular fractionation studies. Nuclear, mitochondria, and cytosolic fractions were obtained by the following procedures. Adherent cells (2×10^7) were harvested and washed with PBS. Nuclear and mitochondrial fractions were isolated with the use of a FOCUS™-Cytoplasmic & Nuclear Protein Extraction kit and a FOCUS™ Mitochondria Kit (Geno Technology Inc., St. Louis, MO). The nuclear and mitochondrial pellets were lysed in SDS sample buffer, and the samples were concentrated with a Microcon filtration device (Millipore, Bedford, MA) before separation by SDS–PAGE and immunoblotting. For immunoblotting, 50 μg protein samples were separated by SDS–PAGE (4–20% polyacrylamide gradient

Table 1
Oligonucleotides used for p53BS ChIP analysis

Target	Forward	Reverse
MAP4K4 p53BS I	5′-TCGCCAAATTAAATGGTGATTG	5′-AAGCTCTGGCTAATTCACTTGGA
MAP4K4 p53BS II	5′-TCACCATGTTGGCTAGGCTG	5′-TGCAGATCACTTGAGGCCAG
MAP4K4 p53BS III	5′-TTCATTAGGAGGAGATTGTGTCAGC	5′-TGAACATGCCTCTCAGTGGC
MAP4K4 p53BS IV	5′-GGAATTCCCTTGTATAATAGCCAGC	5′-TGCAGTCTAGCATCACATGGTAAAT
MAP4K4 p53BS V	5′-CGGGCATGATGGCACAT	5′-TGCCTCAGCCTCCCTAGTAGC
NOXA p53BS	5′-CTCGAGACCTGCTCCACTTC	5′-CGCTGGAATCCTCTCTGTTC
BAX p53BS1	5′-GGAGTCTCTCTCGGTTGCAC	5′-CCAAGCTACTCGGGAGACTG
BAX p53BSII	5′-GGGCAGATTGTGTGGAGTTT	5′-CTTGAGGCCAGGAGTTTGAG
PUMA p53BSI	5′-ACAGATCCACACCCCCAGC	5′-ACTTTGTGGACCCTGGAACG
PUMA p53BSII	5′-AGCCGAGATAGTGCCATTGC	5′-CGGAGTTTGCTCTTGTTGCC
β-Globin	5′-GGCAAGGTGAACGTGGATGAAGTTGGTG	5′-GGAGTGGACAGATCCCCAAAGGACTCAAAG

gel) and transferred on nitrocellulose (Millipore, Bedford, MA). The membrane was developed according to a protocol provided by LI-COR, Inc. (Lincoln, NE) as previously reported [12]. Blots were blocked in 50% blocking buffer diluted in TBS-T (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h and incubated with primary antibodies. The dilutions of all primary antibodies were used according to the manufacturer's instructions. Antibody binding was detected using a secondary antibody: the goat anti-mouse, anti-rat, and anti-rabbit secondary antibodies conjugated with IRDye™ 38. The reactive bands were revealed and detected with the Odyssey™ Infrared Imaging System (LI-COR, Inc.). Protein bands were quantified with the provided image analysis software program. Statistical analysis was based on three independent experiments.

Chromatin immunoprecipitation (ChIP) assay. *In vivo* chromatin immunoprecipitation (ChIP) assay was performed according to the instruction manual of ChIP-IT kit (Active Motif, Carlsbad, CA). Briefly, formaldehyde was added to cells treated with or without 100 μM PRIMA-1 for 10 min. The cells were pelleted and resuspended in 200 ml of SDS lysis buffer (1% SDS/10 mM EDTA/50 mM Tris–HCl, pH 8.1) and incubated on ice. Lysate samples were sonicated and debris was removed by centrifugation. The chromatin solution was obtained and salmon sperm DNA/Protein A agarose slurry was added and incubated for 2 h at 4 °C. Beads were pelleted by centrifugation, and supernatants were incubated with 2 mg of p53 (DO-1) antibody overnight at 4 °C. The resultant DNA complexes were collected using a salmon sperm DNA/Protein A agarose slurry. After reverse crosslinking of histone–DNA with glycine followed by RNase A treatment, DNA was purified using mini-columns provided with kit. The purified DNA was amplified by promoter-specific primers and PCR products were subjected to agarose gel electrophoresis in the presence of SYBR Safe™ DNA gel stain (Invitrogen, Eugene, OR). The primer sequences from the promoter region of each gene were shown in Table 1. The primer pairs of MAP4K4-p53BSI-V were used as described by Miled et al. [13]. The PCR bands were quantified using Bio-Rad Quantity one software (Bio-Rad, Hercules, CA).

Small interfering RNA system and transfection. Small interfering RNAs were synthesized by Dharmacon Research Inc. (Lafayette, CO). The p53siRNA consisted of a mixture of four siRNA duplexes targeting four different regions of the p53 mRNA (siGENOME SMARTpool tumor protein p53 (M-003329-01)). A pool of four non-targeting siRNA duplexes was used as a negative control (siRNA CONTROL non-targeting siRNA pool, D-001210-02). Transfection of cells with siRNA duplexes was performed using DharmaFECT2 siRNA transfection reagent (Dharmacon, Inc.). To determine the optimum conditions for p53 downregulation, cells were transfected with 0, 25, 50, and 100 nM of control siRNA and p53siRNA for 0, 24, 48 or 72 h as recommended by Dharmacon. The downregulation of p53 protein was determined by Western blot. Subsequently, cells were transfected with 100 nM of control siRNA or p53siRNA for 48 h and following a change of media, treated for a further 24 h with 100 μM PRIMA-1. To assess whether the effect of PRIMA-1 on Bax and PUMA expression is p53 dependent, cells were harvested and lysed for Western blotting using primary antibodies for Bax and PUMA as previously reported [12]. Triplicate samples were collected for each experiment.

