Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound

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The tumor suppressor p53 inhibits tumor growth primarily through its ability to induce apoptosis. Mutations in p53 occur in at least 50% of human tumors. We hypothesized that reactivation of mutant p53 in such tumors should trigger massive apoptosis and eliminate the tumor cells. To test this, we screened a library of low-molecular-weight compounds in order to identify compounds that can restore wild-type function to mutant p53. We found one compound capable of inducing apoptosis in human tumor cells through restoration of the transcriptional transactivation function to mutant p53. This molecule, named PRIMA-1, restored sequence-specific DNA binding and the active conformation to mutant p53 proteins *in vitro* and in living cells. PRIMA-1 rescued both DNA contact and structural p53 mutants. *In vivo* studies in mice revealed an antitumor effect with no apparent toxicity. This molecule may serve as a lead compound for the development of anticancer drugs targeting mutant p53.

The tumor suppressor p53 triggers cell-cycle arrest and apoptosis in response to diverse stress stimuli, including DNA damage, oncogene activation and hypoxia[1–4]. At least 50% of human tumors carry mutant p53 (ref. 5). The fact that the specific DNA binding function of p53 is disrupted in most tumor-derived p53 mutants indicates that this function is critical for p53-mediated tumor suppression. p53-dependent apoptosis seems to have a major role for the efficacy of cancer chemotherapy[6], and tumors carrying mutant p53 are often more resistant to chemotherapy than tumors carrying wild-type p53 (ref. 7).

Tumor cells are likely to be particularly sensitive to p53 reactivation. First, tumor cells are sensitized to apoptosis due to oncogene activation[8]. Second, mutant p53 proteins tend to accumulate at high levels in tumor cells, mainly due to failure of mutant p53 to transactivate mouse double minute-2 (MDM2) whose product induces p53 degradation. Therefore, reactivation of abundant mutant p53 may trigger a massive apoptotic response in tumor cells, whereas normal cells that express minute levels of p53 should not be affected.

Various strategies have been designed to restore function to mutant p53 (ref. 9). The introduction of second-site suppressor mutations can at least partially restore specific DNA binding and/or stabilize the folding of the protein[10–12]. Synthetic peptides derived from the p53 C-terminus can restore the specific DNA binding and transactivation function to mutant p53 and induce p53-dependent apoptosis in tumor cells[13–15]. We sought to identify small molecules with similar activity by screening of a chemical library, and present here a novel compound, PRIMA-1, that can restore sequence-specific DNA-binding, wild-type conformation and transcriptional transactivation to mutant p53. PRIMA-1 induced apoptosis in human tumor cells in a p53-dependent manner and suppressed the growth of human tumor xenografts carrying mutant p53.

Identification of PRIMA-1

To identify compounds that could suppress the growth of human tumor cells in a mutant p53–dependent manner, we established an assay based on Saos-2-His-273 cells carrying tetracycline-regulated mutant p53 (Tet-Off). We treated cells with 25 μM of compounds in the presence or absence of doxycycline and monitored cell growth using the WST-1 proliferation reagent. We identified one compound that suppressed the growth of Saos-2-His-273 cells in a mutant p53–dependent manner (Fig. 1*a*). This compound, 2,2-bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one, was designated PRIMA-1 (p53 reactivation and induction of massive apoptosis) (Fig. 1*b*). In subsequent experiments, we showed that PRIMA-1 could inhibit growth of several other human tumor-cell lines carrying tetracycline-regulated mutant p53, including SKOV-His-175, SKOV-His-273 and H1299-His-175 (Fig. 1*c*). As the effective concentration of PRIMA-1 is proportional to the number of treated cells, we used different concentrations in the assays described below.

