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PRIMA-1 Reactivates Mutant p53 by Covalent Binding to the Core Domain

Jeremy M.R. Lambert,[1,2] Petr Gorzov,[1] Dimitry B. Veprintsev,[3] Maja Söderqvist,[1] Dan Segerbäck,[4] Jan Bergman,[4] Alan R. Fersht,[3] Pierre Hainaut,[2] Klas G. Wiman,[1,*] and Vladimir J.N. Bykov[1]
[1]Department of Oncology-Pathology, Cancer Center Karolinska, Karolinska Institutet, SE-171 76 Stockholm, Sweden
[2]International Agency for Research on Cancer, 150 Cours Albert Thomas, 69372 Lyon Cedex 08, France
[3]Centre for Protein Engineering, Medical Research Council, Cambridge CB2 2QH, UK
[4]Department of Biosciences and Nutrition, Karolinska Institutet, Novum, SE-141 57, Huddinge, Sweden
*Correspondence: klas.wiman@ki.se
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SUMMARY

Restoration of wild-type p53 expression triggers cell death and eliminates tumors in vivo. The identification of mutant p53-reactivating small molecules such as PRIMA-1 opens possibilities for the development of more efficient anticancer drugs. Although the biological effects of PRIMA-1 are well demonstrated, little is known about its molecular mechanism of action. We show here that PRIMA-1 is converted to compounds that form adducts with thiols in mutant p53. Covalent modification of mutant p53 per se is sufficient to induce apoptosis in tumor cells. These findings might facilitate the design of more potent and specific mutant p53-targeting anticancer drugs.

INTRODUCTION

The transcription factor p53 is activated in response to cellular stress (Lane, 2004; Vousden and Lu, 2002) leading to cell cycle arrest, senescence, and/or apoptosis. p53 mutation occurs frequently in human tumors (Hollstein et al., 1991; Olivier et al., 2002) and plays a critical role in tumor evolution by allowing evasion from p53-dependent arrest or cell death. Mutation of p53 might also increase resistance to chemotherapy (Olivier et al., 2006). These observations, and the finding that mutant p53 is often expressed at high levels, make mutant p53 an important target for cancer therapy. Screening studies have identified small molecules, including CP-31398, WR-1065, PRIMA-1, and MIRA-1, that reactivate mutant p53 (Bykov et al., 2002a, 2002b, 2005a; Foster et al., 1999). PRIMA-1 and its methylated version PRIMA-1^{MET} induce several p53 target genes and mutant p53-dependent apoptosis in human tumor cells (Bykov et al., 2002b, 2005a, 2005b; North et al., 2002). Systemic administration of PRIMA-1 or PRIMA-1^{MET} inhibits human xenograft tumor growth in mice (Bykov et al., 2002b; Foster et al., 1999). PRIMA-1 also restores mutant p53-mediated transcription-independent apoptosis (Chipuk et al., 2003). Moreover, PRIMA-1^{MET} synergizes with chemotherapeutic drugs to induce tumor cell apoptosis (Bykov et al., 2005b; Nahi et al., 2004, 2006; Rehman et al., 2005). Nevertheless, the exact molecular mechanism by which PRIMA-1 and PRIMA-1^{MET} and other mutant p53-targeting compounds restore wild-type function to mutant p53 and/or affect other cellular targets has remained unknown. A better understanding of the molecular mechanisms should facilitate rational drug design of more potent and specific molecular scaffolds for mutant p53 reactivation.

Here we have investigated the chemical properties of PRIMA-1 and PRIMA-1^{MET} and their potential binding to mutant p53. We propose that covalent modification of thiol groups in the mutant p53 core domain is responsible for the observed mutant p53 reactivation by PRIMA-1 and PRIMA-1^{MET}.

RESULTS

Decomposition of PRIMA-1 and PRIMA-1^{MET} and Its Role for Biological Activity

To examine potential degradation of PRIMA-1 and the analog PRIMA-1^{MET}, we incubated each compound at 37°C at pH 7.4

SIGNIFICANCE

Given the frequent mutations of p53 in almost all types of human tumors, mutant p53-targeting drugs should have a wide clinical applicability. Moreover, because mutant p53-carrying tumors are usually more resistant to currently used chemotherapy, there is an urgent need for more effective treatment of such tumors. We demonstrate that modification of thiol groups in mutant p53 by PRIMA-1 conversion products is sufficient to restore its tumor suppressor activity. This might open possibilities for the design of more potent mutant p53-specific compounds based on the same or a similar molecular mechanism, and eventually the development of efficient anticancer drugs.

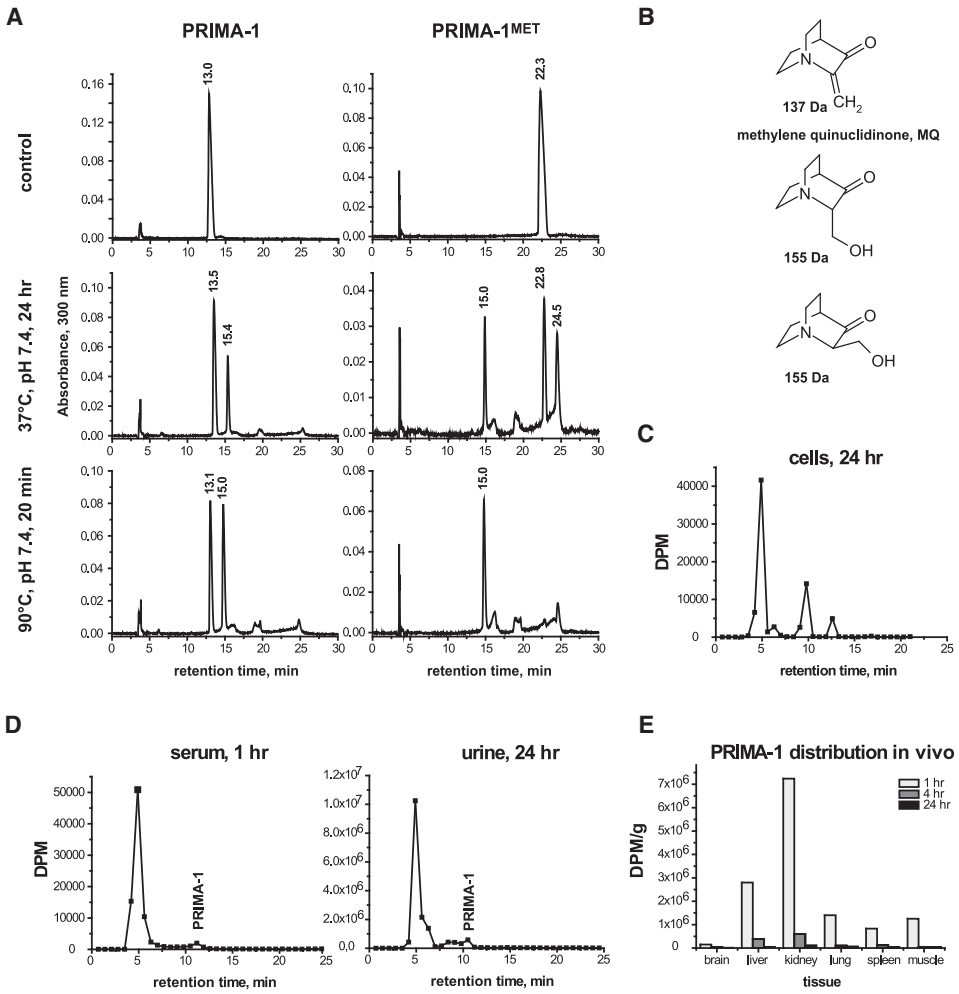

Figure 1. Chemical Stability of PRIMA-1 and PRIMA-1^{MET} In Vitro and in Living Cells

(A) HPLC profiles of untreated (upper panel) and preheated (lower panel) PRIMA-1 and PRIMA-1^{MET}. Preheating was performed in PBS for 24 hr at 37°C or 20 min at 90°C. The major decomposition products of PRIMA-1 and PRIMA-1^{MET} have identical retention times on HPLC and coeluted with each other.

(B) Structural formulas of the identified decomposition products of PRIMA-1 and PRIMA-1^{MET}.

(C) Chromatogram of lysate from cells treated with 50 μM [^{14}C]-PRIMA-1 for 24 hr.

(D) Chromatograms of serum and urine from [^{14}C]-PRIMA-1-treated mice at 1 and 24 hr after intravenous injection. The estimation of the retention time of PRIMA-1 on the chromatograms is based on coelution with the internal standard of cold PRIMA-1 spiked with actual samples and traced by UV-detector.

(E) Distribution of [^{14}C]-PRIMA-1 in organs in vivo at 1, 2, and 24 hr after intravenous injection of radiolabeled PRIMA-1.

in phosphate-buffered saline (PBS) for various periods and analyzed samples by high-pressure liquid chromatography (HPLC). Incubation resulted in a time-dependent conversion of the parental compounds and the elution on HPLC yielded two major peaks at 15.0 and 24.0 min (Figure 1A). Half of the starting material of PRIMA-1 and PRIMA-1^{MET} was converted to other products after 41.4 and 32.6 hr, respectively. Incubation at 90°C for 20 min resulted in an identical pattern of degradation as that obtained by incubation at 37°C for 3 days (Figure 1A and data not shown). Analysis of the collected peaks by mass spectrometry (MS) and ^{1}H nuclear magnetic resonance (NMR) showed that the fraction eluted at 15 min contains a mixture of three compounds with molecular masses of 137 and 155 Da (Figure 1B). The former is denoted methylene quinuclidinone (MQ). Other detected products could not be identified.