JNK signaling inhibition assay. Cells were pre-treated with 10 μM adriamycin (ADR) for 2 h, washed twice with PBS, and exposed to either 50 μM SP600125 or 100 μM PRIMA-1 for 24 h. Cell lysates were prepared as described previously [3]. Western blot was performed as described above using anti-c-Jun, anti-p-c-Jun, and anti-Bax primary antibodies. The reactive bands were revealed and detected with the Odyssey™ Infrared Imaging System (LI-COR, Inc.) as previously reported [12].

Statistical analysis. Data were represented as means ± SEM using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).[1] In all cases, *n* refers to the number of independent experiments. Statistical analyses were preformed by the Student *t*-test and analysis of variance (ANOVA) where $p < 0.05$ was considered significant.

[1] http://www.graphpad.com.

Results and discussion

Mutant p53-dependent apoptotic activity by PRIMA-1

We recently reported [12] that the restoration of the transcriptional transactivation function of p53 target genes in breast cancer cells by PRIMA-1 is dependent on p53 mutation; and that the α-isoform of the heat shock protein 90 (Hsp90α) is associated with mutant p53 reactivation. We further showed that both p53 and Hsp90α are translocated to the nucleus of tumor cells for the transcriptional transactivation of p53 target genes. Thus, PRIMA-1 unlike other low molecular weight p53 rescue compounds, promotes the refolding of mutant form of p53 into an active confirmation *via* protein–protein interaction with Hsp90α. To investigate the apoptotic effect of PRIMA-1 on breast cancer cells, we used the Annexin-V assay that is commonly used for detecting cells undergoing apoptosis [14]. Cells were treated with or without 100 μM PRIMA-1 for 24 h. After drug treatment, cultures were rinsed with PBS and re-incubated in fresh medium for another 72 h. Flow-cytometric profiles of Annexin-V and propidium iodide-stained MDA-231, GI-101A, and MCF-7 cells are shown in Fig. 1A. Breast cancer cells with p53 mutations (MDA-231 and GI-101A) showed 30–38% increase in the number of apoptotic cells after treatment with PRIMA-1 as compared to controls. In contrast, MCF-7 cells treated with PRIMA-1 showed relatively minor cell death (8%). These data indicate that PRIMA-1 induces apoptosis only in breast cancer cells with mutated p53, similar to other reports although with different cell types [9]. Next, we checked the time course for the induction of apoptosis in these cells. Data shown in Fig. 1B indicated that the GI-101A cells had a delayed apoptotic response at 72 h, whereas MDA-231 cells responded earlier at 48 h. This could reflect differences in p53 mutation sites in both cells. The p53 mutation sites in MDA-231 cells are A278P, R280K, and M385T whereas GI-101A has Y236C, A278P, and *R72P (polymorphism) mutant p53. These differences in p53 mutation sites may impact on the equilibrium for DNA binding restoration of p53 and hence the activation of p53 target genes that involved in apoptotic cell death.

Effect of PRIMA-1 on p53 phosphorylation

p53 phosphorylation is commonly used as a surrogate marker for p53 activation including that of the induction of apoptosis. For this purpose, Western blot analysis was used to detect the phosphorylation status of p53 following exposure of breast cancer cells to PRIMA-1. Cells were treated with 100 μM PRIMA-1 for 2, 4, 8, 12, and 24 h, and the expression of p53 protein (total and phosphorylated) in the subcellular fractions of cells was examined. As shown in Fig. 2A–C, treatment of cells with PRIMA-1 resulted in significant increase in the levels of p53 in the nuclear fraction (NF) of p53 mutant cell lines, but not in

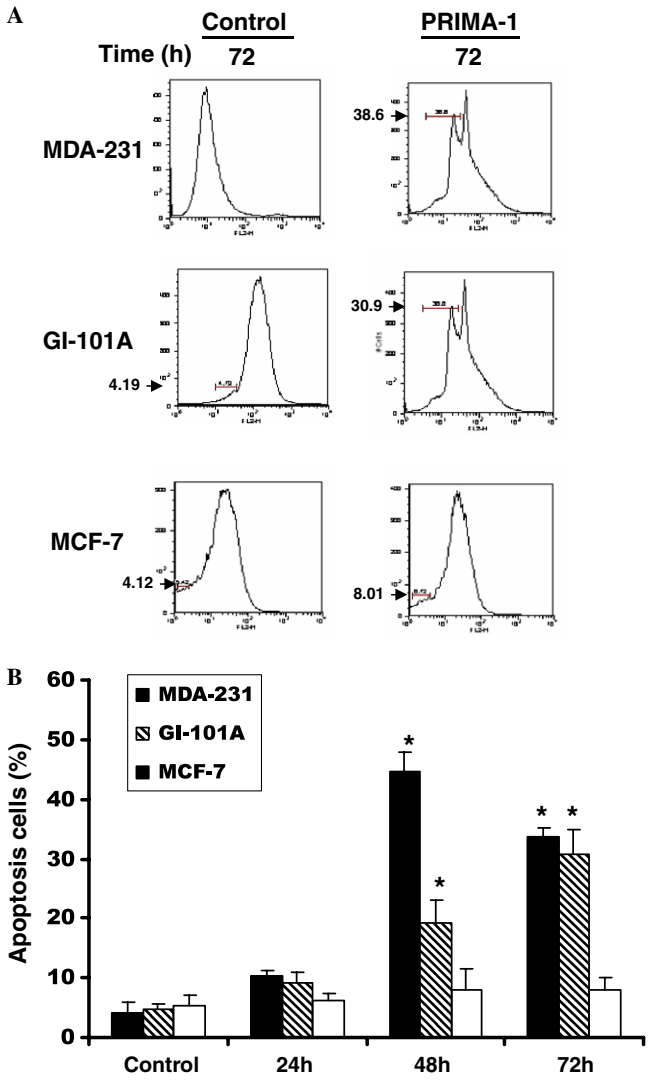

Fig. 1. The apoptotic activities of PRIMA-1 in breast cancer cells as assessed by Annexin V and determined by the FACS analysis. (A) Breast cancer cell lines were treated with or without 100 μM PRIMA-1 for 24 h. Cells were washed and harvested at 72 h following treatments, stained with Annexin V-FITC/PI, and analyzed with flow cytometry. (B) The percentage of apoptotic cells following drug treatment with or without 100 μM PRIMA-1 for 24 h, then harvested at 24, 48, and 72 h following treatments and analyzed as indicated in (A). Data are presented as means ± SEM of three independent experiments. The level of significance is indicated: $*p < 0.05$.

