Journal of Cellular Biochemistry 104:2363–2373 (2008)

PRIMA-1 Synergizes With Adriamycin to Induce Cell Death in Non-Small Cell Lung Cancer cells

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Abstract p53-dependent apoptosis is important for the efficacy of cancer treatment, and tumors carrying mutant p53 are often resistant to chemotherapy. Non-small cell lung cancer (NSCLC) cells generally exhibit resistance to apoptosis following treatment with many cytotoxic drugs. The new molecule PRIMA-1 appears to kill human tumor cells by restoring the transcriptional activity to mutated p53. We investigated the induction of apoptosis in response to this drug in three NSCLC cell lines carrying different p53 proteins: A549 (p53wt), LX1 (p53R273H), and SKMes1 (p53R280K). PRIMA-1 alone did not trigger apoptosis but significantly reduced cell viability. However, in combination with adriamycin, PRIMA-1 strengthen the adriamycin-induced apoptosis in A549 and LX1. Interestingly, even in SKMes1 cells, the combined treatment triggered a strong PARP cleavage without DNA fragmentation. Our data suggest that in NSCLC cells, PRIMA-1 may induce cell death through pathways other than apoptosis but may synergize with adriamycin to trigger an apoptotic response. J. Cell. Biochem. 104: 2363–2373, 2008.

Key words: PRIMA-1; p53; apoptosis; cytotoxicity; non-small cell lung cancer

Lung cancer is the leading cause of cancer mortality in the Western world, and the incidence continues to rise [Jemal et al., 2005]. Based on the biology, therapy and prognosis, lung cancer is broadly divided into two classes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Squamous cell carcinoma, adenocarcinoma and large cell carcinoma are classified as NSCLC and account for 75–80% of all lung cancer cases. Current therapies for NSCLC are often unsuccessful, with an overall 5-year survival of about 15% [Spira and Ettinger, 2004]. Such a resistance to therapy can be attributed to disfunction in the apoptotic program, since resistance to conventional radio- and chemotherapy-induced apoptosis in NSCLC cells has been widely reported [Volm and Rittgen, 2000; Joseph et al., 2001; Kaufmann and Vaux, 2003; Fesik, 2005].

Apoptosis is a crucial cellular function for the maintaining of normal tissue homeostasis and the elimination of severely damaged or mutated cells. It is defined by a pattern of molecular changes that generally result in the activation of caspases, which orchestrate the removal of the dying cell [Coultas and Strasser, 2000; Crighton and Ryan, 2004; Norbury and Zhivotovsky, 2004; Fennell, 2005]. The induction of apoptosis is central to the tumor-suppressive activity of the p53 gene. Following activation by different types of stress, p53 activates many genes involved in cell cycle arrest and apoptosis, mainly through its transcription-dependent activity [Vogelstein et al., 2000; Horn and Vousden, 2007]. Furthermore, a

R. Magrini and D. Russo contributed equally to this work.

Grant sponsor: Associazione Italiana per la Ricerca sul Cancro (AIRC); Grant sponsor: Ministero della Salute; Grant sponsor: Fondo per gli Investimenti della Ricerca di Base (FIRB); Grant sponsor: Alleanza Contro il Cancro (ACC).

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Received 25 January 2008; Accepted 26 March 2008
DOI 10.1002/jcb.21794

transcription-independent p53 function in apoptosis regulation has been recently highlighted [Chipuk et al., 2005; Chipuk and Green, 2005, 2006]. p53 mutations, frequently occurring in human cancer (about 50% in NSCLC), may severely compromise p53-dependent apoptosis and, indeed, several studies in human tumor cells have shown that inactivation of p53 impaired the apoptotic response to chemotherapy [Levine, 1997; Soussi and Lozano, 2005; Hall and McCluggage, 2006].

In the last years, the use of small molecules that rescue the tumor suppressor functions of p53 is emerging as a new strategy to treat cancer. Several molecules have been tested for their ability to restore the wild-type p53 conformation and functions [North et al., 2002; Takimoto et al., 2002; Bykov et al., 2002b, 2005a; Issaeva et al., 2003; Peng et al., 2003; Selivanova and Wiman, 2007]. A rationale behind this approach is that the restoration of the p53 pathway in tumor cells expressing abundant mutated protein could trigger a massive apoptotic response. PRIMA-1 (for *p*53-*r*eactivation and *i*nduction of *m*assive *a*poptosis) has been identified as a low molecular weight compound that selectively inhibits the growth of tumor cells expressing mutated p53 [Bykov et al., 2002b]. This molecule is able to restore the sequence-specific DNA-binding and transcriptional transactivation of mutant p53 in vitro and to suppress tumor-cells growth in mice by inducing apoptosis [Bykov et al., 2002b]. Interestingly, PRIMA-1 inhibited the growth of cell lines derived from various human tumor types in a mutant p53-dependent manner [Bykov et al., 2002a]. It has also been shown that PRIMA-1^{Met}, a PRIMA-1 derivative, can function synergistically with cisplatin, or other commonly used anticancer drugs, to induce tumor cell apoptosis and inhibition of human tumor xenograft growth in vivo in SCID mice [Bykov et al., 2005b].

The apoptotic potential of PRIMA-1, demonstrated in different cell types, prompted us to investigate its ability to induce apoptosis in three NSCLC cell lines carrying endogenous wild-type (A549: WT) or mutated (LX1: R273H; SKMes1: R280K) p53s. In a previous study, we showed that in LX1 and SKMes1 the transactivation activity of p53 towards different target genes (including the pro-apoptotic *BAX*, *PUMA*, and *AIP1*) was abrogated [Magrini et al., 2007]. Here we examined the induction of apoptosis by PRIMA-1, alone or in combination with adriamycin, in A549, LX1, and SKMes1 cells lines in relation to the functional status of their endogenous p53.