To determine if decomposition of PRIMA-1 occurs in living cells, we treated H1299-His175 cells with [^{14}C]-labeled PRIMA-1 for 24 hr and analyzed the cell lysates by HPLC. We observed significant decomposition after 4 hr, and only a minor portion of the starting material could be detected in cells after 24 hr (Figure 1C). We conclude that conversion of PRIMA-1 occurs even more efficiently in living cells than in vitro. Furthermore, we treated Balb/c mice with [^{14}C]-PRIMA-1 by intravenous injection and analyzed serum and urine by HPLC. In agreement with our data from experiments in cultured cells, we detected nearly complete decomposition of PRIMA-1 already at 1 hr after treatment (Figure 1D). The emerged radioactive products in both serum and urine eluted faster than the PRIMA-1 decomposition products shown in Figure 1A. These metabolites can be expected to be short-lived in complex environments such as

biological fluids and tissues. Therefore it is likely that the products of their reaction with other molecules carrying thiol groups, rather than the decomposition products themselves in their free form, are detected in vivo (Figure 1D). Also, we examined the distribution of PRIMA-1 in vivo in various organs (Figure 1E). Only a small portion of the radioactivity was detected in the brain, suggesting that PRIMA does not efficiently penetrate the blood-brain barrier. The highest amount of radioactivity was detected in kidneys, indicating that PRIMA-1 is excreted from the body via the urine and that the excretion is very rapid. Only around 3% of the radioactivity remained in serum after 24 hr as compared with 1 hr after the injection, and more than 99% of the radioactivity was found in urine as compared to serum at this time point.

The putative decomposition of PRIMA-1 and PRIMA-1^{MET} is shown in Figure 2A. Conversion according to this scheme should generate formaldehyde. Using the Purpald reagent, we confirmed that both PRIMA-1 and PRIMA-1^{MET} released formaldehyde (data not shown). Exposure of H1299-His175 cells to a range of formaldehyde concentrations up to 50 μM did not induce any growth suppression (data not shown). Thus, formaldehyde is unlikely to be responsible for PRIMA-1-induced cell death.

If conversion of PRIMA-1/PRIMA-1^{MET} is required for its biological effects, PRIMA-1 analogs that cannot be decomposed as shown in Figure 2A should be inactive. To test this, we synthesized a molecule termed PRIMA-D that closely resembles PRIMA-1 but cannot be converted to MQ (Figure 2C). PRIMA-D was completely inactive with respect to induction of apoptosis in H1299-His175 cells at concentrations up to 100 μM (data not shown). No decomposition of PRIMA-D was detected after incubation in PBS for 24 hr at 37°C (Figure 2C), and no MQ was detected after boiling for 1 hr (data not shown). This supports our hypothesis that decomposition of PRIMA-1 and PRIMA-1^{MET} is critical for their biological activity.

We isolated HPLC fractions corresponding to MQ (p15 min) and the peak at 24 min that contains the unidentified products. Concentrations of these products were determined based on the decrease of the HPLC peak of the parent compound during decomposition and on the relative yield of the products based on the ultraviolet irradiation (UV) spectrometry. The obtained fractions were concentrated under vacuum and tested in Saos-2 and Saos-2-His273 cells using the WST-1 assay. For MQ (p15), we obtained IC_{50} values of 20.6 ± 0.4 μM in Saos-2 and 14.8 ± 3.9 μM in Saos-2-His273 cells. For p24, the IC_{50} values were 14.8 ± 4.0 and 8.8 ± 0.1 μM, respectively. These fractions were also assayed for induction of DNA fragmentation by fluorescence-activated cell sorter propidium iodide (FACS-PI) (Figure 2D). Thus, these results show that both major decomposition products of PRIMA-1 have mutant p53-dependent activity.

Role of Thiol Modification for the Biological Effect of PRIMA-1 and PRIMA-1^{MET}

One of the decomposition products of PRIMA-1 and PRIMA-1^{MET}, MQ, has a chemically active double bond (Figure 2A) that is prone to participate in reactions of nucleophilic addition. In a cellular environment, protein thiol groups will be primary targets for such reactions. To investigate whether covalent modification of thiols plays a role for the observed biological effects of PRIMA-1 and PRIMA-1^{MET}, we pretreated H1299-His175 cells with 5 mM N-acetylcysteine (NAC), followed by addition of PRIMA-1. NAC completely blocked PRIMA-1-induced growth suppression and apoptosis (Figures 3A and 3B). NAC also blocked the effect of MIRA-3 (Bykov et al., 2005a) and STIMA-1 (Zache et al., 2008), two substances that suppress growth of human tumor cells in a mutant p53-dependent manner similar to PRIMA-1. In contrast, NAC only partially blocked the effect of the chemotherapeutic drugs vinblastin, camptothecin, and paclitaxel (Figure 3A). Analysis by WST-1 and FACS-PI showed that NAC does not block wild-type p53-induced apoptosis in the absence of PRIMA-1 in BL41-tsp53 and J3D-tsp53 cells upon activation of wild-type p53 expression at 30°C (data not shown). Incubation of NAC with PRIMA-1^{MET} overnight at 37°C resulted in complete depletion of the parent compound and the appearance of other products as shown by HPLC (Figure 3C). MS analysis of the isolated fractions confirmed adduct formation between NAC and PRIMA-1^{MET} via substitution of a methoxy group and mainly by nucleophilic addition to a carbon-carbon double bond (Figure 3C). The reaction between NAC and MQ is very rapid and is nearly complete in 1 min at 37°C (data not shown).

Because glutathione is an important component of the cellular redox control and xenobiotic neutralizing system, we speculated that inhibition of glutathione synthesis by buthionine sulfoximide (BSO) might potentiate the effect of PRIMA-1. Indeed, BSO dramatically increased the growth suppression effect of PRIMA-1 in both H1299 and H1299-His175 cells. However, BSO itself did not induce significant growth suppression in these cells. The selectivity for mutant p53-expressing cells was retained (Figure 3D). Altogether, these results provide strong evidence that modification of thiols is involved in the biological activity of PRIMA-1 and PRIMA-1^{MET}.

PRIMA-1^{MET} Promotes an Oxidative Environment in Tumor Cells

If PRIMA-1 and PRIMA-1^{MET} exert their biological effects through regulation of the redox status in tumor cells, for instance by alkylating thiols, then treatment with PRIMA-1 or PRIMA-1^{MET} should induce an oxidative cellular milieu. To test this prediction, we treated Saos-2 and Saos-2-His273 cells with PRIMA-1^{MET} for 24 hr, stained cells with DCF, a reduced form of fluorescein that fluoresces upon oxidation, and analyzed the cells by FACS (Bass et al., 1983). This experiment revealed that PRIMA-1^{MET} causes increased oxidation in a mutant p53-dependent manner (Figure 3E). No oxidation was observed in p53 null cells even at 100 μM of PRIMA-1^{MET}. Similar results were obtained with parental p53 null H1299 cells and the mutant p53-expressing H1299-His175 cells. Interestingly, cisplatin did not cause any increased oxidation in Saos-2-His273 even at cytotoxic concentrations, although it induced reactive oxygen species (ROS) in HCT116 colon carcinoma cells expressing wild-type p53 (data not shown). These data suggest that PRIMA-1^{MET} exerts its effect in part via restoring ROS-inducing ability to mutant p53, rather than indiscriminately alkylating thiol groups in proteins.

PRIMA-1 and PRIMA-1^{MET} React Covalently with Thiol Groups in Mutant p53

Because at least one degradation product of PRIMA-1 and PRIMA-1^{MET} possesses thiol-modifying activity, we tested whether PRIMA-1 modifies thiol groups in proteins in vitro.