the wild-type p53 tumor cells. In MDA-231 cells, total p53 protein increased to a peak at 12 h compared to control; while in GI-101A cells, the expression of p53 was delayed till 24 h, which is similar to the time required for the apoptotic response shown by both cell lines (Fig. 1B). In contrast, the expression of total p53 in MCF-7 cells showed decreasing trend in both whole cell lysates (WCLs) and the nuclear fractions (NFs). In addition to the increase in total p53 protein levels, serine 15 (Ser-15) phosphorylation was also induced by PRIMA-1 in both MDA-231 and GI-101A. Serine 15 phosphorylation of p53 was significantly higher in the nuclear fractions of cells treated with PRIMA-1 for 12 h. Other phosphorylation sites such as Ser20 and Ser46 were not significantly changed upon PRIMA-1 treatment (data not shown). As shown in Fig. 2C, there is no obvious phosphorylation of p53 on serine 15 in MCF-7 cells treated with PRIMA-1. It is well known that there are at least eight phosphorylation sites within the N terminal subdomain of p53: serines 6, 9, 15, 20, 33, 37, and 46 and threonine 18 [15]. The biological functions of several of these phosphorylations are not clear. However, it appears that the Ser-15 phosphorylation plays a crucial role in the transactivation process of p53 [16,17]. Unlike other compounds known to rescue p53 function without altering the phosphorylation status of p53 like CP-31398 [18], phosphorylation of p53 at serine 15 by PRIMA-1 seemed to be involved in the reactivation of p53 transcriptional function. It is also possible that the phosphorylation of Ser-15 leads to an enhancement of the acetylation of C-terminal lysines and has some effect on p53-mediated apoptosis [19]. Furthermore, stabilization of p53 also occurs through phosphorylation of Ser-15 and binding to Hsp90 [20]. Our recent study showed that the reactivation of p53 transcriptional function involved the binding of p53 to Hsp90 [12]. Thus multiple effects of a single phosphorylation site (Ser-15) suggest that this phosphorylation may be causing a conformational change in the transactivation domain of p53 protein following treatment with PRIMA-1 that allows for the proper induction of p53 target genes.

Differences in p53 binding to the promoter regions of MAP4K4, Bax, and PUMA genes

Downstream target genes of p53 are thought to mediate its tumor suppressive activity as well as initiate apoptotic cell death. Therefore, questions should be raised on whether differential transactivation of these genes is regulated at the level of p53 binding to their promoters. For examples, previous reports indicated that PRIMA-1-induced apoptosis in tumor cells occurred by the activation of Bax, a proapoptotic Bcl-2 family member [8,9], and also through the activation of c-Jun-NH2-kinase (JNK) signaling pathway [10]. However, whether the induction of these genes is regulated at the level of p53 binding to their promoters was not addressed or investigated in these studies. To address this issue, *in vivo* p53 binding to consensus sites in the Bax, PUMA, and MAP4K4 promoters was investigated in cells exposed to PRIMA-1. p53-DNA complexes were crosslinked *in vivo* by treating breast cancer cells with formaldehyde and processed by chromatin immunoprecipitation-PCR. Since p53 has more than one binding site on the promoters of the above-mentioned genes [21], multiple primers were used in the PCR amplifications as shown in Table 1. PRIMA-1 treatment of both MDA-231 (Fig. 3A) and GI-101A (Fig. 3B) led to the induction of p53 binding to its binding sites on both Bax-I and PUMA-I promoters. Since the affinity of p53 binding to its binding site on the Bax promoter is known to be weaker than its binding to p21 and PUMA promoters [13], the