We next analyzed the response to PRIMA-1 of a panel of human tumor-cell lines with different p53 status (p53-null, wild-type p53 and mutant p53), representing various tumor types including colon, lung, ovarian and renal carcinoma, and Burkitt lymphoma (see Supplementary Table A on the supplementary information page of *Nature Medicine* online). The

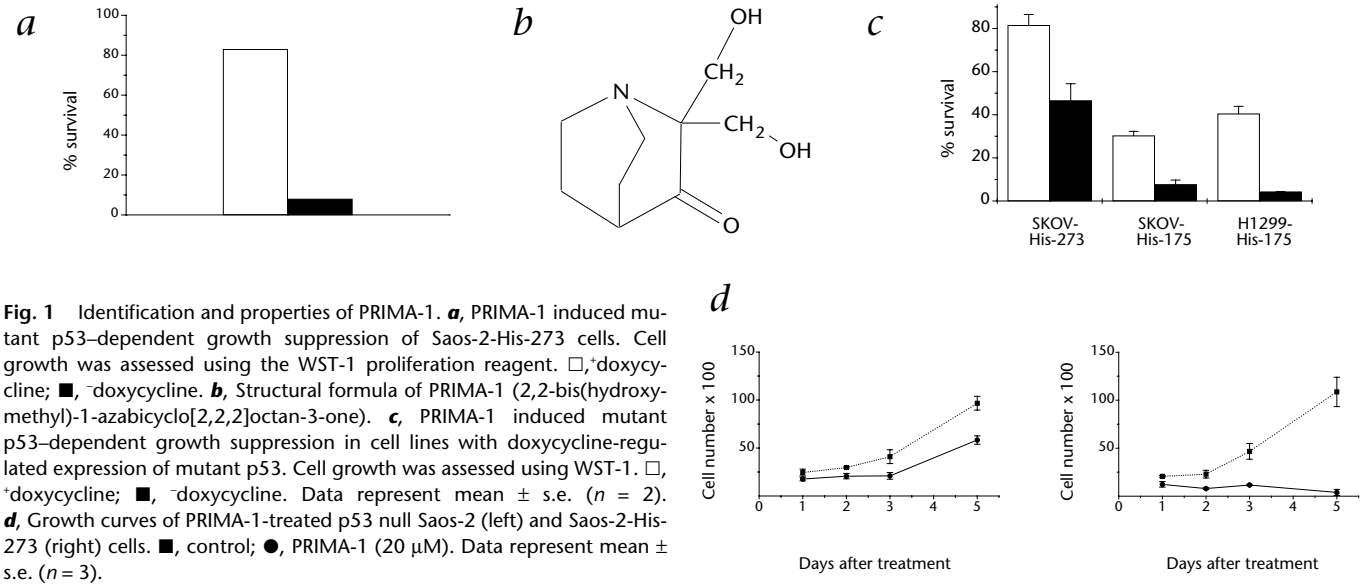


Fig. 1 Identification and properties of PRIMA-1. ***a***, PRIMA-1 induced mutant p53–dependent growth suppression of Saos-2-His-273 cells. Cell growth was assessed using the WST-1 proliferation reagent. □,⁺doxycycline; ■, ⁻doxycycline. ***b***, Structural formula of PRIMA-1 (2,2-bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one). ***c***, PRIMA-1 induced mutant p53–dependent growth suppression in cell lines with doxycycline-regulated expression of mutant p53. Cell growth was assessed using WST-1. □, ⁺doxycycline; ■, ⁻doxycycline. Data represent mean ± s.e. (n = 2). ***d***, Growth curves of PRIMA-1-treated p53 null Saos-2 (left) and Saos-2-His-273 (right) cells. ■, control; ●, PRIMA-1 (20 μM). Data represent mean ± s.e. (n = 3).

growth suppression effect of different concentrations of PRIMA-1 was assessed using the WST-1 proliferation reagent and compared with that of two anticancer drugs, 5-fluorouracil (5-FU) and doxorubicin. The IC_{50} (concentration of a drug that causes 50% growth inhibition) values for PRIMA-1 varied depending on tumor type. However, comparison between lines of a given tumor type showed that the effect of PRIMA-1 was dependent on mutant p53. In contrast, 5-FU inhibited growth more efficiently in tumor cells carrying wild-type p53 (ref. 18). The effect of doxorubicin was independent of p53 status. Notably, PRIMA-1 had no significant growth-inhibitory effect on non-transformed diploid human fibroblasts.

Analysis of the growth of treated p53 null Saos-2 and Saos-2-His-273 cells over five days showed that PRIMA-1 completely inhibited growth of cells expressing mutant p53, but only caused a minor reduction in growth rate in the absence of mutant p53 expression (Fig. 1*d*).