MATERIALS AND METHODS

Cell Culture and Reagents

A549 (human epithelial-like lung adenocarcinoma; p53wt) and SKMes1 (human epithelial-like lung squamous carcinoma; p53R280K) cell lines were grown in D-MEM (GIBCO Invitrogen, Milano, Italy) containing 10% and 5% fetal bovine serum (Sigma–Aldrich, Milano, Italy), respectively. LX1 (human epithelial-like lung squamous carcinoma; p53R273H) cells were cultured in RPMI 1640 (GIBCO Invitrogen) supplemented with 10% fetal bovine serum. For all cell lines, the medium contained 100 UI/ml of penicillin and streptomycin (MP Biomedicals, Irvine). Cells were maintained at 37°C in 5% CO_2 at 100% humidity. Adriamycin was dissolved at 3.5 mM in H_2O. PRIMA-1 (Calbiochem) was dissolved in H_2O at 10 mM.

Western Blots

To prepare total extracts, adherent cells were collected and washed twice with cold PBS. Lysis was performed in buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP40, 10% glycerol, 10 mM EDTA, 1 mM DDT, and protease inhibitors (0.5 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A). Cell lysates were incubated 20 min at 4°C in rocking and centrifugated at 14,000 rpm at 4°C for 5 min. Supernatants were collected and protein concentration was determined using the Bradford assay (Bio-Rad, Milano, Italy). Usually 20–30 μg of total proteins were resolved on 7.5–12% SDS–PAGE and transferred to PVDF Hybond-P membrane (Amersham, Milano, Italy). Membranes were blocked with 5% non-fat dry milk in 0.1% Tween-20 in PBS for 1 h, then incubated 1 h (or overnight) at 4°C with the appropriate primary antibody. The following antibodies were employed: anti-p53 (CM1, Novocastra, Newcastle upon Tyne, UK); anti β-actin (AC-74, Sigma–Aldrich); anti-p21 (C-19); anti-mdm2 (smp14) (both from Santa-Cruz Biotechnology, Milano, Italy) and anti-PUMA (Cell Signaling Technology, Inc.). Subsequently, membranes were incubated with peroxidase-conjugate anti-mouse

or anti-rabbit secondary antibodies (Sigma–Aldrich). Detection was carried out with Supersignal West Pico chemiluminescent substrate (Pierce, Rockford) or ECL-Plus Western Blotting Detection System (Amersham, GE Healthcare, UK). Expression of β-actin was used as loading control.

Detection of Apoptosis

Apoptosis was investigated by Western blot, through PARP fragmentation, flow cytometry and by DNA laddering. Fragmentation of PARP was analyzed using anti-PARP mouse monoclonal antibody (C-2, BD-Pharmingen, Milano, Italy). The apoptotic sub-G_1 fractions were determined by flow cytometry. Briefly, cells were harvested by trypsinization at different times after treatment and pooled with the floating cells, washed twice with PBS and fixed in 70% ethanol. Cells were washed with PBS and resuspended in a solution containing propidium iodide (50 μg/ml) in 0.1% triton X-100 in PBS with 200 μg/ml RNaseA. Stained nuclei were analyzed using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA), equipped with a doublet discrimination module. ModFit LT2 software (Veruty Software House, Topsham, ME) was used to assess the cell cycle status. Apoptotic nuclei appeared as a broad hypodiploid DNA peak before the G1 phase of the cell cycle. For DNA laddering analysis, floating and adherent cells were collected after treatment, washed with 1× PBS and lysed in lysis buffer (5mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% SDS, 100 μg/ml Proteinase K) overnight at 37°C. RNA was removed by 3 h incubation with 1 mg/ml RNase at 37°C. Genomic DNA was isolated by phenol/chloroform extraction, precipitated with absolute ethanol and dissolved in water. DNA was run on 2% agarose gel for 3 h at 80 V and stained with 0.5 μg/ml ethidium bromide.

MTT Assay

Cells were plated in 96-well plates at a density of 5,000 cells per well in 100 μl of medium, cultured 24 h and treated according to the indicated schedule. After treatment, 10 μl of 5 mg/ml Thiazol Blue Tetrazolium Blue (MTT, Sigma–Aldrich), dissolved in PBS, was added to each well and plates were returned to the incubator for 4 h. Subsequently, 100 μl of 10% SDS; 0.01 M HCl was added, and plates were further incubated overnight to solubilize the purple MTT-formazan crystals. Absorbance was measured at 570 nm with a reference at 650 nm.

RESULTS

Three NSCLC Cell Lines Show different Ability to Undergo Apoptosis Following UV-C or Adriamycin

We have shown that in NSCLC cells LX1 and SKMes1 expressing p53R273H and p53R280K, respectively, the transactivation activity of p53 towards the apoptotic targets *BAX* and *PUMA* was abrogated [Magrini et al., 2007]. Here, we investigated whether, in these cell lines, apoptosis could be induced by two different stimuli, such as UV-C irradiation and adriamycin treatment, regardless the lack of transactivation activity of their resident p53s toward some pro-apoptotic targets.