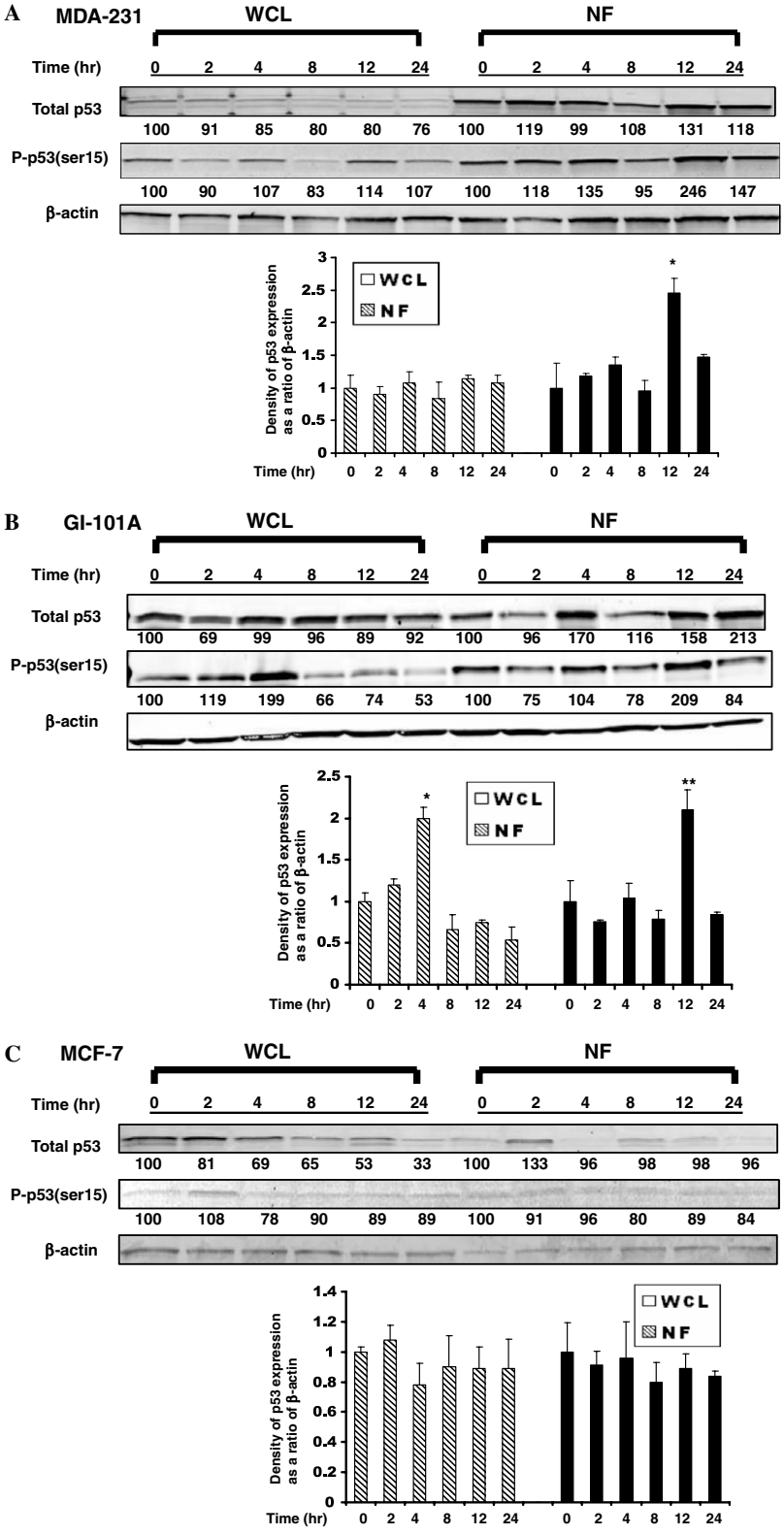

Fig. 2. Effect of PRIMA-1 on p53 phosphorylation in breast cancer cells. (A) MDA-231, (B) GI-101A, and (C) MCF-7 cells. Cells were treated 100 μM PRIMA-1 for 2, 4, 8, 12, and 24 h, and were subjected to fractionation with the FOCUS™ kit (GenoTechnology Inc., St. Louis, MO). Whole cell lysate (WCL) and nuclear fraction (NF) were prepared and protein concentration was determined using the Bio-Rad determination kit. Aliquots (50 μg) of lysates were subjected to immunoblotting with anti-p53 and anti-p53Ser15 primary antibodies. β-Actin was used as loading controls. Data are presented as means ± SEM of three independent experiments. The level of significance is indicated: $*p < 0.05$.