Restoration of p53-dependent apoptosis

We used FACS and TdT-mediated dUTP nick-end labeling (TUNEL) analyses to determine whether PRIMA-1-induced growth suppression was due to induction of apoptosis. Treatment with 125 μM PRIMA-1 for 48 hours caused a substantial increase in the fraction of cells with a sub-G1 DNA content in the presence of mutant p53 (Fig. 2*a* and *b*), indicating DNA fragmentation and cell death. TUNEL staining of PRIMA-1-treated Saos-2-His-273-cells revealed TUNEL-positive nuclei, indicating cell death by apoptosis (Fig. 2*c*). Moreover, pretreatment with the caspase inhibitor Z-DEVD-FMK reduced PRIMA-1-induced cell death 3-fold and the caspase inhibitor BOC-D-FMK completely abolished PRIMA-1-induced cell death, strongly suggesting that PRIMA-1 triggers cell death by apoptosis.

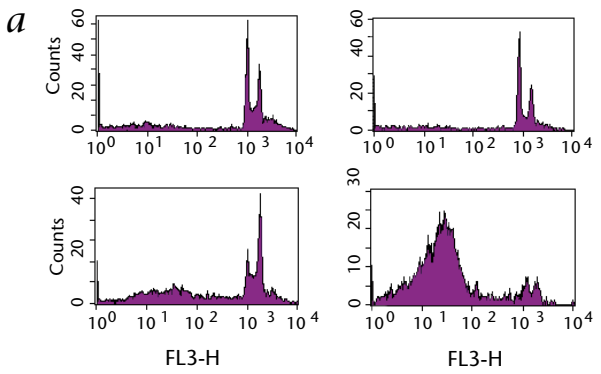
Restoration of wild-type p53 conformation and DNA binding

We next investigated whether PRIMA-1 can restore the proper folding of p53, using the conformation-specific monoclonal antibodies PAb1620 and PAb240 in an ELISA. After incubation of recombinant Glutathione-S-Transferase (GST)–wild type p53 protein with PRIMA-1, we observed a 34% increase in the PAb1620-positive fraction accompanied by a 10% decrease in the PAb240-positive fraction (Fig. 3*a*). Moreover, PRIMA-1 preserved the PAb1620 epitope in the recombinant wild-type and His-175 mutant p53 proteins during incubation at 37 °C for 30 minutes (Fig. 3*b*). Importantly, our results demonstrated that PRIMA-1 prevented unfolding of p53 proteins as measured by the appearance of the PAb240 epitope upon heating at 37 °C. The non-conformational DO-1 epitope in the N-terminus of p53 remained unchanged during PRIMA-1 treatment.

Furthermore, we observed a 46% increase in the PAb1620-positive p53 fraction in lysates from SKOV-His-175 cells treated with 150 μM of PRIMA-1 (data not shown). PRIMA-1 treatment of the same cells also caused the appearance of PAb1620-positive immunostaining (Fig. 3*c*). These results demonstrate that PRIMA-1 can restore the wild-type conformation to mutant p53 both *in vitro* and in living cells. Notably, PRIMA-1 treatment of SKOV-His-175 cells resulted in a decrease in total p53 levels according to staining with polyclonal anti-p53 antibodies.

We investigated whether PRIMA-1 could modulate the specific DNA binding of wild-type and mutant p53 proteins. Increasing concentrations of PRIMA-1 were capable of preserving the specific DNA binding of recombinant GST-wild-type p53 during incubation of the protein at 37 °C in a bandshift assay (Fig. 4*a*). PRIMA-1 also reactivated the sequence-specific DNA binding of endogenous His-175 mutant p53 protein in a CW678-cell extract (Fig. 4*b*). Likewise, the sequence-specific DNA binding of endogenous Trp-282 mutant p53 in BL-60 cells was rescued by PRIMA-1 (Fig. 4*c*). The monoclonal antibodies PAb421 and PAb1801 were added to supershift p53–DNA complexes. Neither antibody alone caused any stimulation of specific DNA binding of mutant p53. The position of the wild-type p53–DNA–PAb421 complex is shown in Fig. 4*b* (lane 7).

We further tested the ability of PRIMA-1 to restore the specific DNA binding of a broad series of p53 mutants using cell extracts of human tumor-cell lines as the source of p53. PRIMA-1 restored the specific DNA binding to 13 of 14 p53 mutants tested in bandshift assays (Supplementary Table B). The only exception was the Phe-176 p53 mutant from KRC/Y cells.