In response to UV, LX1 cells, as well as A549 underwent apoptosis, while SKMes1 did not. At 24 h after irradiation with 30 and 60 J/m^2, PARP protein was cleaved in A549 and LX1. In LX1, PARP cleavage was already evident at 16 h post 60 J/m^2 (Fig. 1A). Cell cycle analysis confirmed induction of apoptosis in both cell lines after irradiation at 60 J/m^2, with the appearance of the subG_1 peak at 16 and 24 h (Fig. 1B). SKMes1 did not show PARP cleavage nor increase of the subG_1 fraction after high doses of UV. Similarly, adriamycin triggered PARP cleavage in A549 and LX1 cells, but not in SKMes1 (Fig. 1C). Interestingly, the cleavage of PARP appeared at lower adriamycin concentrations in LX1 compared to A549. In contrast, SKMes1 cells were refractory to apoptosis induction even after 40 h of treatment or higher adriamycin doses (10 μM) (data not shown). The evaluation of apoptosis induction through the percentage of subG_1 cells was consistent with PARP results for all cell lines (Fig. 1D). Thus, heterogeneity of apoptotic response was detected in our panel of NSCLC cell lines, with SKMes1 being particularly refractory to apoptosis when evaluated by FACS analysis and PARP cleavage.

PRIMA-1 is Cytotoxic but Unable to Trigger Apoptosis

To assess the effect of PRIMA-1 on different NSCLC cell lines, cell survival after 24 and 48 h treatment was determined using the MTT assay (Fig. 2). LX1 cells appeared to be more sensitive than SKMes1 and A549 cells both after 24 and

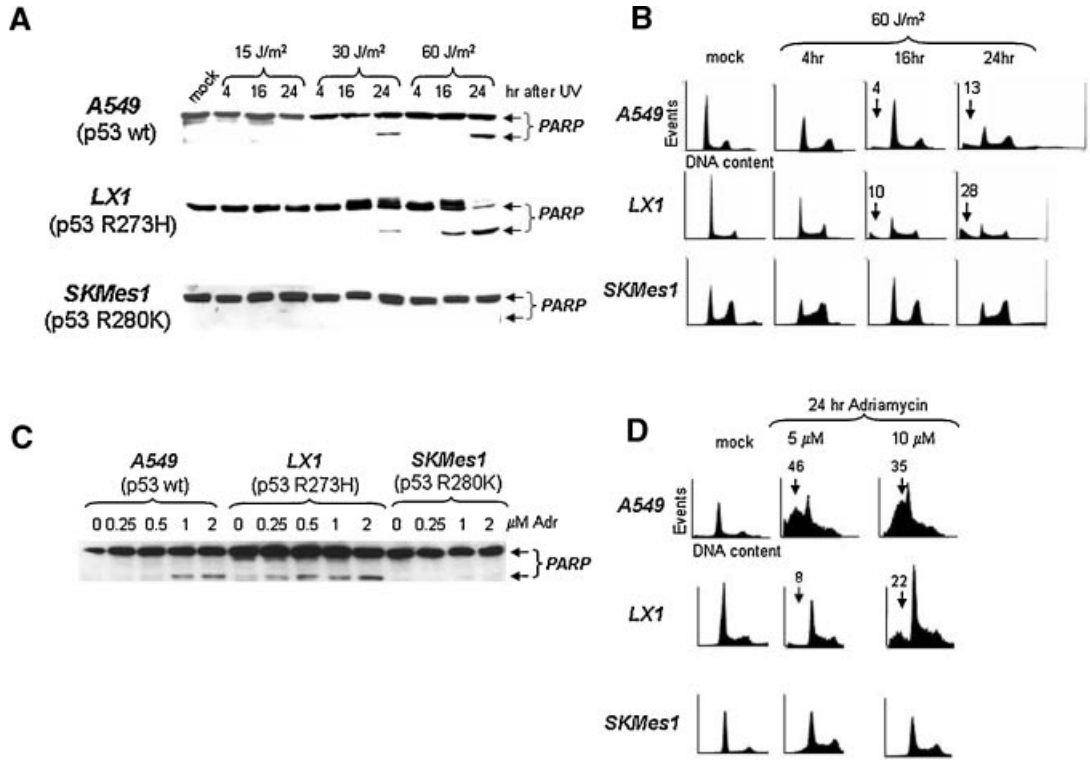


Fig. 1. Induction of apoptosis after UV-C and adriamycin treatment. The extent of PARP cleavage (arrows) was measured in cells subjected to UV-C irradiation (**A**) and 24 h adriamycin (**C**). FACS profiles of mock and treated cells stained with propidium iodide after 60 J/m^2 (**B**) and 5–10 μM adriamycin (**D**). Arrows indicate subG$_1$ fractions and the corresponding percentages are reported.

48 h of treatment. MTT experiments performed after adriamycin treatment revealed a similar sensitivity for all cell lines. These results indicated that PRIMA-1 has a different growth inhibitory activity on tumor cell lines, which might be dependent on p53 status and cell type.

The ability of PRIMA-1 to induce apoptosis was examined through the determination of PARP cleavage and induction of subG$_1$ cells. Neither cleavage of PARP (Fig. 3A) nor increase in the subG$_1$ fraction of cells (Fig. 3B) was appreciable. These results showed that per se PRIMA-1 was not able to trigger an apoptotic response in these cells even at doses that induce a strong cytotoxicity, suggesting that these cells may die through pathways other than apoptosis.