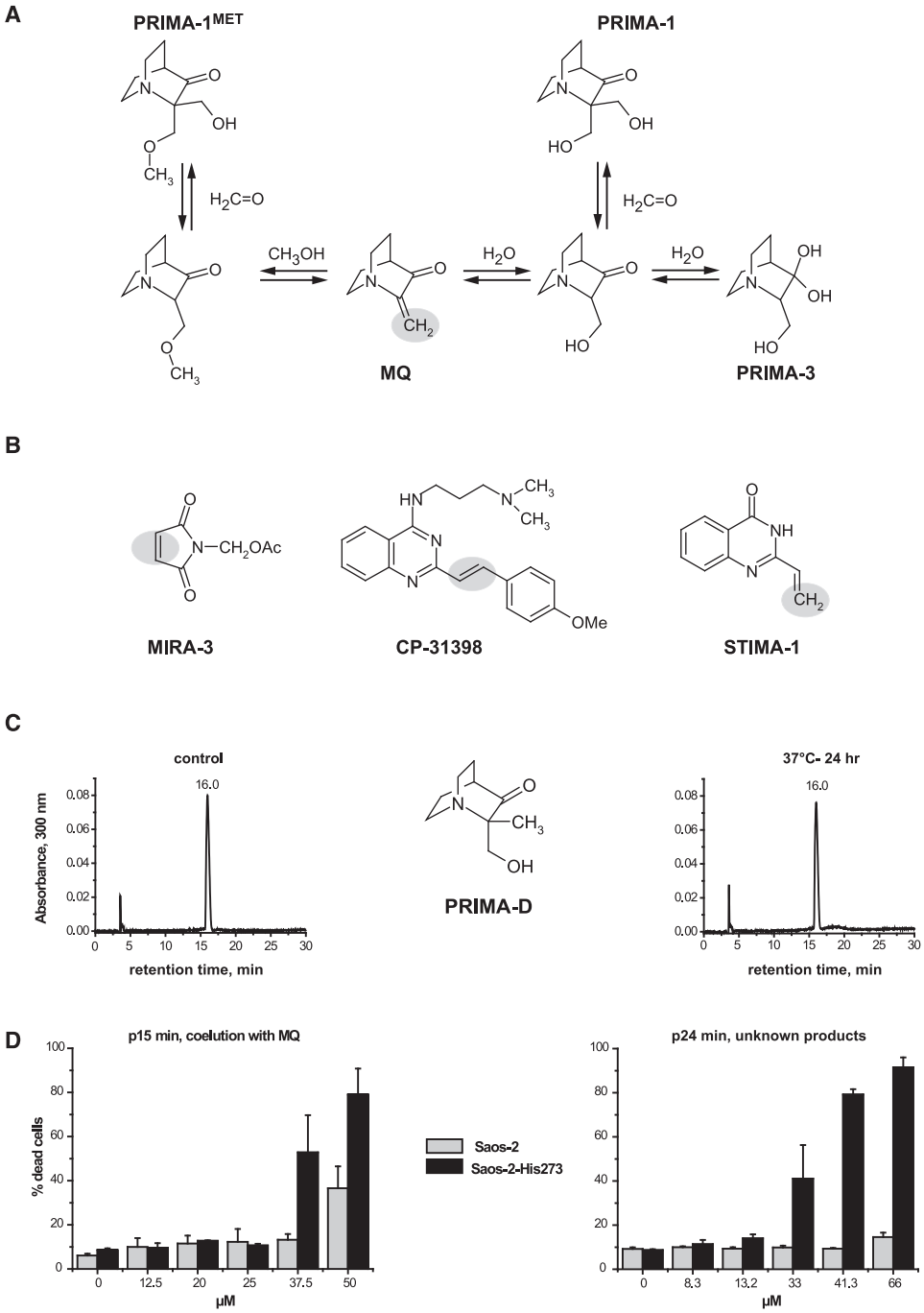

Figure 2. Active Chemical Groups in Mutant p53-Reactivating Compounds

(A) Conversion of PRIMA-1 and PRIMA-1^{MET}. A reactive chemical group, i.e., a double bond coupled to an electron-withdrawing group forming a classical Michael acceptor, is indicated in MQ, a common decomposition product of PRIMA-1 and PRIMA-1^{MET}.

(B) Similar reactive groups are indicated in MIRA-3, CP-31398, and STIMA-1.

(C) Structural formula and HPLC profiles of untreated (left panel) and preheated (right panel) PRIMA-D. Preheating was performed in PBS for 24 hr at 37°C.

(D) FACS-PI analysis demonstrating induction of mutant p53-dependent cell death in Saos-2 osteosarcoma cells by major decomposition products of PRIMA-1. Data are shown as mean and standard error of the mean (±SEM).

PRIMA-1 and PRIMA-1^{MET} preincubated at 37°C for 3 days decreased the number of free thiols in bovine serum albumin (BSA) in a concentration-dependent manner as determined by the Ellman reagent (Ellman, 1959) (data not shown). To examine alkylation of thiols in mutant p53, we treated recombinant glutathione S-transferase (GST) His175 or GST-Gln248 mutant p53 with PRIMA-1 or PRIMA-1^{MET} that had been preincubated at either 90°C for 20 min or 37°C for 3 days, and assessed thiol

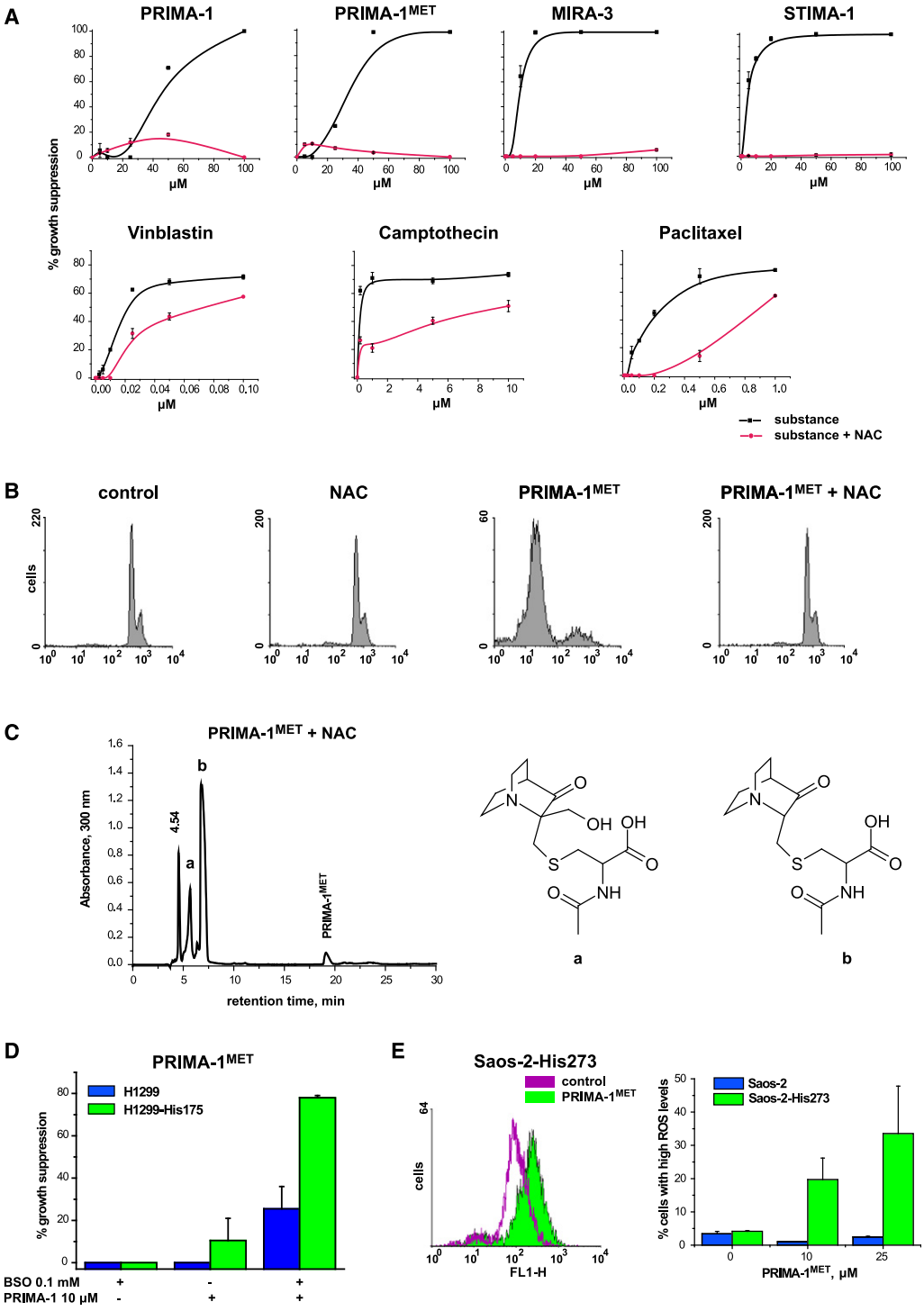

Figure 3. The Biological Effect of PRIMA-1 Is Inhibited by NAC

(A) NAC completely blocks the cytotoxic effect of PRIMA-1, PRIMA-1^{MET}, MIRA-3, and STIMA-1 in H1299-His175 cells, as shown by the WST-1 proliferation assay, whereas only partial protection against vinblastin, camptothecin, and paclitaxel was observed. Data are shown as mean ± SEM.

(B) Treatment with NAC prevents PRIMA-1^{MET}-induced cell death in H1299-His175 cells according to PI staining and FACS.

(C) Left panel shows reverse-phase HPLC profile of PRIMA-1^{MET} incubated with NAC at 37°C for 3 days in 50 mM Tris-HCL (pH 7.5). The new products *a* and *b* were collected on HPLC and analyzed by MS. Right panel shows the identified structures of PRIMA-1^{MET}-NAC adducts based on MS data.

(D) Glutathione depletion by BSO increases sensitivity of H1299 and H1299-His175 cells to PRIMA-1^{MET}. Data are shown as mean ± SEM.

(E) PRIMA-1^{MET} treatment induces ROS in Saos-2-His273 cells in a concentration-dependent manner as shown by DCF staining and FACS. Data are shown as mean ± SEM.