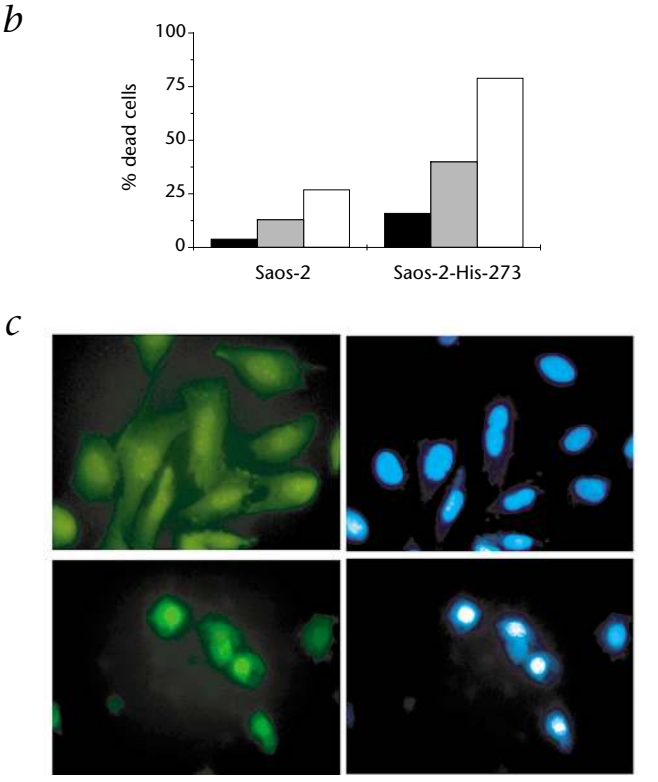


Fig. 2 PRIMA-1 induces cell death by apoptosis. ***a***, FACS profiles of control (top) and PRIMA-1-treated (bottom) Saos-2 (p53-null; left) and Saos-2-His-273 (His-273 mutant p53; right) cells. ***b***, Cell death induced by different concentrations of PRIMA-1 is mutant p53-dependent as shown by FACS analysis. ■, 50 μM; ■, 75 μM; □, 125 μm. ***c***, TUNEL (left) and Hoeschst (right) staining of control (top) and PRIMA-1-treated (bottom) Saos-2-His-273 cells.

Notably, KRC/Y cells were resistant to PRIMA-1-induced growth suppression (Supplementary Table A).

Transcriptional transactivation restored to mutant p53

We addressed the question whether PRIMA-1 could also restore the transcriptional transactivation function to mutant p53 in living cells. Treatment of A431 cells that carry endogenous His-273 mutant p53 and a stably transfected p53-responsive LacZ reporter with 50 μM PRIMA-1 for 20 hours resulted in the appearance of LacZ-positive cells whereas untreated cells were LacZ-negative (Fig. 5*a*). A strong induction of a p53-responsive enhanced green fluroscent protein (EGFP) reporter was seen in SKOV-His-175 cells expressing mutant p53 after treatment with PRIMA-1 for 24 hours (Fig. 5*b*). In contrast, PRIMA-1-treated SKOV-His-175 cells grown in the presence of doxycycline (p53 off) did not express detectable levels of EGFP.

PRIMA-1 induced the expression of two endogenous p53 target genes, p21 and MDM2, in H1299-His-175 cells expressing mutant p53 and in SW480 colon carcinoma cells expressing endogenous His-273 mutant p53 (Fig. 5*c* and *e*). PRIMA-1 did not cause any induction of MDM2 nor p21 in the absence of mutant p53 expression (Fig. 5*d*). PRIMA-1 did not induce MDM2 nor p21 expression in HCT116 colon carcinoma cells that express wild-type p53 (Fig. 5*e*). 5-FU did not affect p21 nor MDM2 levels in H1299-His-175 (Fig. 5f) and SW480 cells (data not shown).

PRIMA-1-induced apoptosis depends on p53 transactivation

To further ascertain that PRIMA-1 exerts its effect through p53-mediated transcriptional transactivation and *de novo* protein synthesis, we tested the effect of cycloheximide on PRIMA-1-induced growth inhibition. Pretreatment of SKOV-His-175 cells with cycloheximide before addition of PRIMA-1 caused a four-fold increase in cell survival according to the WST-1 proliferation assay. Moreover, SKOV cells carrying tetracycline-regulated His-175-22/23 mutant p53 that has an inactivated transactivation domain were resistant to PRIMA-1 at a range of concentrations, in contrast to SKOV-His-175 cells (Fig. 5*g*). These results indicate that transcriptional transactivation by p53 is critical for PRIMA-1-induced cell death.