PRIMA-1, in Combination With Adriamycin, Triggers an Apoptotic Response

It has been shown that PRIMA-1^{Met} synergizes with cisplatin to induce apoptosis in tumor cells expressing mutated p53 [Bykov et al., 2005b]. In order to investigate a possible synergy on apoptosis induction between PRIMA-1 and a commonly used anticancer drug, cells were treated with 0.5–2 μM adriamycin for 24 h, then PRIMA-1 was added (100–200 μM) and PARP cleavage was determined after 16 h. An increase in cleavage after the addition of PRIMA-1 to adriamycin-treated cells was observed in all cell lines (Fig. 4A, arrows). Interestingly, SKMes1 cells that did not exhibit PARP cleavage following adriamycin (Fig. 1), showed an even stronger PARP cleavage compared to LX1. Furthermore, both in LX1 and SKMes1 cells carrying mutated p53, the cleavage occurred already at 0.5 μM adriamycin and 200 μM PRIMA-1 when no cleavage was detected for A549. Figure 4B, where the average proportion of PARP cleavage based on three independent experiments is reported, explicates even better this observation. Clearly, at 0.5 μM adriamycin, the addition of 200 μM PRIMA-1 induced a significant increase in cleavage in SKMes1 ($P < 0.001$) and, to a lesser extent, in LX1. At 1 μM adriamycin, cleavage increased after the addition of 200 μM PRIMA-1 in all cell lines, although only in SKMes1 the increase was statistically

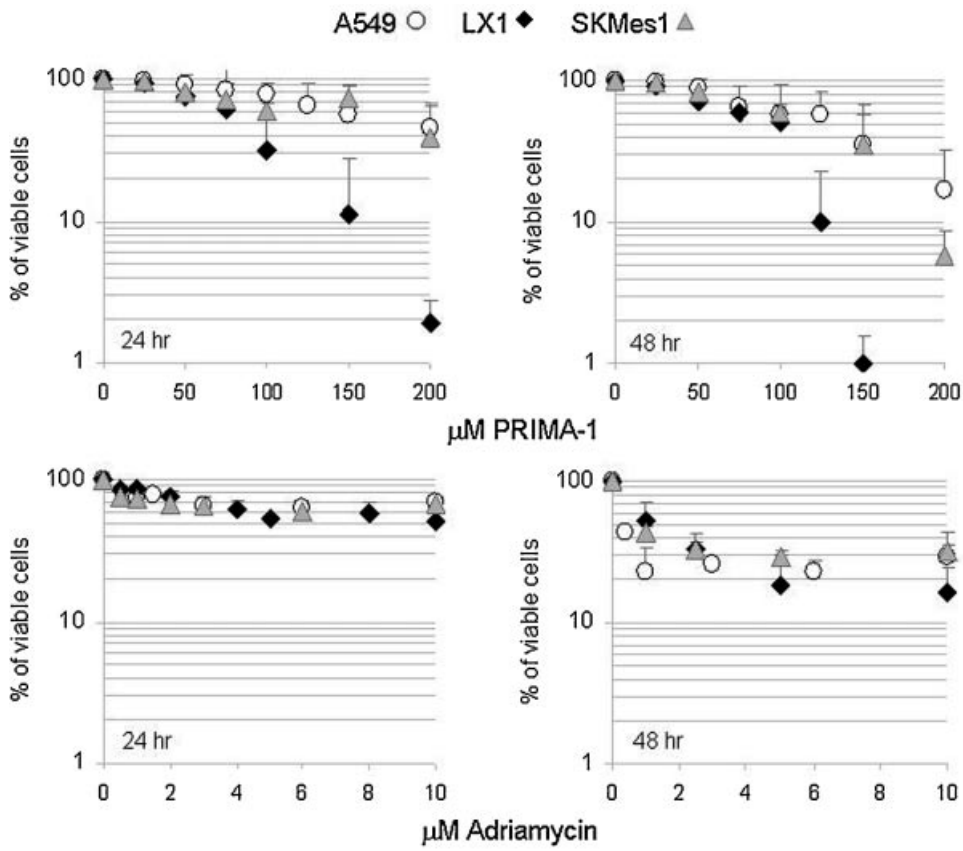
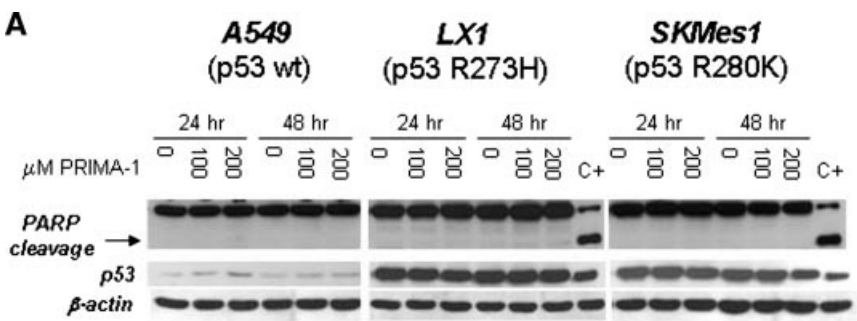


Fig. 2. Cell viability after PRIMA-1 or adriamycin treatments. Cells were seeded in 96-well and treated with different PRIMA-1 (**upper panels**) or adriamycin (**lower panels**) concentrations in triplicates. MTT assay was used to measure the percentage of viable cells after 24 h (**left panels**) and 48 h (**right panels**) of treatment. The average and the standard deviations of at least three independent experiments are reported.



B

	A549				LX1				SKMes1			
PRIMA-1 μM	sub G1	G1	S	G2/M	sub G1	G1	S	G2/M	sub G1	G1	S	G2/M
0	0	61	27	12	8	34	39	19	0	44	36	20
50	5	64	21	10	4	34	42	20	0	47	33	20
100	0	64	24	12	8	23	40	29	2	38	32	28
150	-	-	-	-	5	24	42	29	0	34	41	25
200	7	57	23	13	8	25	35	32	0	36	43	21

Fig. 3. Evaluation of PRIMA-1 induced apoptosis in NSCLC cells. **A**: PARP cleavage in cells treated with 0, 100, 200 μM PRIMA-1 for 24 and 48 h. The level of p53 after the treatment is also shown. β-actin was used as loading control. These Western blots are representative of experiments repeated at least three times. **B**: Cell cycle distribution of cells after 24 h PRIMA-1 treatment. The percentage of sub G_1 cells is highlighted. FACS experiments were repeated from 2 to 4 times giving comparable results.