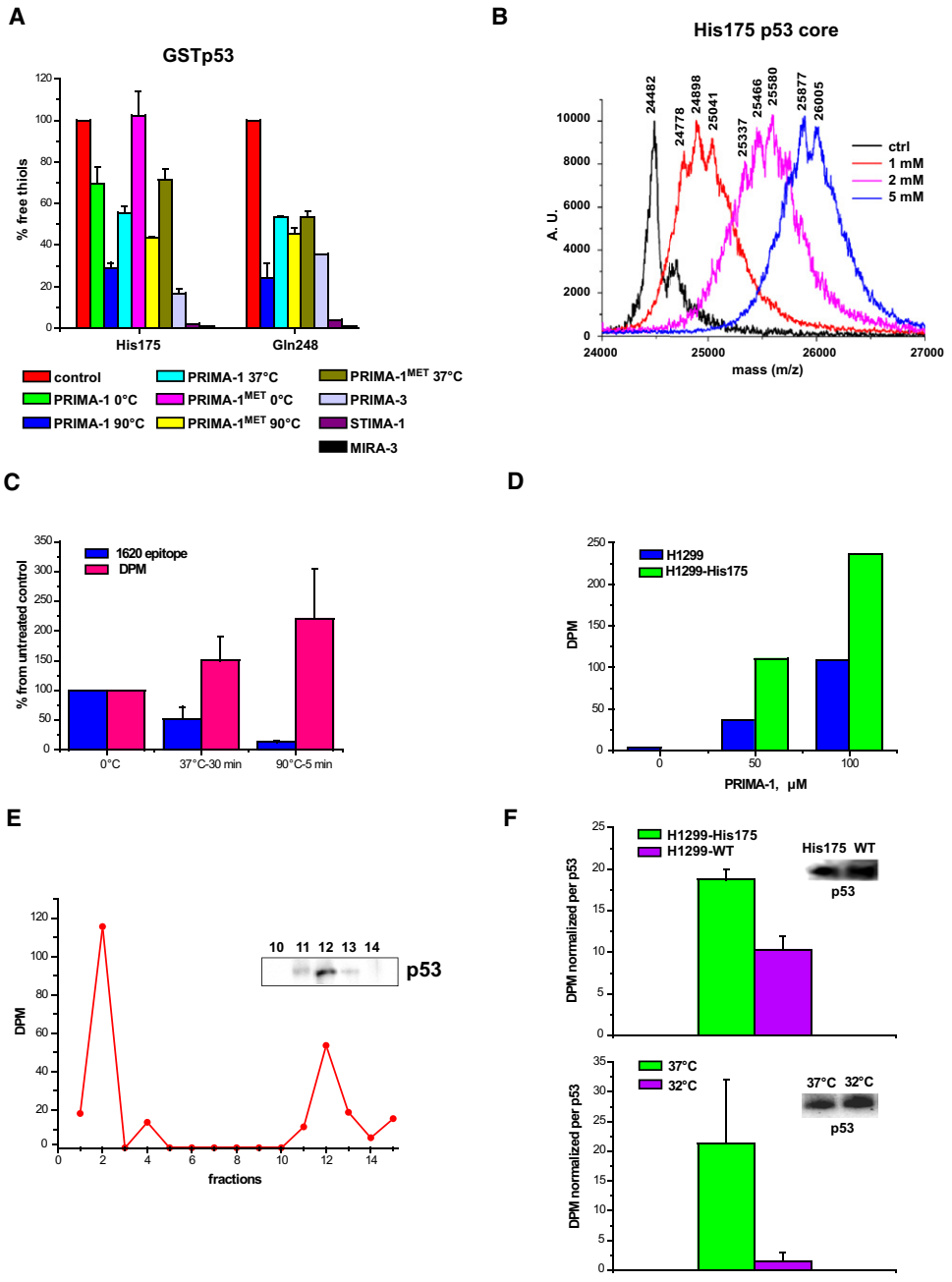

Figure 4. PRIMA-1 Decomposition Products Bind to p53 In Vitro and in Living Cells

(A) PRIMA-1, PRIMA-1^{MET}, PRIMA-3, STIMA-1, and MIRA-3 reduce free thiols in recombinant GST-His175 and Gln248 mutant p53. Data are shown as mean ± SEM.

(B) Treatment with preheated PRIMA-1 increases the molecular mass of the His175 mutant p53 core domain. The expected gain of molecular weight is 137 and 166 Da, depending on the type of modification.

(C) Analysis of [^{14}C]-PRIMA-1 binding to recombinant GST-WT p53 showing more efficient binding with increasing p53 unfolding. Data are shown as mean ± SEM.

(D) Quantification of radioactivity in p53 immunoprecipitates from extracts of cells treated with [^{14}C]-PRIMA-1.

(E) Elution profile of immunoprecipitated mutant p53 from H1299-His175 cells on an mRP-C_{18} HPLC column. Western blot analysis demonstrated that the radioactivity comigrated with cellular mutant p53.

(F) Analysis of radioactivity in p53 immunoprecipitates from extracts of H1299-His175, H1299-WT, and J3D-tsp53 cells treated with [^{14}C]-PRIMA-1. Data are shown as mean ± SEM. Western blotting shows levels of immunoprecipitated p53 in representative samples.

modification using biotinylated maleimide. PRIMA-1 and PRIMA-1^{MET} were used at concentrations that exceed the concentration of the antioxidant DTT (10 mM) in the protein preparations. This analysis revealed that decomposition products of PRIMA-1 and PRIMA-1^{MET} can react covalently with thiols in both mutant p53 proteins (Figure 4A). The structural analog

PRIMA-3 blocked thiols in mutant p53 with similar efficiency as PRIMA-1 and PRIMA-1^{MET}. As a positive control, we used the mutant p53-targeting compounds MIRA-3 and STIMA-1, which were even more efficient in blocking thiols in mutant p53 (Figure 4A).

To study binding of PRIMA-1 decomposition products to p53, we performed MS analysis of recombinant wild-type and His175 mutant p53 core domains treated with PRIMA-1 preheated at 90°C for 20 min. Again, we used PRIMA-1 at concentrations that were high enough to override the reducing agent (TCEP) in the p53 preparation. Treatment with 1 mM PRIMA-1 increased the molecular weight of the mutant p53 core domain by 296 Da, corresponding to approximately 2 molecules of MQ (Figure 4B). Increasing concentrations of PRIMA-1 resulted in a dose-dependent increase in the molecular weight of the p53 core up to 1395 and 1523 Da at 5 mM PRIMA-1. This corresponds approximately to 10 molecules of MQ (Figure 4B). Experiments with the wild-type p53 core domain yielded similar results (data not shown). Based on our experiments with NAC, we expect at least two PRIMA-1 and PRIMA-1^{MET} adducts with thiols: a dominant one produced by MQ that will result in a 137 Da gain of molecular weight, and another adduct that could be produced via substitution of a hydroxyl group in PRIMA-1 or a methoxy group in PRIMA-1^{MET}, resulting in a 166 Da increase in molecular weight (Figure 3C). Also, formation of protein-formaldehyde adducts cannot be excluded. In order to examine the relationship between p53 conformation and PRIMA-1 binding, we incubated GST wild-type (WT) p53 at 37°C for 30 min or at 90°C for 5 min to induce increasing degrees of p53 unfolding, as verified by enzyme-linked immunosorbent assay (ELISA) using PAb1620 antibodies that recognize wild-type conformation of p53 (Figure 4C). Binding of [^{14}C]-PRIMA-1 to GST-WT p53 was proportional to the degree of p53 unfolding (Figure 4C).

To assess binding of PRIMA-1 decomposition products to mutant p53 in living cells, we treated H1299-His175 and H1299 cells with [^{14}C]-PRIMA-1 for 24 hr and immunoprecipitated p53. We observed significant radioactivity in immunoprecipitates with anti-p53 antibodies from the His175-expressing cells as compared with the p53 null cells (Figure 4D). Next we fractionated immunoprecipitated p53 on an mRP-C_{18} HPLC column and assessed radioactivity in each fraction. The peak of radioactivity at 12 min comigrated with cellular His175 mutant p53 protein according to results of western blotting (Figure 4E). We conclude that PRIMA-1 decomposition products also bind to mutant p53 in living cells.

We also investigated efficiency of [^{14}C]-PRIMA-1 binding to wild-type and His175 mutant p53 in living cells by immunoprecipitation of p53 from cell lysates and comparison of radioactivity in the immunoprecipitates with amount of p53 according to western blotting results. We did not detect any radioactivity by immunoprecipitation in HCT116 WT p53$^{+/+}$ cells, presumably due to low levels of wild-type p53. To induce levels of wild-type p53, we treated the cells with 1 μg/ml cisplatin 24 hr before adding [^{14}C]-PRIMA-1. We found that His175 mutant p53 binds about twice as much [^{14}C]-PRIMA-1 as wild-type p53 in cisplatin-treated HCT116 wild-type p53$^{+/+}$ cells (data not shown).

To allow an even more stringent comparison of binding in the same cellular background, we used H1299 cells carrying either exogenous wild-type or mutant p53. We treated the cells with 100 μM [^{14}C]-PRIMA-1 for 7 hr, immunoprecipitated p53, and measured immunoprecipitated radioactivity by liquid scintillation counting and amount of p53 in the immunoprecipitates by western blotting. We detected almost 2-fold higher PRIMA-1 binding to His175 mutant p53 than to wild-type p53 after normalizing to amount of immunoprecipitated p53 (Figure 4F). We performed similar experiments in J3D-tsp53 cells that express unfolded mutant p53 at 37°C. Temperature shift to 32°C induces expression of wild-type p53 and apoptosis. Upon treatment with [^{14}C]-PRIMA-1 and immunoprecipitation with anti-p53 antibodies, we detected a significant binding to mutant p53 (37°C) but only low levels of binding to wild-type p53 (32°C) after normalizing to amount of immunoprecipitated p53 (Figure 4F). However, we cannot exclude that slower conversion at the lower temperature (32°C) could reduce the amount of generated reactive product, which would underestimate binding to wild-type p53.