Antitumor activity of PRIMA-1 *in vivo*

Intravenous (i.v.) injections of PRIMA-1 in mice did not cause any obvious changes in weight or behavior compared with untreated animals. The average weight of mice treated with PRIMA-1 at the highest dose (100 mg/kg) was 20 g after 1 month of observation, the same as that of control mice. To assess the effect of PRIMA-1 on human tumor xenografts, we inoculated mice with Saos-2-His-273 cells expressing mutant p53. The animals received intratumor (20 mg/kg) or i.v. (20 or 100 mg/kg) injections of PRIMA-1 twice a day for three days. In the control group, the average tumor volume after 59 days was 555.7 mm^3. Mice that received i.v. injections of PRIMA-1 at a dose of 100 mg/kg or 20 mg/kg had an average tumor volume of 11.7 mm^3 and 53 mm^3, respectively (Fig. 6a). Intratumor injections of 20 mg/kg of PRIMA-1 caused a decrease in average tumor volume to 5.3 mm^3. The differences in tumor volumes are statistically significant (P = 0.0026 for intratumor injection, P = 0.003 for i.v. injection). Thus, PRIMA-1 has *in vivo* antitumor activity in this animal tumor model.

To assess *in vivo* reactivation of mutant p53, we examined whether PRIMA-1 could induce p53 target genes in human tumor xenografts. Treatment of SW480 tumor xenografts with PRIMA-1 resulted in induction of both MDM2 and p21 at 16 hours post-treatment (Fig. 6b), in accordance with our *in vitro* results (Fig. 5e).

We next compared the tumor suppressor effect of PRIMA-1 *in vivo* in the presence or absence of mutant p53 expression using mice inoculated with Saos-2 and Saos-2-His-273 cells in the right and left flanks, respectively. We observed a significant decrease in the volumes of Saos-2-His-273 xenografts after PRIMA-1 treatment; the average tumor volume was 48 mm^3 compared to 322 mm^3 in control mice. In contrast, Saos-2 xenografts were not significantly affected; average

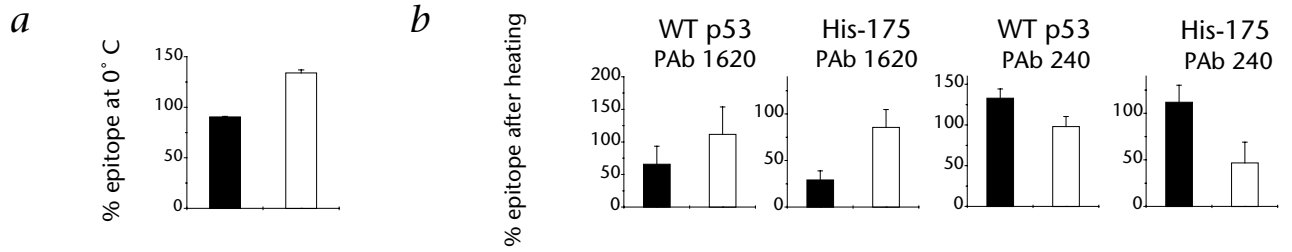
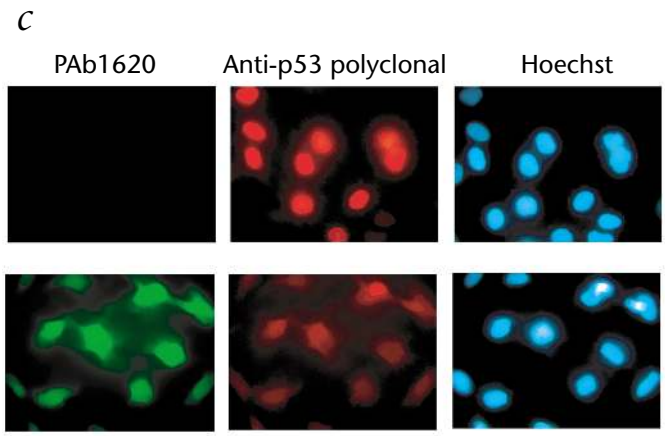