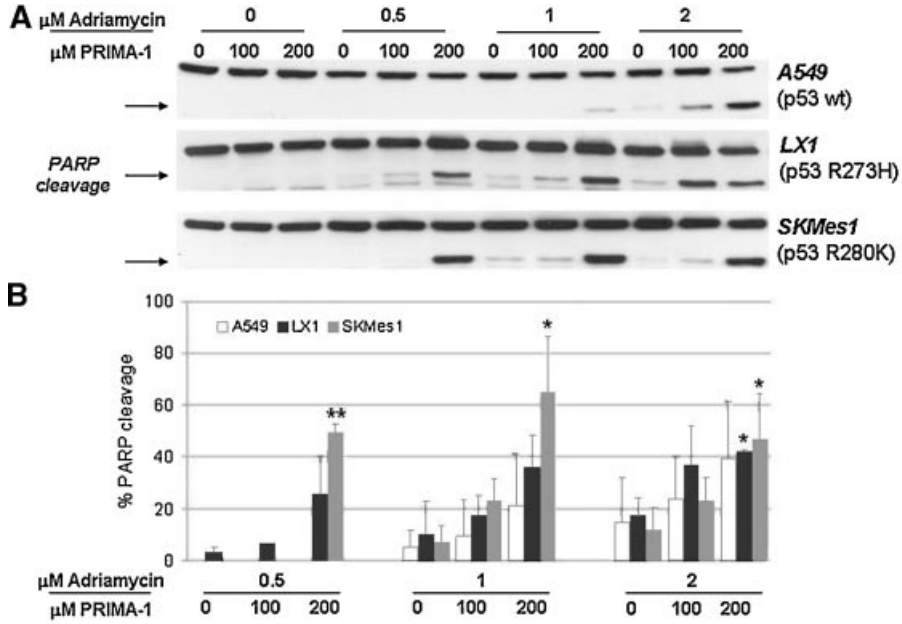


Fig. 4. Apoptosis triggered by adriamycin/PRIMA-1 treatment in NSCLC cells. **A**: Cells were treated with 0, 0.5, 1, and 2 μM adriamycin for 24 h. PRIMA-1 was added at different concentrations, as indicated, and the treatment was continued for 16 h. The percentage of PARP cleavage was determined by Western blot. Blots are from one experiment repeated at least three times with comparable results. **B**: The average and the standard deviations for three PARP cleavage experiments is represented. Within each set of adriamycin-treated samples, the two-tailed *P* values refer to the comparison between 200 μM PRIMA-1 treated samples and matched controls (0 μM PRIMA-1). $^{*}P < 0.044$; $^{**}P < 0.001$.

significant ($P = 0.0443$). At 2 μM adriamycin and after the addition of PRIMA-1, cleavage increased significantly only in SKMes1 and LX1 ($P = 0.0346$ and $P = 0.0367$, respectively). Thus, adriamycin and PRIMA-1 combined were synergistic in triggering a marker of apoptosis in LX1 and particularly in SKMes1.

Two other hallmarks of apoptosis, namely, DNA fragmentation and the percentage of sub G_1 cells (Fig. 5) were also investigated. In LX1

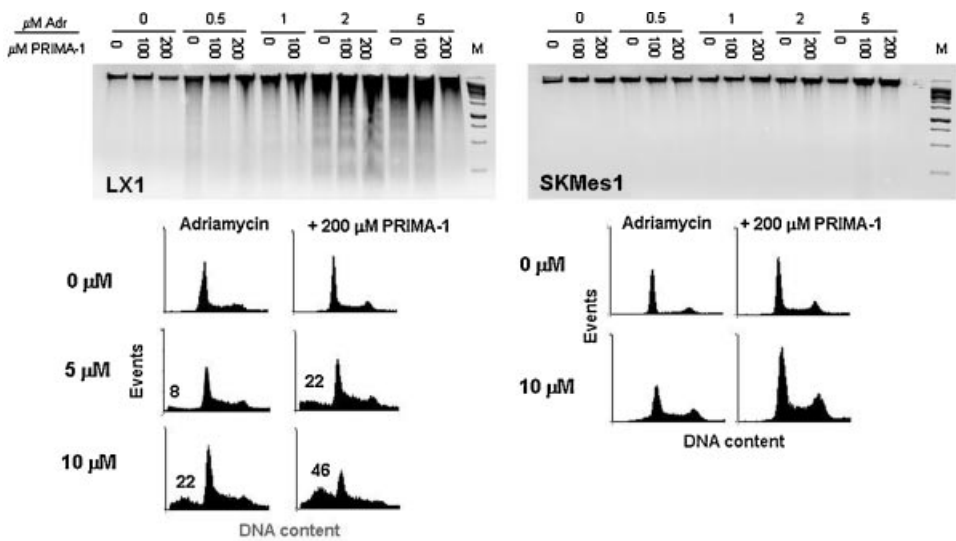


Fig. 5. LX1 and SKMes1 differ in DNA laddering and FACS experiments. DNA laddering (**upper panels**) and percentage of subG_1 cells (**lower panels**) were determined for LX1 and SKMes1 after the adriamycin/PRIMA-1 combined treatment. For FACS analysis, only the percentage of subG_1 cells is indicated.

we observed DNA fragmentation already at low adriamycin doses, but the addition of PRIMA-1 did not significantly increased the DNA laddering. On the other hand, the FACS analysis revealed an increased percentage of sub G_1 cells. In SKMes1, no DNA laddering nor sub G_1 cells were detected even at high adriamycin concentration. Furthermore, morphologic analysis of DAPI stained cells treated with PRIMA-1 alone or in combination with adriamycin did not show apoptotic nuclei (less than 1‰) in all cell lines (data not shown). Overall, these results indicated that the combined treatment can initiate the apoptotic response, as indicated by the strong PARP cleavage observed in both cell lines, but other steps necessary to successfully complete apoptosis are still severely impaired, at least in SKMes1.