Thus, our results with recombinant and cellular p53 show that unfolded mutant p53 is modified by PRIMA-1 more efficiently than the correctly folded wild-type protein.

PRIMA-1-Treated Recombinant Mutant p53 Triggers Apoptosis in p53 Null Cells

To determine the role of alkylation of mutant p53 for the biological effect of PRIMA-1, we treated recombinant mutant p53 proteins with PRIMA-1 prewarmed at 90°C for 20 min, followed by dialysis in order to remove unbound PRIMA-1. We then introduced the treated protein directly into p53 null Saos-2 cells using the Chariot protein transfer reagent. Mutant p53 transfer into Saos-2 cells resulted in strong nuclear and cytoplasmic p53 immunostaining in a majority of the cells after 2 hr (Figure 5A). p53 remained in the cells 24 hr after protein transfer (data not shown). Introduction of untreated GST-WT p53 reduced Saos-2 cell survival by 28%, and PRIMA-1-treated wild-type p53 induced cell death with significantly higher efficiency (Figure 5B). PRIMA-1-treated GST-His175 and GST-Gln248 mutant p53 were equally efficient in inducing cell death upon Chariot transfer, whereas PRIMA-1-treated BSA was entirely nontoxic (Figure 5B). Importantly, transfer of GST-Gln248 mutant p53 treated with PRIMA-1 that had not been prewarmed failed to induce cell death (data not shown). As expected, dialyzed prewarmed PRIMA-1 without protein had no effect on Saos-2 cells (Figure 5B). Treatment of the cells with 5 mM NAC before and during protein transfer provided only minor protection against cell death induced by PRIMA-1-treated mutant p53 (data not shown), confirming that PRIMA-1-modified mutant p53 rather than soluble PRIMA-1 is responsible for the biological effect. PRIMA-1-treated GST-Gln248 and GST-His175 mutant p53 induced expression of 14-3-3 protein in Saos-2 cells after 6 hr according to immunostaining results (Figure 5C and data not shown). At 24 hr, we observed a considerable increase in the fraction of cells with G2/M DNA content and a substantial sub-G1 cell population, indicating G2 cell cycle arrest and apoptosis (Figure 5D).

Both PRIMA-1-treated recombinant mutant p53 proteins induced expression of PUMA in Saos-2 cells after 6 hr (Figure 6A). Introduction of PRIMA-1-modified mutant p53:s induced significant Bax protein expression after 6 hr in contrast to untreated mutant p53 or dialyzed prewarmed PRIMA-1 alone

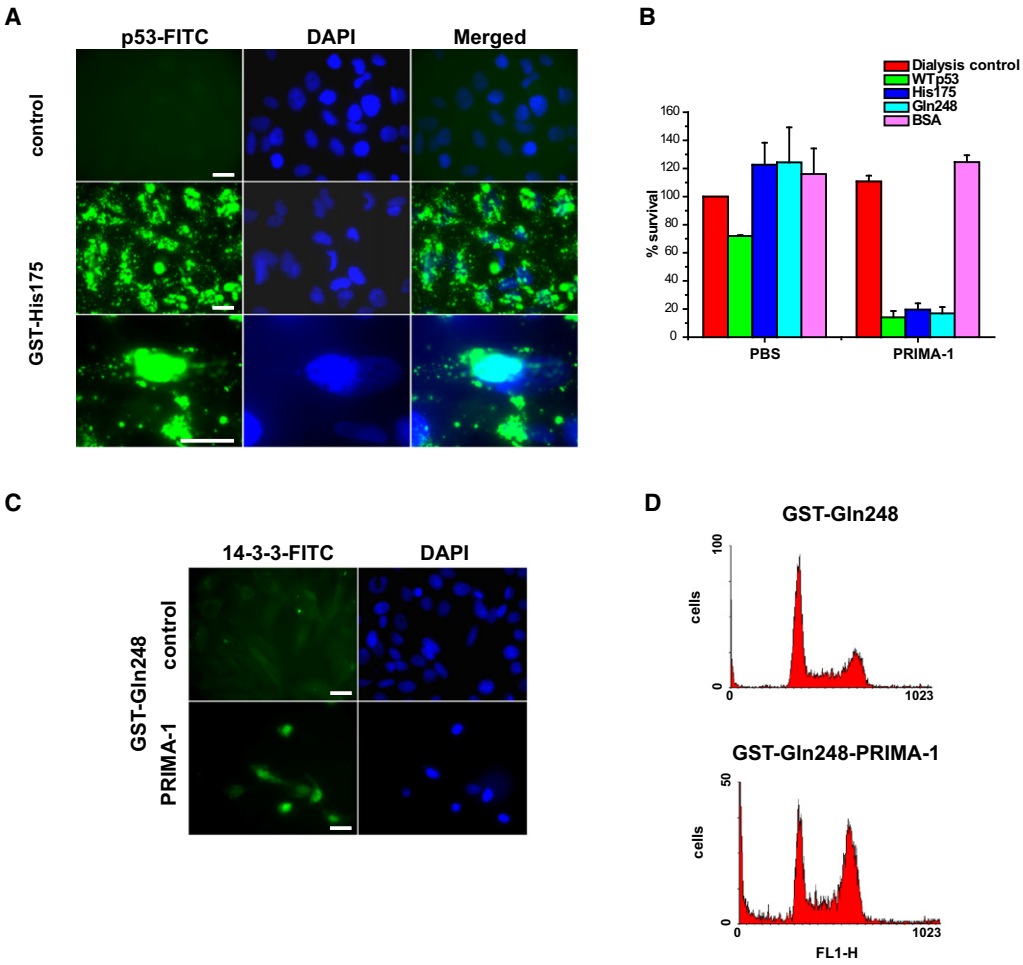

Figure 5. PRIMA-1-Modified Mutant p53 Induces G2/M Arrest and Cell Death in p53 Null Cells

(A) p53 immunostaining of Saos-2 cells upon transfer of GST-His175 mutant p53 protein. Untreated Saos-2 cells were used as control. Scale bars = 100 μm.

(B) Cell survival as determined by counting viable cells after transfer of GST-WT p53, GST-His175, or GST-Gln248 mutant p53 or BSA treated either with PBS or preheated PRIMA-1. Proteins were dialyzed before transfer to remove any unbound PRIMA-1. Data represent average number (mean ± SEM) of unfragmented DAPI-stained cell nuclei from four vision fields (40×) in three independent experiments. GST-WT p53 showed weak cytotoxicity in Saos-2 cells. Transfer of PRIMA-1-treated GST-WT p53, GST-His175, and GST-Gln248 mutant p53 into Saos-2 cells induced substantial cell death, whereas PRIMA-1-treated BSA had no effect.

(C) Induction of 14-3-3 protein by PRIMA-1-treated GST-His175. Scale bars, 100 μm.

(D) FACS analysis of Saos-2 cells after transfer of GST-Gln248 mutant p53 protein treated with either PBS or preheated PRIMA-1 revealed that PRIMA-1-modified recombinant mutant p53 induces DNA fragmentation and G2/M cell cycle arrest.

(Figure 6B). As shown in Figure 6C, reverse-transcriptase polymerase chain reaction (RT-PCR) confirmed induction of Bax at the mRNA level. Introduction of GST-His175 mutant p53 resulted in a strong upregulation of NOXA and a somewhat weaker induction of PUMA and Bax mRNA after 2 hr according to RT-PCR results. GST-WT p53 induced only Bax mRNA, but not as efficiently as GST-His175 mutant p53 (Figure 6D). In agreement with these results, we observed an ~2-fold increase in the capacity of GST-His175 mutant p53 to bind to a p53 consensus DNA binding site after PRIMA-1 treatment, transfer into p53 null cells, and analysis of total cell extracts by the TransAM assay (data not shown). Interestingly, recombinant PRIMA-1-treated GST-His175 mutant p53 protein alone did not show any increased DNA binding as compared with mock-treated recombinant GST-His175 mutant p53. This suggests that cellular factors and/or additional modifications of p53 that are lacking in bacteria are crucial for mutant p53 restoration by PRIMA-1. Indeed, we observed that PRIMA-1-treated GST-His175 is phosphorylated on Ser15 upon introduction into Saos-2 cells according to immunostaining with an antibody specific for Ser15-phosphorylated p53 (data not shown).