Fig. 3 Effect of PRIMA-1 on the conformation of mutant p53 proteins. ***a***, PRIMA-1 induced the wild-type p53-specific PAb1620 (□) epitope in GST-wild-type p53 protein incubated at 0 °C as detected by ELISA and slight decrease in the PAb240 (■) epitope. ***b***, PRIMA-1 prevented loss of the PAb1620 epitope and gain of the PAb240 epitope upon heating of GST-wild-type p53 and GST-His-175 mutant p53. ■, untreated; □, PRIMA-1 treated. ***c***, PRIMA-1 (100 μM) induced the wild-type p53-specific PAb1620 epitope in SKOV-His-175 cells as shown by PAb1620 immunostaining.



tumor volumes in the PRIMA-1-treated and control mice were 703 mm^3 and 981 mm^3, respectively (Fig. 6*c*). The differences in tumor volumes are statistically significant ($P = 0.008$ for Saos-2-His-273, $P = 0.04$ for Saos-2). Histological examination of tumor sections revealed induction of morphological changes in PRIMA-1-treated Saos-2-His-273 xenografts, including tumor-cell depletion and absence of vascularization, whereas PRIMA-1 did not cause any significant morphological changes in Saos-2 xenografts (Fig. 6*d*). Thus, PRIMA-1 suppressed *in vivo* tumor growth in a mutant p53–dependent manner.

Discussion

Our previous studies provided compelling evidence that the specific DNA binding, transactivation and apoptosis-inducing functions of several mutant p53 proteins could be reactivated by a synthetic peptide derived from the p53 C-terminal domain[13,15]. These findings prompted us to search for even more potent p53 reactivating molecules. We report here the identification of a small molecule, PRIMA-1, that can restore the tumor suppressor function to tumor-derived mutant p53 proteins.

We identified PRIMA-1 by screening a chemical library for compounds that selectively inhibit the growth of tumor cells in the presence of mutant p53 expression. Our approach allowed identification of compounds that can target p53 in the context of multiple cellular proteins. Moreover, compounds were selected for their ability to rescue the growth suppressor function