We next examined whether PRIMA-1 treatment increases the sensitivity of these cells to adriamycin. Figure 6 shows that the addition of 200 μM PRIMA-1 reduced cell viability in all cell lines and at every adriamycin dose tested. However, while for A549 the decrease in cell viability after PRIMA-1 addition did not reach statistical significance, in LX1 and SKMes1 a statistically significant decrease in cell viability was measured at each adriamycin doses when 200 μM PRIMA-1 was added ($P < 0.01$ for LX1; $P < 0.03$ for SKMes1), although LX1 appeared the most and SKMes1 the least sensitive cells.

p21, MDM2, and PUMA were not Induced by Adriamycin/PRIMA-1 Combined Treatment

The potential of PRIMA-1 as an apoptosis inducer has been correlated to the ability of this drug to reactivate the transactivation activity of the mutated p53 towards target genes. It has been reported that in H1299-His-175 cells expressing mutant p53 and in SW480 colon carcinoma cells expressing endogenous p53R273H, PRIMA-1 induced the expression of p21 and Mdm2 [Bykov et al., 2002b]. Since this induction can be interpreted as a sign of mutant p53 reactivation, we tested the effect of the adriamycin/PRIMA-1 treatment on p21, mdm2, and PUMA protein modulation. We have previously shown that in LX1 and SKMes1 the resident mutated p53s (p53R273H and p53R280K, respectively) were not able to activate transcription of *p21* and *MDM2* and the corresponding proteins were not detected after UV irradiation [Magrini et al., 2007]. Here we showed that after PRIMA-1 treatment, p21 was not induced in all three cell lines (Fig. 7). In contrast, p21 was induced by adriamycin alone, but protein amounts decreased after the addition of PRIMA-1, particularly at the highest dose of both drugs. The Mdm2 levels increased in A549 at 0.5 μM adriamycin plus 200 μM PRIMA-1 (consistent with the up-regulation of wt p53), but at higher adriamycin concentrations PRIMA-1 induced a down-regulation of Mdm2. In SKMes1 and LX1, Mdm2 was not

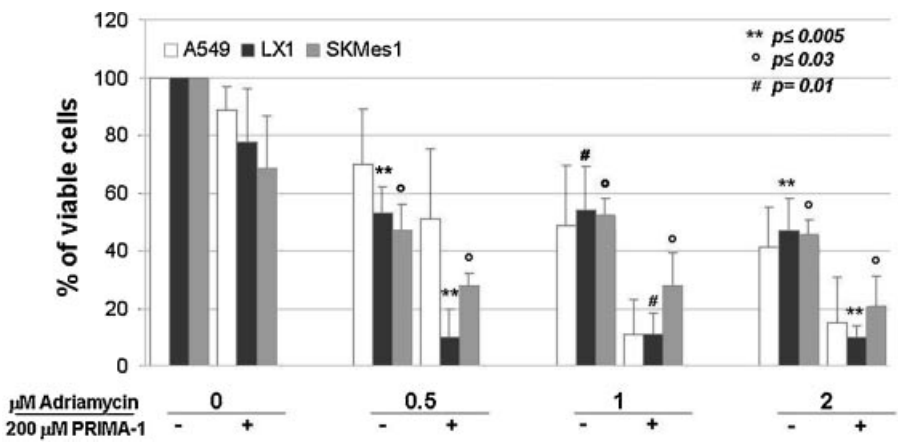


Fig. 6. Reduced viability after the adriamycin/PRIMA-1 treatment. Cells were seeded in 96-well and treated with adriamycin/PRIMA-1 as above. MTT assay was used to measure the percentage of viable cells. The average and the standard deviation of at least three independent experiments, each performed in three technical replicates, are reported. Within each set of adriamycin-treated samples, the two-tailed *P*-value is referred to the comparison between samples without or with 200 μM PRIMA-1.

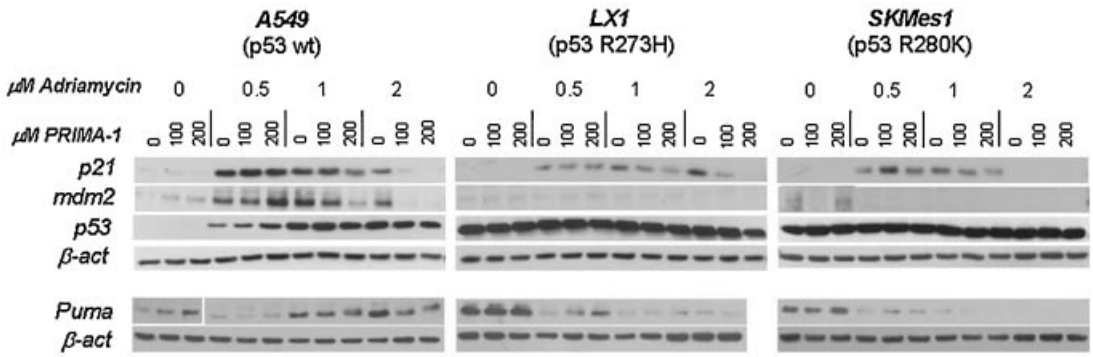


Fig. 7. Expression of p53 target proteins after adriamycin/PRIMA-1 treatment in NSCLC cells. Western blots showing the level of p21, Mdm2, PUMA, and p53 proteins following the standard combined treatment. β-actin was used as loading control.

induced by treatments. The pro-apoptotic PUMA was also investigated. In A549, PUMA was induced by adriamycin alone, but protein amounts again decreased after the addition of PRIMA-1. In LX1 and SKMes1, PUMA was present in mock and PRIMA-1 treated cells, but its level was almost undetectable following adriamycin and did not increase in the combined treatment. Thus, the activation of PARP cleavage, observed after adriamycin/PRIMA-1 treatment, did not correlate with p21, MDM2, and PUMA activation.