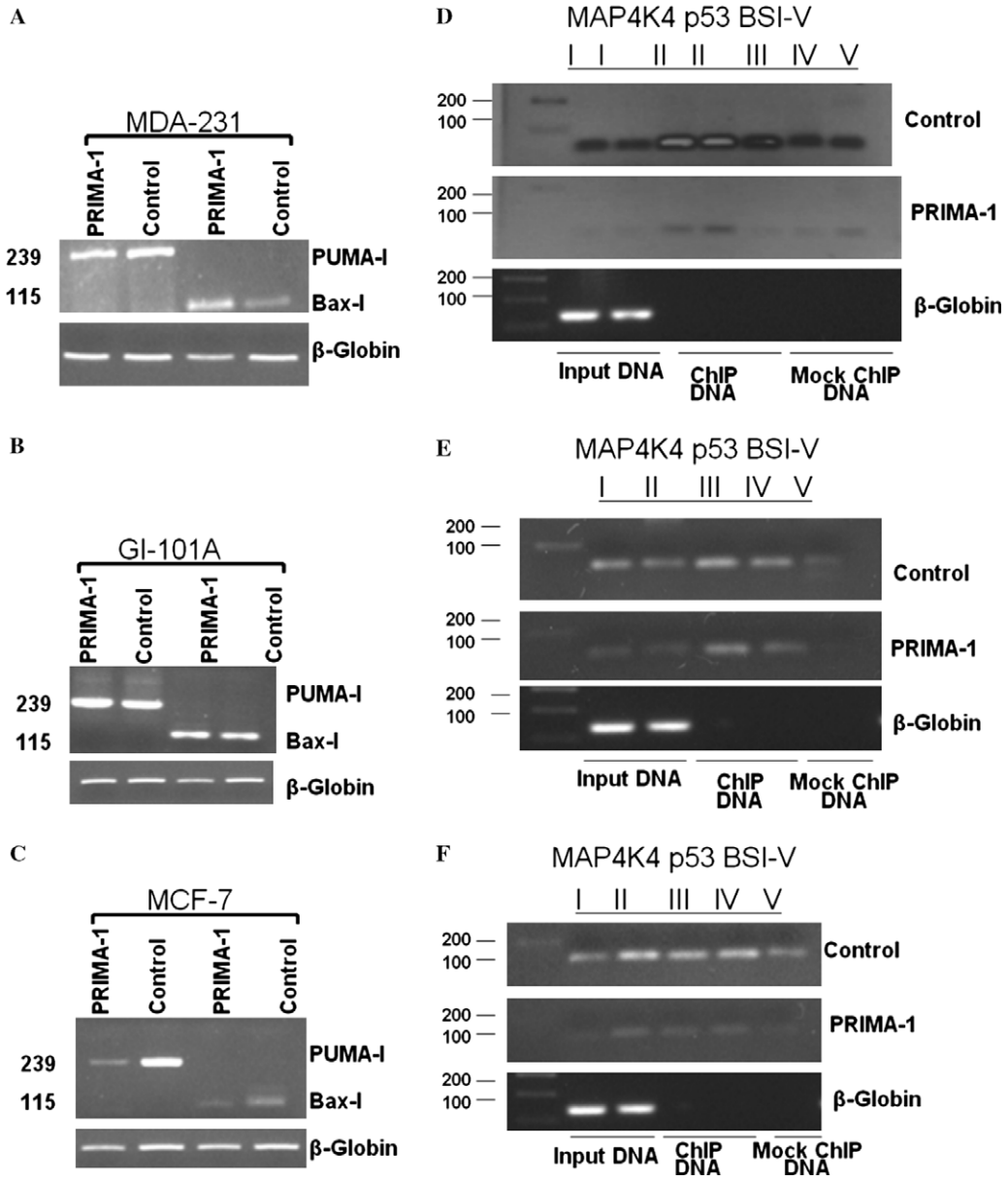

Fig. 3. *In vivo* p53 binding to the promoters of Bax, PUMA, and MAP4K4 binding sites. ChIP analysis was performed on MDA-231, GI-101A, and MCF-7 cells following treatment with 100 μM PRIMA-1 for 24 h. Crosslinked chromatin was immunoprecipitated with DO-1 antibody against total p53 and samples were analyzed by PCR using primers (Table 1) specific for Bax, PUMA, and MAP4K4 (I, II, III, IV, and V) p53 binding sites. β-Globin was used as control. (A–C) ChIP from MDA-231, GI-101, and MCF-7 cells, respectively, for PUMA-I and Bax-I p53 binding sites. (D–F) ChIP from MDA-231, GI-101, and MCF-7 cells, respectively, for MAP4K4 p53 binding sites. Data are representative of two to three independent experiments.

increase in p53 binding on PUMA-1 binding site seen in Fig. 3A and B is not as obvious as in case of Bax-I binding compared to controls. In contrast, PRIMA-1 did not promote DNA binding of p53 to both Bax-I and PUMA-I promoters in MCF-7 cells (Fig. 3C), in fact the binding of p53 to Bax-I and PUMA-I promoters is depressed in these cells after treatment with PRIMA-1 compared to control. The data indicate that PRIMA-1 treatment restored the transcriptional binding affinity of p53 to the proapoptotic target genes only in cells with mutated p53. Similar results were obtained with the transcriptional binding activation of p53 on both Bax-II and PUMA-II promoters after treatment of both MDA-231 and GI-101A cells with PRIMA-1 and the lack of their activation in MCF-7 cells (data not shown).

The MAP4K4 gene encodes an upstream activator of JNK signaling pathway [22]. JNK signaling has been implicated in the cellular response to stress and apoptosis [23,24]. However, evidence has also been accumulating to suggest the involvement of JNK in cell survival or anti-apoptosis. The anti-apoptotic function of JNK is related to the status of p53. For example, specific antisense oligonucleotides of JNK (JNKS) were shown to inhibit the growth of certain p53-deficient tumor cells but not p53-positive

tumor cells [25]. This effect was attributed to the fact that the activation of the JNK pathway inhibits p53-induced cell cycle arrest and therefore promotes p53-induced apoptosis [26]. Thus, JNK may only exert its anti-apoptotic function in p53-deficient tumor cells [27]. To test whether PRIMA-1-induced apoptosis in breast cancer cells is also the result of MAP4K4 gene activation as well, we performed the ChIP assays on the five p53 binding sites (MAP4K4p53BSI-V). Fig. 3D–F show that PRIMA-1 inhibited the binding of p53 to its binding sites on MAP4K4 gene in all breast cell lines regardless of the status of its p53 function. The data clearly indicate that the JNK signaling pathway may not be involved in PRIMA-1-induced apoptosis in breast cancer cells as attested by the lack of p53 binding to its promoter on MAP4K4 gene. Although the previous report by Li et al. [10] showed the involvement of JNK signaling in PRIMA-1-induced apoptosis in colorectal carcinoma cells, the authors did not perform *in vivo* studies of p53 binding to the promoter sites on MAP4K4 gene in these cell lines. In addition to our *in vivo* p53 binding studies to the promoter sites of Bax, PUMA, and MAP4K4, we also investigated the binding of p53 to the promoter sites of other p53 proapoptotic genes such as Noxa, which encodes a BH3-only protein and hence is likely to contribute to p53-mediated apoptosis in a similar manner to PUMA and Bax. Our ChIP analysis failed to support the involvement of Noxa in PRIMA-1-induced apoptosis in breast cancer cells (data not shown). Thus, it