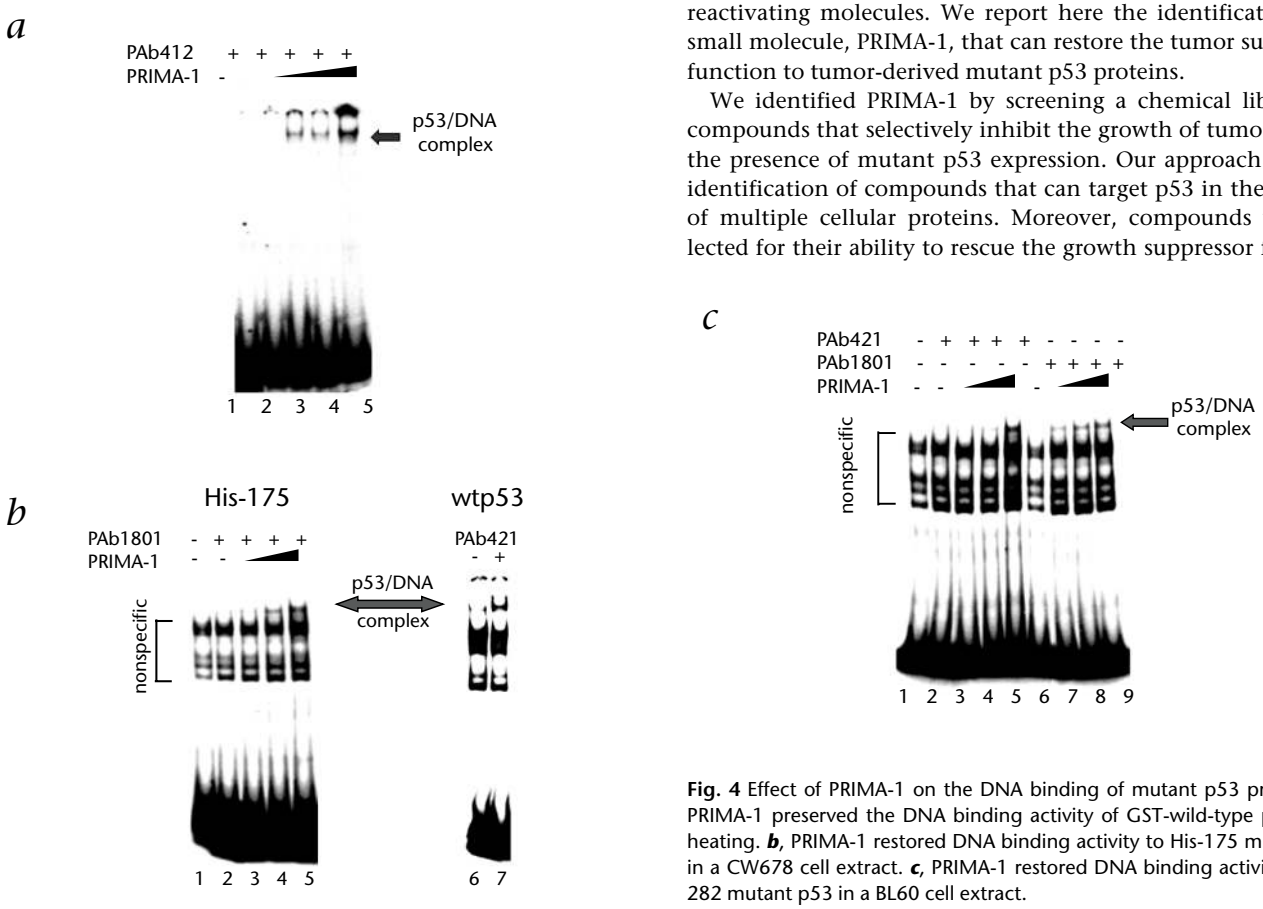


Fig. 4 Effect of PRIMA-1 on the DNA binding of mutant p53 proteins. ***a***, PRIMA-1 preserved the DNA binding activity of GST-wild-type p53 upon heating. ***b***, PRIMA-1 restored DNA binding activity to His-175 mutant p53 in a CW678 cell extract. ***c***, PRIMA-1 restored DNA binding activity to Trp-282 mutant p53 in a BL60 cell extract.