DISCUSSION

Cells derived from lung cancer are often very resistant to apoptosis induction by many drugs used in chemotherapy. The recent identification of small molecules that reactivate the mutated p53 and induce apoptosis in tumor cells constitutes a promising tool to design more potent and specific anticancer therapies. In this article we addressed the possibility to use PRIMA-1 to rescue the mutant p53 endogenously expressed in NSCLC cell lines and induce apoptosis. To this aim, we used three NSCLC cell lines carrying mutated (LX1: R273H; SKMes1: R280K) or wild-type (A549) p53s. Recently, we showed that the p53 transactivation activity towards some pro-apoptotic target genes (*BAX*, *PUMA*, *AIP1*) were completely abolished in LX1 and SKMes1, while it was retained in A549 cells [Magrini et al., 2007]. Here we showed that, although p53 transactivation activity was severely impaired, the two lung cancer cell lines carrying mutant p53 showed different ability to undergo apoptosis following UV or adriamycin. While LX1 (p53R273H) presented several hallmarks of apoptosis, like PARP cleavage, DNA fragmentation and subG$_1$ cells following both stimuli, the SKMes1 cell line (p53R280K) appeared very resistant to apoptosis induction and presented no such hallmarks. This difference can depend on several factors including specific features of the two mutated p53s, the net balance between pro- and anti-apoptotic BH3 proteins. Since a specific mutant p53 protein may retain some wild-type functions or acquire new transactivation activities [Sigal and Rotter, 2000; Weisz et al., 2004; Bossi et al., 2006] we cannot exclude that pro-apoptotic genes, still not analyzed, are activated in LX1, but not in SKMes1 and promote apoptosis. For instance, the ability to up-regulate the promoters of *MDR-1*, *PCNA*, *ASN*, and *hTERT*, not activated by wt p53, have been described for the p53R273H mutant [Deb et al., 1992; Dittmer et al., 1993; Scian et al., 2004]. In addition, there is evidence showing that p53 may have transcription-independent activities [Yee and Vousden, 2005] and one such reported activity is the direct activation of Bax by cytosolic p53 [Chipuk et al., 2004] which could be impacted by the specific nature of the p53 protein. On the other hand, anti-apoptotic Bcl-2 family members have been found overexpressed in lung cancer cells and this may cause a deregulation of the apoptotic pathway [Kaufmann and Vaux, 2003; Haura et al., 2004; Fesik, 2005]. Although the level of anti- and pro-apoptotic proteins were not determined in this article, A549 and SKMes1 cells are positive for Bcl-2 over expression, while LX1 are negative according to published results [Loprevite et al.,

1997]. Thus, the resistance of SKMes1 cells to undergo apoptosis might be related both to Bcl-2 and mutated p53 over expression.

In order to sensitize NSCLC cells under study to the induction of apoptosis, we used PRIMA-1, a low molecular weight compound shown to be able to induce apoptosis in some human tumor cells through the reactivation of the transactivation activity of mutated p53 [Bykov et al., 2002b]. Interestingly, this molecule has been shown to be more effective in inhibiting growth of mutant p53- than wild-type p53-carrying cells, especially for lung and colon carcinoma cell lines [Bykov et al., 2002a]. Such a preference was not found for other known anticancer drugs like cisplatin and 5-fluorouracil which, on the contrary, were more effective towards wild-type p53-carrying cell lines. For adriamycin, no preference towards wild-type p53- or mutant p53-carrying cell lines in any of the tumor types analyzed was found [Bykov et al., 2002a]. Our MTT experiments showed that LX1 were significantly more sensitive to PRIMA-1 than A549 already after 24 h of treatment, while longer exposure and higher doses were necessary to reduce SKMes1 viability under 10%. These results indicated that the presence of a mutated p53 may be related but cannot be the solely determinant for PRIMA-1 cytotoxicity. The analysis of apoptosis markers revealed that, when PRIMA-1 was given alone, no PARP cleavage nor subG_1 cells were found in all cell lines, regardless their p53 status. Thus, PRIMA-1 was cytotoxic, but it failed to trigger apoptosis in these NSCLC cells. When PRIMA-1 was given after an adriamycin treatment, a strong PARP cleavage was observed in all cell lines. Interestingly, a remarkable cleavage was observed in SKMes1 that had been refractory to other treatments in terms of PARP cleavage and/or induction of DNA fragmentation. Since caspase-dependent pathways are often impaired in NSCLC cells [Fennell, 2005] and caspase-3 is the major responsible for PARP cleavage, these results may suggest that such an adriamycin/PRIMA-1 treatment schedule was probably able to eliminate or inactivate endogenous caspase inhibitors in SKMes1. An interesting observation from our results is that the PARP cleavage measured after the adriamycin/PRIMA-1 treatment was stronger in mutated p53- than in wild-type p53-carrying cell lines. Indeed, in LX1 and SKMes1 a 25% and 50% of cleavage, respectively, was measured after low adriamycin and PRIMA-1 concentrations, while no PARP cleavage was detected in A549 at the same dose. However, following adriamycin/PRIMA-1 treatment, the two cell lines carrying mutated p53 maintain differences in their capacity to fully execute apoptosis, with LX1 showing DNA fragmentation and subG_1 cells unlike SKMes1. This result indicates that caspase activation and PARP fragmentation are not sufficient events to successfully execute apoptosis in these cells. Nevertheless, the adriamycin/PRIMA-1 combination reduced cell viability, suggesting again that PRIMA-1 may kill these NSCLC cells with alternative pathways to apoptosis.