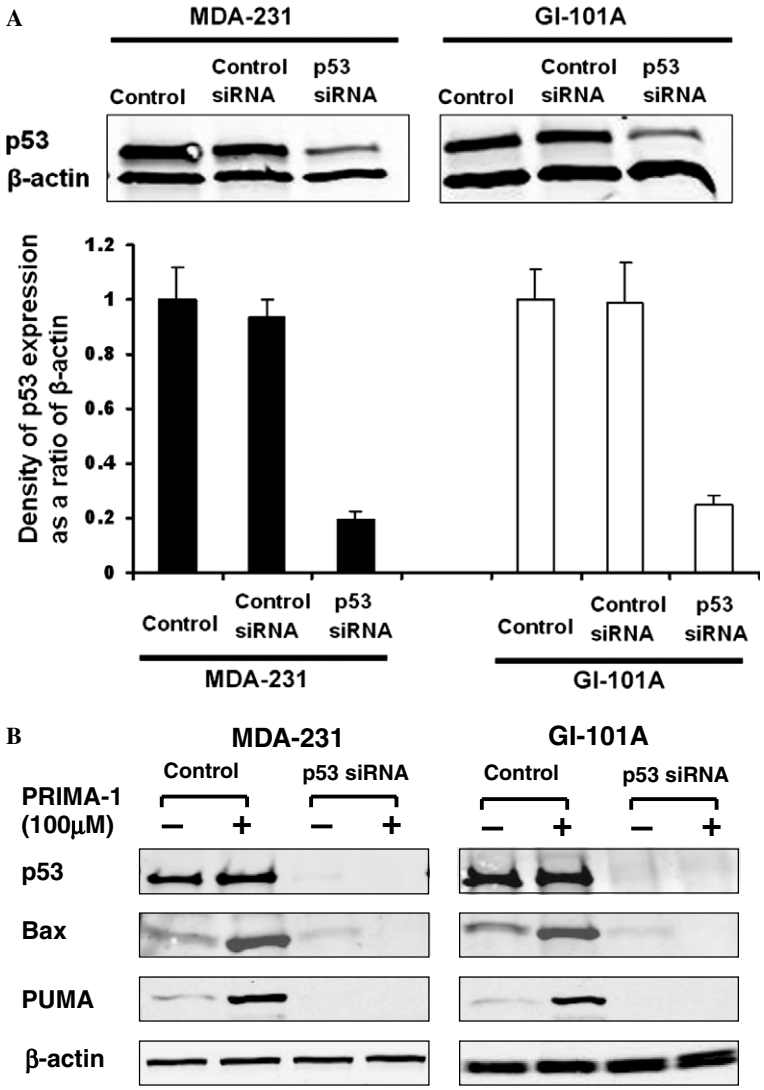

Fig. 4. p53-dependent induction of Bax and PUMA by the treatment of PRIMA-1. (A) MDA-231 and GI-101A cells were treated with siRNA directed against p53 (p53siRNA; 100 nM) for 48 h. Scrambled siRNA duplexes were utilized as a control (control siRNA). Expression of p53 following treatment with control siRNA or p53siRNA was compared to levels in the untreated cell lines by Western blot analysis utilizing β-actin as a loading control. The percentage of knockdown was quantified by densitometry and normalized according to β-actin levels. (B) MDA-231 and GI-101A cells transfected with control siRNA or p53siRNA were treated with 100 μM PRIMA-1 for a further 24 h. The expression of p53, Bax, and PUMA was determined by Western blot utilizing β-actin as a loading control. Data in (A) are presented as means ± SEM of three independent experiments. Western blots are representative of three independent experiments.

appears that, in response to PRIMA-1 restoration of p53 transcriptional transactivation function, p53 activates the "intrinsic" mitochondrial apoptotic pathway by inducing the expression of at least two Bcl-2 proapoptotic family members—PUMA and Bax shifting the balance towards proapoptotic effect. Our recent expression proteomics study has supported these findings and showed the activation of mitochondrial intrinsic pathway upon PRIMA-1 treatment of breast cancer cells [28]. Based on these data, we concluded that the involvement of JNK signaling in PRIMA-1-induced apoptosis in breast cancer cells seems unlikely.