We detected a robust caspase activation at 24 hr (Figure 7A). Consistent with our earlier findings, PRIMA-1-treated GST-Gln248 mutant p53 generated ROS in Saos-2 cells (Figure 7B). To extend our results to mutant p53 derived from human tumor cells, we purified His175 mutant p53 from H1299-His175 cells by size-exclusion chromatography, exposed it to preheated PRIMA-1, and dialyzed and transferred the protein into p53

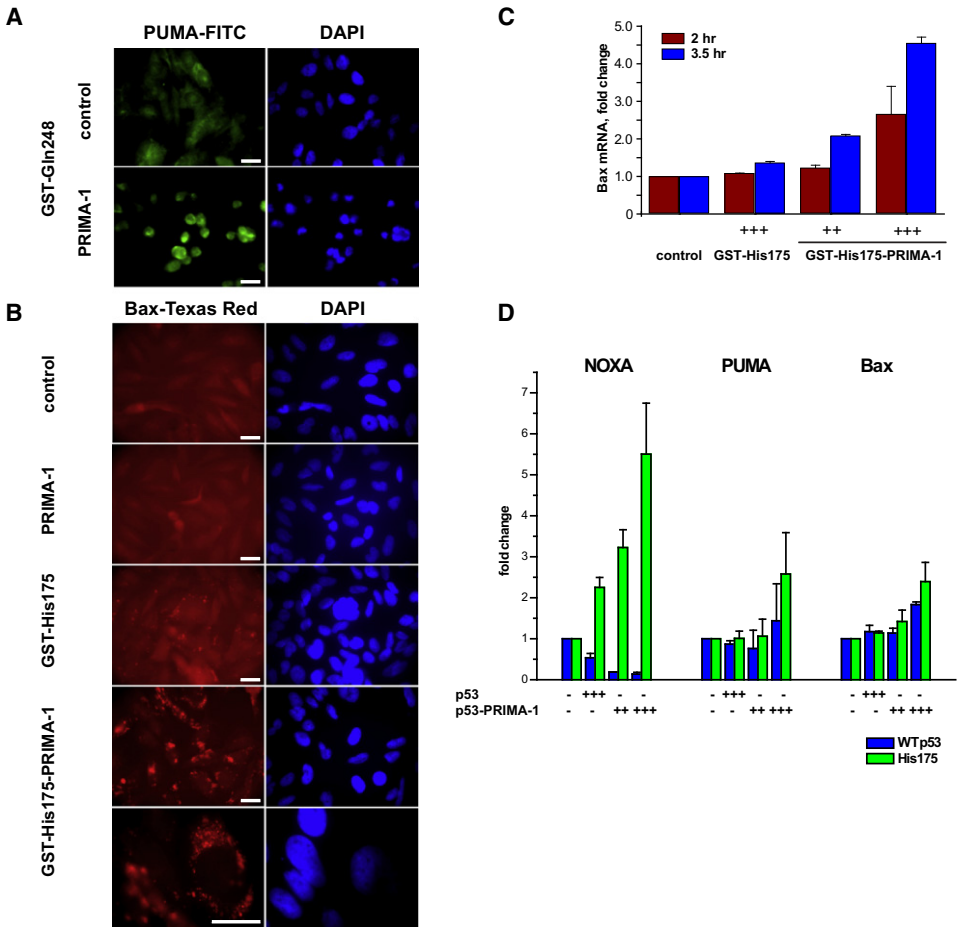

Figure 6. PRIMA-1-Modified Mutant p53 Triggers Activation of Proapoptotic p53 Target Proteins Leading to Apoptosis in p53 Null Cells

(A) Induction of PUMA protein by PRIMA-1-treated GST-Gln248 mutant p53 as shown by immunofluorescence staining. Scale bars = 100 μm.

(B) Bax immunostaining of Saos-2 cells treated with 50 mM dialyzed preheated PRIMA-1, 2 μg GST-His175 mutant p53, or GST-His175 mutant p53 treated with preheated PRIMA-1 demonstrates induction of Bax expression by preheated PRIMA-1-treated GST-His175 mutant p53 but not untreated GST-His175 mutant p53 or preheated PRIMA-1 alone. Scale bars, 100 μm.

(C) GST-His175 mutant p53 treated with preheated PRIMA-1 induces Bax mRNA in Saos-2 cells as shown by RT-PCR. This effect is dependent on the amount of mutant p53 protein. ++ and +++ indicate 5 and 10 μg protein, respectively. Data are shown as mean ± SEM.

(D) PRIMA-1-modified GST-His175 mutant p53 upregulates NOXA, PUMA and Bax mRNA in Saos-2 cells, whereas PRIMA-1-modified GST-WT p53 only induces Bax mRNA. Data are shown as mean ± SEM.

H1299 cells. PI staining and FACS analysis showed that PRIMA-1-treated His175 mutant p53 induced significant cell death in a concentration-dependent manner at 24 hr (Figure 7C). Therefore, modification of mutant p53 per se is sufficient for PRIMA-1-induced tumor cell death.

DISCUSSION

Pharmacological reactivation of mutant p53 should trigger massive cell death and efficiently eliminate tumors. This notion is supported by studies demonstrating that restoration of functional p53 in mouse tumors in vivo rapidly eliminates tumors through apoptosis and/or senescence (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). Screening of chemical libraries has led to the identification of small molecules that restore tumor suppressor activity to mutant p53 (Bykov et al., 2002b, 2005a; Foster et al., 1999), raising hopes for the development of novel efficient cancer therapy. However, the exact molecular mechanism(s) of mutant p53 reactivation by such compounds remains unclear. It is not known whether they actually interact physically with mutant p53.

Our previous studies showed that the ability of the maleimide-derived molecule MIRA-1 to reactivate mutant p53 is linked to its thiol-alkylating activity (Bykov et al., 2005a). Here we demonstrate that PRIMA-1 is converted to reactive products that react with nucleophiles and thus alkylate thiol groups in proteins. CP-31398 harbors a reactive carbon-carbon double bond, raising the possibility that it too can participate in such reactions (Figure 2B). Our finding that growth suppression induced by PRIMA-1 and PRIMA-1^{MET} was completely blocked by the thiol group donor NAC strongly supports the idea that thiol modification is important for their biological activity. We confirmed that the PRIMA-1 and PRIMA-1^{MET} decomposition product MQ, as well as MIRA-1, CP-31398, and STIMA-1 (Zache et al., 2008), form adducts with NAC via its free thiol group, indicating that

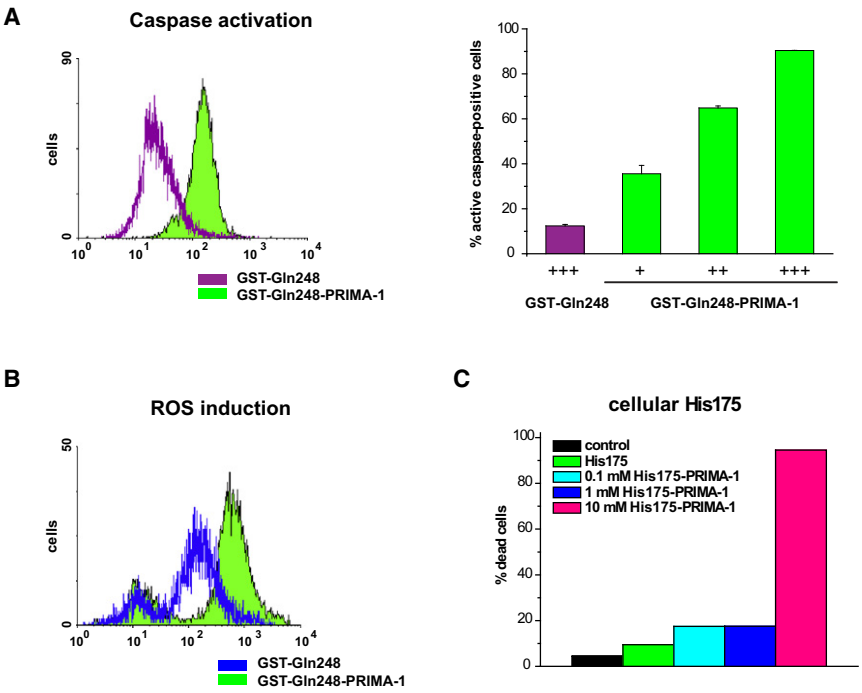

Figure 7. PRIMA-1-Modified Mutant p53 Induces ROS and Triggers Apoptosis in p53 Null Cells

(A) FACS analysis showing that GST-Gln248 mutant p53 treated with preheated PRIMA-1 activates caspases in Saos-2 cells. This effect is dependent on the amount of mutant p53. +, ++, and +++ indicate 0.5, 1, and 2 μg protein, respectively. Data are shown as mean ± SEM.

(B) GST-Gln248 mutant p53 treated with preheated PRIMA-1 induces ROS in Saos-2 cells as shown by DCF staining.

(C) Purified His175 mutant p53 from H1299-His175 cells was treated with PBS or different concentrations of preheated PRIMA-1 and transferred into H1299 cells. FACS-PI analysis showed that transfer of preheated PRIMA-1-treated cellular mutant p53 induces significant cell death.

this reaction accounts for the observed NAC-mediated protection. In contrast, NAC provided only minor protection against chemotherapeutic drugs that do not possess alkylating activity. Thus, although the identified mutant p53-targeting compounds appear structurally different at first glance, they share a common chemical reactivity as Michael acceptors, suggesting a similar molecular mechanism of action.

We confirmed covalent modification of the p53 core domain in vitro by MQ and other decomposition products using MS. Importantly, our immunoprecipitation experiments with radioactively labeled PRIMA-1 indicated that covalent modification of mutant p53 by MQ and/or other degradation products of PRIMA-1 occurs in living cells. Although it is yet to be shown that PRIMA-1 can modify mutant p53 in tumor cells also in vivo, such modification is likely to occur based on our in vitro results and data on in vivo decomposition and distribution of PRIMA-1. We have previously shown that treatment of recombinant mutant p53 with PRIMA-1 restores wild-type conformation (Bykov et al., 2002b). Taken together, these results indicate that covalent modification of one or several cysteine (Cys) residues in the core domain is responsible for restoration of wild-type p53 conformation.