The mechanism(s) of PRIMA-1 activity is (are) not yet clear. It has been shown that in breast cancer cells, PRIMA-1 induced the expression of heat shock protein 90 (Hsp90), restored the p53-Hsp90 interaction and enhanced the translocation of the p53-Hsp90 complex to the nucleus [Rehman et al., 2005]. According to the authors, this interaction could play a role in the reactivation of p53 transcriptional activity by mediating the mutated p53 refolding. Furthermore, PRIMA-1 and its derivative PRIMA-1^{Met} induced a p53 redistribution to nucleoli, together with pro-myelocytic leukemia protein (PML) nuclear body-associated proteins, cAMP responsive binding protein (CBP) and Hsp70 [Rokaeus et al., 2007]. This evidence suggested nucleolar translocation of mutated p53 and its interaction with proteins like Hsp90 and Hsp70 as possible mechanisms for p53 reactivation by PRIMA-1.

Since the majority of tumors with p53 mutations over-express mutant p53, the reactivation of the abundant mutated protein might then trigger a massive p53-dependent apoptosis. It has been reported that in H1299-His-175 cells, expressing exogenous mutant p53, and in SW480 colon carcinoma cells, expressing endogenous mutant p53R273H, PRIMA-1 induced the expression of p21 and MDM2 [Bykov et al., 2002b]. Increased level of p21 after PRIMA-1 was also found in H1299 cells expressing a temperature-sensitive p53 mutant [Li et al., 2005]. Furthermore, cisplatin and PRIMA-1^{Met} resulted in a synergistic increase in the level of Bax and PUMA in a mutant p53-dependent manner, while no increase was found in p53-null cells [Bykov et al., 2005b]. To address the question whether the adriamycin/PRIMA-1 treatment was able to restore the

transactivating function of mutated p53s towards some effector genes, we determined the level of p21, mdm2, and PUMA following adriamycin/PRIMA-1, but we did not find significant increase of these proteins. In particular, the level of p21 protein increased after adriamycin treatment, but it dropped when PRIMA-1 was added, while there was no significant increase in mdm2 and PUMA protein levels after treatment. This result suggests that in NSCLC cell lines expressing mutated p53 PRIMA-1 or the combined adriamycin/PRIMA-1 treatments did not rescue the p53-transactivation activity. Consistent with our results, it has been very recently reported that PRIMA-1 did not reactivate the DNA-binding and transcriptional activities of p53ser249 or p53gln248 mutant expressed in Hep3B cells towards *p21*, *PUMA*, and *COX-2* genes [Shi et al., 2007]. However, p53 could play a role in PRIMA-1 and/or adriamycin/PRIMA-1 response throughout transcription-independent mechanisms. Indeed, it has been reported that PRIMA-1 restoration of the p53 function triggers Bax-dependent apoptosis, involving cytochrome *c* release and caspase activation, in the absence of transcription or de novo synthesis in NSCLC cell lines [Chipuk et al., 2003]. Another mechanism, cited in Shi et al. [2007] may be related to the ability of PRIMA-1 to target mutant p53 and release it from interactions with other factors that may govern its activity. Thus, multiple mechanisms can be envisaged for PRIMA-1 induced apoptosis and/or cytotoxicity. Notably, a caspase-independent cell death has been described in which an apoptotic inducer can initiate a suicide program that does not involve caspase activation [Chipuk and Green, 2006]. Further investigations are necessary to better understand the influence of the p53 status and cell type in PRIMA-1 response.

Overall our results indicate that in NSCLC cells, PRIMA-1 can induce cell death also when apoptosis is impaired. However, it may enhance the apoptotic potential of drugs already used in chemotherapy and trigger some hallmarks of apoptosis (Table I). These findings may be of interest for unraveling new checkpoints in the apoptotic pathways downstream of caspases and may help developing new strategies that could inactivate endogenous caspase inhibitors in NSCLC or kill tumor cells particularly resistant to apoptosis induction.

TABLE I. Summary of Biological Responses of NSCLC Cell Lines Under Study to Different Treatments

	A549	LX1	SKMes1
UV			
Cytotoxicity	+	+	+
PARP cleavage	+	+	−
SubG1	+	+	−
Adriamycin			
Cytotoxicity	+	+	+
PARP cleavage	+	+	−
SubG1	+	+	−
PRIMA-1			
Cytotoxicity	+	++	+
PARP cleavage	−	−	−
SubG1	−	−	−
Adriamycin/PRIMA-1			
Cytotoxicity	++	+++	++
PARP cleavage	+	+	++
SubG1	+	+	−

ACKNOWLEDGMENTS

We thank Dr. Galina Selivanova for helpful discussions and comments on the manuscript. Dr. G. Cutrona and Dr. S. Matis are acknowledged for their experimental expertise in FACS analysis. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), Ministero della Salute, Fondo per gli Investimenti della Ricerca di Base (FIRB) e Alleanza Contro il Cancro (ACC).

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