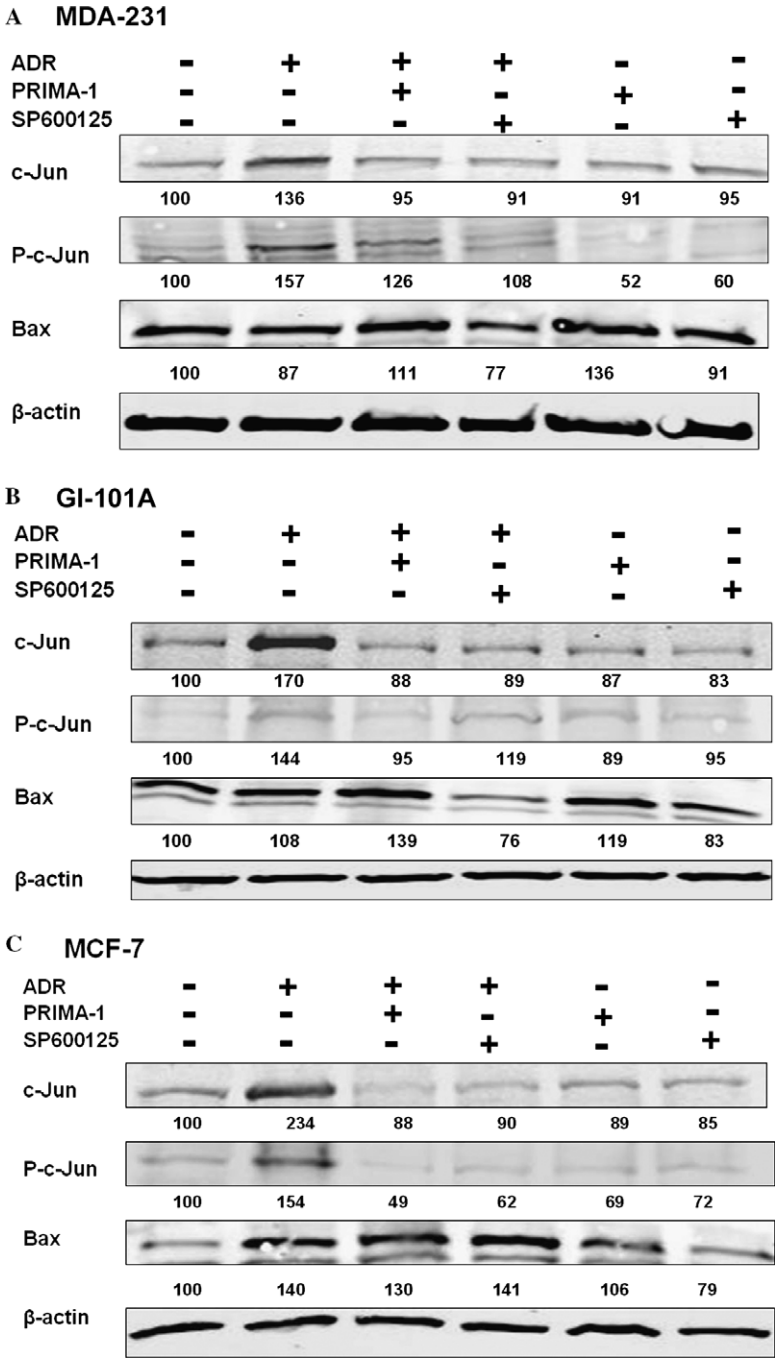

Fig. 5. Downregulation of JNK signaling pathway by PRIMA-1. MDA-231 (A), GI-101A (B), and MCF-7 (C) cells were pre-treated with 10 μM ADR for 2 h, washed with PBS then treated with either 50 μM SP600125 or 100 μM PRIMA-1 for 24 h. Fifty micrograms of protein samples of cell lysates from treated and control samples was separated by SDS–PAGE (4–20% polyacrylamide) and Western blotted with antibodies directed against c-Jun, p-c-Jun, and Bax. β-Actin was used as a loading control. The reactive bands were detected with the Odyssey™ Infrared Imaging System. The ratio of the integrated absorbance of c-Jun, p-c-Jun, and Bax bands to that of the actin band was used as an index of protein expression in each cell line. Western blots are representative of three independent experiments.

p53-dependent induction of Bax and PUMA by the treatment of PRIMA-1

To confirm our ChIP data that *in vivo* p53 binding to its promoter on Bax and PUMA resulted in the activation of Bax and PUMA proteins, we utilized siRNA specific to p53 to downregulate the expression of p53 gene. Incubation of MDA-231 and GI-101A cells with p53siRNA (100 nM) for 48 h resulted in the reduction of p53 expression by approximately 80% compared to cells treated with siRNA control (Fig. 4A). Induction of PRIMA-1-mediated expression of Bax and PUMA in MDA-231 and GI-101A breast cancer cells was completely inhibited by p53siRNA when compared to control siRNA-treated cells (Fig. 4B). These data clearly indicate that PRIMA-1-mediated activation of Bax and PUMA is p53 dependent and thus confirmed our ChIP data (Fig. 3A and B). Collectively these data also indicate that the activation of proapoptotic targets, Bax and PUMA, plays a major role in the induction of PRIMA-1-induced apoptosis [8,9].

Downregulation of JNK signaling pathway by PRIMA-1

c-Jun NH_2-terminal kinase (JNK), a member of the mitogen-activated kinase (MAPK) family, is a key regulator of apoptosis [23,24]. Modulation of its activity can either promote or inhibit apoptotic process, depending on cell system and the modulator. The kinase acts on a variety of targets, including, in addition to c-Jun, other transcription factors such as p53 and c-Myc and proapoptotic and anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-xl, thereby influencing levels and activities of molecules that participate in cell death [27]. To further confirm that PRIMA-1 inhibited the binding of p53 to its binding sites on the promoters of MAP4K4 (Fig. 3), we investigated JNK pathway activation induced by adriamycin (ADR), a DNA-damaging agent known to activate JNK signaling [29], in the presence or absence of PRIMA-1 or SP600125, a specific JNK inhibitor. Cells were pre-treated with 10 μM ADR for 2 h then exposed to either 50 μM SP600125 or 100 μM PRIMA-1 for 24 h. Western blot analysis was performed with c-Jun, phosphorylation c-Jun (p-c-Jun), and Bax antibodies. A representative result of this study is illustrated in Fig. 5. Treatment of MDA-231 and GI-101A cells with ADR resulted in increased expression of both c-Jun and p-c-Jun protein levels that was decreased in the presence of either PRIMA-1 or SP600125 (Fig. 5A and B). In contrast to this inhibitory effect on JNK activation, PRIMA-1 increased ADR-induced Bax expression in these cells. These data indicate that PRIMA-1 promoted the activation of Bax but abrogated the activation of JNK in breast cancer cells with p53 mutation. ADR-induced Bax expression was inhibited in the presence of SP600125, which was previously attributed to increased p53 and MDM2 interaction due to the inhibitory effect of SP600125 on p53 phosphorylation [30]. Similarly, both PRIMA-1 and SP600125 inhibited ADR-induced JNK activation in MCF-7 cells (Fig. 5C), again to confirm our previous ChIP data (Fig. 3) that PRIMA-1 inhibited the binding of p53 to its binding sites on the promoters of MAP4K4 gene.

In conclusion, the data presented here demonstrated that PRIMA-1 induces apoptosis in breast cancer cells with mutated p53. Importantly, PRIMA-1 promoted the binding of p53 to its binding sites on the promoters of both Bax and PUMA. Although others have suggested the involvement of Bax in PRIMA-1-induced apoptosis in different model systems, this is the first direct evidence that Bax and PUMA are required for p53-dependent PRIMA-1-induced apoptosis. The current data are also suggestive that JNK activation is not involved in PRIMA-1-induced apoptosis in breast cancer cells.

Acknowledgments

We thank Dr. Yves Pommier for helpful discussion and critical review of the manuscript. This work was supported in part by Grant No. BCTR0402398 from the Susan G. Komen Foundation for Breast Cancer Research to S.S.D.