Our data do not allow any conclusions as to which of the 10 Cys residues in the p53 core domain that could be modified by MQ and other PRIMA-1 decomposition products. However, Cys182, Cys229, Cys242, and Cys277 are exposed on the surface of the core and are thus potential targets for modification (Cho et al., 1994; Sun et al., 2003). Additional cysteines are probably exposed in partially or completely unfolded p53 core domains. Thus, mutant p53 might be more amenable to this type of covalent modification than wild-type p53. Indeed, our data show that unfolded p53 is more extensively modified by PRIMA-1 than correctly folded recombinant p53. Similarly, PRIMA-1 binds more efficiently to His175 mutant p53 than to wild-type p53 in cells.

A key question is how alkylation of thiol groups in mutant p53 could restore native conformation to the core. First, formation of inter- and intramolecular thiol-mediated interactions such as disulfide bonds might cause aggregation of mutant p53 and/or lock the core domain in an unfolded conformation. Alkylation might prevent the formation of such disulfide bonds and thus potentially increase the fraction of the protein that is capable of binding DNA and regulate target gene transcription. Second, the formation of adducts in the core domain might create novel DNA contacts, allowing more efficient DNA binding and hence transactivation of p53 target genes. Third, PRIMA-1 adducts might promote correct folding of the core domain by creating additional contacts with amino acids in the core via hydrogen bonding and/or hydrophobic interactions. Analysis of PRIMA-1-modified mutant p53 by X-ray crystallography should ultimately provide information about the structural consequences of cysteine alkylation.

It should also be kept in mind that alkylation of thiols might regulate the activity of redox modulators of p53. Redox regulation of p53 is supported by data from yeast studies in which lack of thioredoxin reductase activity was shown to result in the accumulation of oxidized thioredoxin, leading to p53 oxidation and inactivation. However, in a double yeast mutant deficient for both thioredoxin reductase and thioredoxin, p53 was reduced and could fold into a DNA-binding transcriptionally active conformation (Merwin et al., 2002). In human cells, Ref-1/APE, a thioredoxin-regulated protein implicated in DNA repair and thiol recycling, was shown to participate in the control of p53 DNA binding (Jayaraman et al., 1997; Seemann and Hainaut, 2005). Therefore, PRIMA-1 conversion products like MQ could act by modifying a cascade of redox interactions that are critical for p53 folding, perhaps by targeting and inhibiting electron transfer reactions between p53 and redox regulators such as Ref-1 or thioredoxin.

The redox mechanism suggested by our data is supported by previous studies showing that the compound amifostine (North et al., 2002) activates wild-type p53 by regulating its redox status. WR-1065, the active form of amifostine, was shown to bind directly to p53 (Shen et al., 2001), resulting in restoration of transcription-transactivation activity to Met272 mutant p53 (North

et al., 2002). Furthermore, an independent screen for molecules that inhibit proliferation of cells expressing mutant p53 but not wild-type p53-expressing cells yielded 3-methylene-2-norbornanone, a compound that restores native conformation to mutant p53 in cells similar to PRIMA-1 (Reddy et al., 2004). Of note, a methylene group in 3-methylene-2-norbornanone is associated with a keto-function that is a characteristic feature of Michael acceptors, and this compound has striking structural similarities to MQ, one of the decomposition products of PRIMA-1.

Introduction of PRIMA-1-treated GST-His175 or GST-Gln248 mutant p53 or cellular His175 mutant p53 in human tumor p53 null cells induced a characteristic "wild-type p53-like" biological response. We observed induction of 14-3-3 protein followed by G2/M cell cycle arrest, as well as induction of PUMA and Bax before the onset of apoptosis according to caspase activation and DNA fragmentation. PRIMA-1-treated mutant p53 showed increased binding to a p53 consensus DNA motif. This is fully consistent with the observed upregulation of the p53 transcriptional targets NOXA, PUMA, and Bax at the mRNA level upon introduction of PRIMA-1-modified mutant p53 into cells. Interestingly, GST-WT p53 modified by PRIMA-1 was somewhat less active in upregulation of p53 target genes at the time point analyzed, possibly due to less efficient PRIMA-1 binding to wild-type p53. We also observed that transfer of PRIMA-1-modified mutant p53 induced ROS, consistent with our findings of ROS induction in PRIMA-1-treated mutant p53-expressing tumor cells. These results are in agreement with previous observations demonstrating that wild-type p53-dependent apoptosis involves induction of ROS production (Johnson et al., 1996; Polyak et al., 1997; Sablina et al., 2005), and strongly support the notion that PRIMA-1 restores wild-type function to mutant p53. Thus, our protein transfer experiments demonstrate that modification of mutant p53 by PRIMA-1 decomposition products per se is sufficient to restore wild-type activity to mutant p53 and induce apoptosis in human tumor cells.

Because PRIMA-1 is converted to products that can modify thiol groups, PRIMA-1 treatment could potentially affect multiple cellular targets. How does PRIMA-1 preferentially target mutant p53-expressing tumor cells? First, alkylation of thiols might depend on the particular structural environment at a given thiol group and might be restricted by steric hindrance. Thus, the total number of cellular protein targets is probably limited and there are presumably preferred targets. Second, it is conceivable that thiol alkylation will have different consequences in different proteins; for many proteins, such modification will be functionally neutral or have relatively minor consequences, whereas thiol modification of abundantly expressed mutant p53 will trigger robust apoptosis. Our observation that PRIMA-1-treated mutant p53 but not PRIMA-1-treated BSA is capable of triggering tumor cell apoptosis supports this idea. The general notion of target-selective thiol-modifying compounds is supported by studies of follow-up molecules to Iressa that target the epidermal growth factor (EGF) receptor. Such compounds, e.g., HKI-272, are Michael acceptors that bind covalently to a cysteine residue in the receptor kinase domain (Kwak et al., 2005). Nonetheless, extensive modification of thiols by PRIMA-1 conversion products might induce changes in the cellular milieu that result in p53-independent cell death. This might account for the observed cytotoxic effect of PRIMA-1 at high concentrations, even in tumor cells carrying wild-type p53 or lacking p53.

Our findings raise the question whether MQ could be used directly as a mutant p53-targeting compound. However, MQ is a reactive compound with limited stability at physiological conditions. Administration of MQ might cause side effects due to adduct formation in the extracellular environment. The delivery of MQ in the form of a prodrug, i.e., PRIMA-1, allows more efficient introduction in tumor cells where conversion will generate the active substance. Furthermore, we cannot exclude a role of other decomposition products in the mutant p53-dependent effects of PRIMA-1.

We have demonstrated that PRIMA-1 is converted to compounds that can modify thiol groups. Strikingly, several other known mutant p53 targeting compounds, including WR-1065, and MIRA-1, have been shown modify thiol groups and affect the cellular redox state. This suggests a common mechanism for mutant p53 reactivation among these compounds. We believe that our identification of thiol modification as a mechanism for mutant p53 reactivation by PRIMA-1 will facilitate the design of even more potent mutant p53-selective compounds and ultimately the development of truly efficient drugs for the treatment of cancer.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Cells

Human Saos-2 osteosarcoma and H1299 lung adenocarcinoma cells are p53 null. The sublines Saos-2-His273 and H1299-His175 carry the indicated tetracycline-regulated mutant p53-expression constructs (Tet-off), and H1299-WT carries WT p53 expression construct (Tet-on). Human BL41-tsp53 Burkitt lymphoma and mouse J3D-tsp53 T-lymphoma cells carry a mouse temperature-sensitive (ts) Val135 mutant p53 construct that is expressed as wild-type p53 at 30°C. Human HCT116 colon carcinoma cells carry wild-type p53 ($p53^{+/+}$), and the isogenic HCT116 ($p53^{-/-}$) cells are p53 null. Polyclonal rabbit anti-p53 and anti-Bax antibodies and monoclonal mouse PAB1620 and anti-p53Ser15 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), fluorescein isothiocyanate-conjugated and Texas-Red-conjugated anti-rabbit immunoglobulin were from Vector Laboratories (Burlingame, CA). Western blotting and ELISA with the PAb1620 antibody were performed according to standard procedures. Chariot protein transfer reagent was from Active Motif (Rixensart, Belgium). [^{14}C]-PRIMA-1, labeled randomly at C11 or C9 at one of the methylol groups, was from Amersham (Sweden). All other reagents were of analytical grade and obtained from Sigma.

HPLC Analysis

HPLC was performed on a Beckman instrument operated with System Gold and coupled to a 156 diode array detector module. A Kromasil C_{18} column 4.5 × 250 mm, 5 μm particle size (Phenomenex, Torrance, CA) was used. The separation was performed at ambient temperature employing a gradient elution in which water was mixed with methanol. The elution started with 7% methanol immediately followed by a linear gradient for 35 min up to 40% methanol. Isocratic elution with 40% methanol continued for 5 min followed by a linear gradient to 100% methanol in 5 min. The flow rate was 0.7 ml/min. Products were detected by absorbance at 300 nm by a UV diode array detector. For the experiments with radiolabeled PRIMA-1, fractions were collected every 0.7 min and radioactivity was assessed by scintillation counting.

For analysis of immunoprecipitated p53 from cells, a macroporous mRP-C_{18} column 4.6 × 50 mm (Agilent Technologies, Santa Clara, CA) was used. The separation was performed at 45°C by employing a gradient elution in which solvent A (0.1% TFA in water) was mixed with solvent B (0.08% TFA in acetonitril). The elution started with 3% B immediately followed by a linear gradient for 2 min up to 10% B, then at 5 min up to 70% B in 5 min and at 10 min up to 100% B in 2 min. The flow rate was 0.7 ml/min. Fractions were collected every minute and radioactivity was assessed by scintillation counting.

A BioSep-SEC-S3000 column 7.8 × 300 mm (Phenomenex, Torrance, CA) was used for size-exclusion chromatography. Separation was performed using isocratic elution with 50 mM NaH_2PO_4 (pH 6.7) at a flow rate of 0.9 ml/min. Fractions were collected every 0.5 min and then analyzed for p53 content by ELISA using a standard protocol.

Mass Spectrometry and NMR

Wild-type and mutant p53 (His175) in the context of thermostable t-p53 core domain (residues 94–312) proteins were purified as described previously (Joerger et al., 2005). Proteins were dialyzed into 25 mM sodium phosphate (pH 7.2), 150 mM NaCl, and 1 mM TCEP. Then 1 M PRIMA-1 was heated in the same buffer at 95°C for 30 min. p53 at a concentration of 25 μM was incubated with 0 (control), 1, 2, 5, and 10 mM preheated PRIMA-1 for 2 hr at room temperature (21°C). Samples were diluted 10:1 into water to reduce ionic strength and analyzed on a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, UK). PRIMA-1 was used at concentrations that exceeded the millimolar concentrations of the antioxidants TCEP and DTT in the protein preparation. NMR studies were carried out on a ^{1}H-NMR Varian Gemini 400 MHz instrument.

Decomposition of [^{14}C]-PRIMA-1 in Living Cells

H1299-His175 cells were plated in 24 well plates, 25,000 cells per well. Next-day cells were treated with 50 μM [^{14}C]-PRIMA-1. After 24 hr cells were harvested by trypsinization, and washed and lysed by five times freezing and thawing in a buffer containing 400 mM NaCl, 10 mM HEPES (pH 7.9), 20% glycerol, and 1 mM EDTA. Samples were cleaned from macromolecules by precipitation in 90% ice-cold ethanol followed by centrifugation at 14,000 rpm for 20 min at 4°C. The supernatant was dried in SpeedVac, redissolved in water, and fractionated by reverse-phase HPLC as described above. Fractions were analyzed by liquid scintillation counting.

Decomposition and Distribution of [^{14}C]-PRIMA-1 In Vivo

All animal studies were approved by the local ethical committee in Stockholm, Sweden, and animal care was in accordance with institutional guidelines. Six Balb/c mice were treated with 4.5 mg/kg [^{14}C]-PRIMA-1 by a single intravenous injection. Mice were divided into three groups, two animals in each. The three groups were killed 1, 4, or 24 hr after the injection. Serum and tissues from brain, liver, lungs, kidneys, spleen, and muscle were collected from each group. Additionally, in the third group, urine was collected during the 24 hr period. Organ tissues were homogenized and radioactivity was measured by liquid scintillation counting. Serum and urine were cleaned from macromolecules by precipitation in 90% ice-cold ethanol followed by centrifugation at 14,000 rpm for 20 min at 4°C. The supernatant was dried in a SpeedVac, redissolved in water, and fractionated by reverse-phase HPLC as described above. Fractions were analyzed by liquid scintillation counting.

Binding of [^{14}C]-PRIMA-1 to p53 in Living Cells

H1299 and H1299-His175 cells were plated at 15,000 cells/cm^2, incubated overnight, and treated with [^{14}C]-PRIMA-1 (106 mCi/mmol) for 24 hr. Cells were harvested and lysed 30 min on ice in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 2 mM PMSF, and 1% protease inhibitor cocktail. One hundred and fifty micrograms of protein for each sample were precleared by adding 10 μl Dynabeads protein A (Invitrogen, Sweden). Anti-p53 rabbit polyclonal antibody (2.5 μg) was added to each sample and incubated overnight at 4°C. Immunoprecipitation was carried out by adding 10 μl beads to each sample and incubating 1 hr on a rotating wheel at 4°C. Beads were washed three times in lysis buffer, and transferred to 10 ml scintillation liquid for counting radioactivity in a Packard 2000CA Tri-Carb Liquid Scintillation Analyzer (United Technologies Packard, CA). Alternatively, p53 was eluted from beads with 0.1 M citric acid (pH 3.1), followed by western blotting and liquid scintillation counting as described above.

Binding of [^{14}C]-PRIMA-1 to Recombinant GST-p53

Recombinant GST-WT p53 was incubated at different temperatures and cooled on ice for 10 min. [^{14}C]-PRIMA-1 was dissolved in 10 mM Tris-HCl (pH 7.5) and heated at 90°C for 20 min, cooled on ice for 15 min, and then added to the protein samples at final concentration of 10 μM. The samples were incubated on ice for 20 min. Reactions were stopped by incubation with 1 mM NAC for 5 min on ice. p53 protein was precipitated with ice-cold ethanol by incubation for 15 min at −20°C. Samples were centrifuged at 14,000 rpm at 4°C for 15 min and the pellets washed twice with ethanol. Finally, samples were dissolved in PBS by incubation at 40°C for 30 min and transferred to the scintillation vials, and radioactivity was measured by liquid scintillation counting.

Formaldehyde Release Assay

One hundred nanomoles of PRIMA-1 and PRIMA-1^{MET} was diluted with PBS and incubated at 37°C for up to 3 days. The amount of formaldehyde was assessed with the Purpald reagent as described elsewhere (Quesenberry and Lee, 1996).

Protein Transfer

Recombinant GST-His175 mutant p53 protein was purified from bacteria using the Pierce protein extraction kit (Pierce, Rockford, IL). The protein was treated for 2 hr at room temperature with either PBS or preheated PRIMA-1, dialyzed against 3 × 800 ml PBS for 24 hr at 4°C and transferred into Saos-2 cells with the Chariot protein transfer reagent (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol. His175 mutant p53 was purified from H1299-His175 cells by SEC-HPLC, the collected fractions were analyzed by ELISA for p53 content. Fractions contained p53 were pooled. pH was adjusted to 7.5 and p53 was treated either with PBS or with pre-warmed PRIMA-1. Then samples were dialyzed against 3 × 800 ml PBS for 24 hr at 4°C. The obtained material was used for Chariot-mediated protein transfer into Saos-2 cells.

Determination of Free Thiol Groups

BSA or recombinant GST-His175 mutant p53 were treated with test compounds or PBS on ELISA plates for 1 hr at RT, washed 3 times with PBS and then incubated with 10 μM of maleimide-biotin conjugate (Pierce, Rockford, IL). The wells were washed 3 times with PBS, blocked with 5% skim milk for 1 hr at RT, probed with avidine-HRP conjugate (Pierce, Rockford, IL) diluted in 5% skim milk for 30 min, and washed 6 times with PBS. The signal was developed with TMB HRP substrate (Pierce, Rockford, IL). Samples were treated with HCl and absorbance was measured at 490 nm in an ELISA reader.

Flow Cytometry

For DNA fragmentation assays, cells were grown on 24-well plates at an initial density of 40000 cells/well, treated, fixed, stained with propidium iodide and analyzed on a FACScan flow cytometer (Becton Dickinson, CA) according to standard procedures. For caspase activation assay cells were labeled with FLICA reagent (CaspaTag TM Pan Fluorescein Caspase activity kit, Intergen, UK) according to the manufacturer's instructions. Samples were analyzed on a FACScan.

TransAM Assay

Cells were placed in six-well plates at a density of 200,000 cells per well, incubated overnight, and then treated with recombinant protein. After 2 hr, cells were harvested by trypsinization. Equal amount of total nuclear protein was loaded on to a 96-well plate coated with an immobilized oligonucleotide containing a p53 consensus binding site (TransAM, p53 Transcription Factor Assay Kit, Active Motif, Belgium). Anti-p53 and anti-rabbit HRP were used to quantify the amount of bound p53 protein. The HRP signal was developed by a substrate provided by the manufacturer and samples were analyzed by ELISA reader at 450 nm.

Real-Time PCR

Cells were treated with PRIMA-1 and harvested after 2 hr or 3.5 hr. RNA was extracted using the RNeasy Mini Kit (QIAGEN, Sweden) according to the manufacturer's instructions. cDNA was synthesized according to standard procedures and 100 ng cDNA was added to 10 μl 2x TaqMan Universal PCR Master Mix (Applied Biosystems, CA) and 1 μl 20x TaqMan Gene Expression Assay Hs00414514_m1 (Bax), Hs00560402_m1 (Noxa/PMAIP1), Hs01080223_m1 (Puma/BBC3), or Hs99999905_m1 (GAPDH). Amplification consisted of a first step with 2 min at 50°C and 10 min at 95°C followed by 40 cycles with 15 s denaturation at 95°C and 1 min annealing/extension at 60°C. Reactions were performed in 96-well optical PCR plates (Applied Biosystems, CA) using the ABI PRISM 7700 Sequence Detection System (Applied

Biosystems, CA). Results were analyzed with the comparative Ct method using GAPDH as the endogenous control.

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