References

[1] B. Vogelstein, D. Lane, A.J. Levine, Surfing the p53 network, Nature (Lond.) 408 (2000) 30710.
[2] M.S. Colman, C.A. Afshari, J.C. Barrett, Regulation of p53 stability and activity in response to genotoxic stress, Mutat. Res. 462 (2000) 179–188.
[3] S.S. Daoud, P.J. Munson, W. Reinhold, et al., Impact of p53 knockout and topotecan treatment on gene expression profiles in human colon carcinoma cells: a pharmacogenomic study, Cancer Res. 63 (2003) 2782–2793.
[4] H. Lai, L. Lin, M. Nadji, S. Lai, E. Trapido, L. Meng, Mutations in the p53 tumor suppressor gene and early onset breast cancer, Cancer Biol. Ther. 1 (2000) 231–236.
[5] S.B. Bull, H. Ozcelik, D. Pinnaduwage, et al., The combination of p53 mutation and neu/erbB2 amplification is associated with poor survival in node-negative breast cancer, J. Clin. Oncol. 22 (2004) 86–96.
[6] G. Selivanova, T. Kawasaki, L. Ryabchenko, K.G. Wiman, Reactivation of mutant p53: a new strategy for cancer therapy, Semin. Cancer Biol. 8 (1998) 369–378.
[7] V.N.J. Bykov, N. Issaeva, A.M. Shilov, et al., Restoration of the tumor suppressor function to mutant p53 by a low molecular weight compound, Nat. Med. 8 (2002) 282–288.
[8] J.E. Chipuk, U. Maurer, D.R. Green, M. Schuler, Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription, Cancer Cell 4 (2003) 371–381.
[9] V.J.N. Bykov, N. Zache, H. Stridh, et al., PRIMA-1^{MET} synergizes with cisplatin to induce tumor cell apoptosis, Oncogene 24 (2005) 3484–3491.
[10] Y. Li, Y. Mao, P.W. Brandt-Rauf, A.C. Williams, R.L. Fine, Selective induction of apoptosis in mutant p53 premalignant and malignant cancer cells by PRIMA-1 through the c-Jun-NH2-kinase pathway, Mol. Cancer Ther. 4 (2005) 901–909.
[11] T. Wang, K. Lee, A. Rehman, Y. Pommier, S.S. Daoud, PRIMA-1-induces apoptosis in p53 mutant breast cancer cell lines by inhibiting the JNK cell survival pathway and promoting the activation of proapoptotic p53 targets Bax and PUMA, Proc. AACR 47 (2006) 2214.
[12] A. Rehman, M.S. Chahal, X. Tang, J.E. Bruce, Y. Pommier, S.S. Daoud, Proteomic identification of heat shock protein 90 as a

candidate target for p53 mutation reactivation by PRIMA-1 in breast cancer cells, Breast Cancer Res. 7 (2005) R765–R774.
[13] C. Miled, M. Pontoglio, S. Garbay, M. Yaniv, J.B. Weitzman, A genomic map of p53 binding sites identifies novel p53 targets involved in an apoptotic network, Cancer Res. 65 (2005) 5096–5104.
[14] A.A. Redkar, P. Mixter, S.S. Daoud, Implication of p53 in growth arrest and apoptosis induced by combined treatment with topotecan and UCN-01 in human mammary epithelial cells, J. Exp. Ther. Oncol. 4 (2004) 213–222.
[15] E. Appella, C.W. Anderson, Signaling of p53: breaking the posttranslational modification code, Pathol. Biol. (Paris) 48 (2000) 227–245.
[16] Z.A. Stewart, L.J. Tang, J.A. Pietenpol, Increased p53 phosphorylation after microtubule disruption is mediated in a microtubule inhibitor- and cell specific manner, Oncogene 20 (2001) 113–124.
[17] L.J. Bean, G.R. Stark, Phosphorylation of serines 15 and 37 is necessary for efficient accumulation of p53 following irradiation with UV, Oncogene 20 (2001) 1076–1084.
[18] W. Wang, R. Takimoto, F. Rastinejad, W.S. El-Deiry, Stabilization of p53 by CP-31398 inhibits ubiquitination without altering phosphorylation at serine 15 or 20 or MDM2 binding, Mol. Cell. Biol. 23 (2003) 2171–2181.
[19] T. Unger, R.V. Sionov, E. Moallem, C.L. Yee, P.M. Howley, M. Oren, Y. Haupt, Mutations in serines 15 and 20 of human p53 impair its apoptotic activity, Oncogene 18 (1999) 3205–3212.
[20] C. Wang, J. Chen, Phosphorylation and hsp90 binding mediate heat shock stabilization of p53, J. Biol. Chem. 278 (2003) 2066–2071.
[21] M.D. Kaeser, R.D. Iggo, Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo, Proc. Natl. Acad. Sci. USA 99 (2002) 95–100.
[22] Z. Yao, G. Zhou, X.S. Wang, et al., HGK that specifically activates the c-Jun-N-terminal kinase signaling pathway, J. Biol. Chem. 274 (1999) 2118–2125.
[23] C.R. Weston, R.J. Davis, The JNK signal transduction pathway, Curr. Opin. Genet. Dev. 12 (2002) 14–21.
[24] A. Lin, Activation of the JNK signaling pathway: breaking the brake on apoptosis, BioEssays 25 (2002) 17–24.
[25] O. Potapova, M. Gorospe, R.H. Dougherty, N.M. Dean, W.A. Gaarde, N.J. Holbrook, Inhibition of c-Jun N-terminal kinase-2 expression suppresses growth and induces apoptosis of human tumor cells in a p53-dependent manner, Mol. Cell. Biol. 20 (2000) 1713–1722.
[26] E. Shaulian, M. Karin, AP-1 as a regulator of cell life and death, Nat. Cell Biol. 4 (2002) E131–E136.
[27] J. Liu, A. Lin, Role of JNK activation in apoptosis: a double-edged sword, Cell Res. 15 (2005) 36–42.
[28] K. Lee, T. Wang, A. Paszczynski, S.S. Daoud, Expression proteomics to p53 mutation reactivation with PRIMA-1 in breast cancer cells, Biochem. Biophys. Res. Commun. 349 (2006) 1117–1124.
[29] F. Li, L. Meng, H. Xing, et al., Essential role of c-Jun-NH_2-terminal kinase on synergy induction of apoptosis by TRAIL plus ADM in ADM resistant MCF-7/ADM cells, Apoptosis 11 (2006) 1239–1246.
[30] Y.L. Hsu, C.Y. Cho, P.L. Kuo, Y.T. Huang, C.C. Lin, Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 in vitro and in vivo, J. Pharmacol. Exp. Ther. 318 (2006) 484–494.