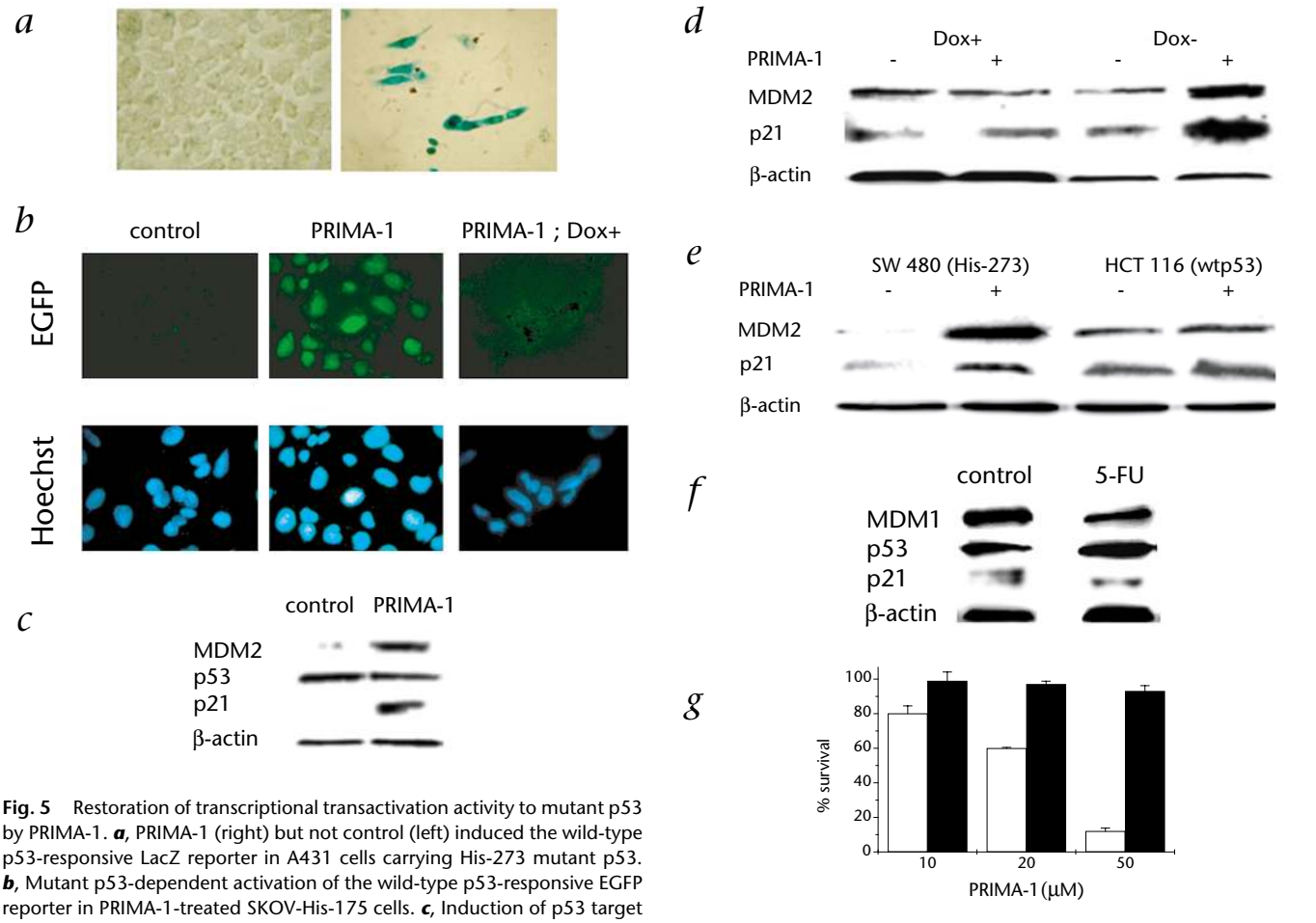


Fig. 5 Restoration of transcriptional transactivation activity to mutant p53 by PRIMA-1. ***a***, PRIMA-1 (right) but not control (left) induced the wild-type p53-responsive LacZ reporter in A431 cells carrying His-273 mutant p53. ***b***, Mutant p53-dependent activation of the wild-type p53-responsive EGFP reporter in PRIMA-1-treated SKOV-His-175 cells. ***c***, Induction of p53 target genes in PRIMA-1-treated H1299-His-175 cells. ***d***, Mutant p53-dependent induction of p53 target genes in H1299-His-175 cells. ***e***, Induction of p53 target genes in PRIMA-1-treated SW480 colon carcinoma cells carrying endogenous His-273/Ser-309 mutant p53. PRIMA-1 did not induce the same p53 target genes in HCT-116 colon carcinoma cells carrying endogenous wild-type p53. ***f***, Treatment with 5-FU did not induce the p53 target genes p21 and MDM2 in H1299-His-175 cells. Protein levels were determined by western-blotting in *c*, *d*, *e* and *f*. ***g***, Effect of PRIMA-1 on SKOV cells carrying the transcriptionally inactive His-175-22/23 mutant p53. ■, SKOV-His-175-22/23; □, SKOV-His-175.

of p53 in living cells. Compounds with nonspecific toxic effects and compounds that do not enter cells should not score in the assay. This screening approach may lead to further identification of compounds that reactivate mutant p53 through previously unknown mechanisms.

We verified the ability of PRIMA-1 to induce apoptosis in a mutant p53–dependent manner using a panel of cell lines with tetracycline-regulated expression of the most common p53 mutant proteins, as well as tumor-cell lines of various origin carrying p53 deletion or different endogenous mutant p53 proteins. The correlation between p53 status and the effect of PRIMA-1 supports the conclusion that PRIMA-1 selectively suppresses tumor-cell growth by inducing apoptosis in cells expressing mutant p53.

Our finding that PRIMA-1 can restore the DNA binding to 13 of 14 p53 mutants tested in bandshift assays corroborates our growth suppression data and demonstrates that both structural and DNA contact mutants can be rescued. It is particularly noteworthy that PRIMA-1 can reactivate His-175 mutant p53, which has extensive structural defects[19]. However, one mutant, Phe-176, was not reactivated by PRIMA-1. A probable explanation is that the substitution of Cys-176, one of the four Zn ligands, results in loss of the Zn atom, which is crucial for shaping the DNA-binding domain of p53 (ref. 19). It seems unlikely that this particular defect can be reversed. The observation that PRIMA-1 did not inhibit growth of renal carcinoma KRC/Y cells carrying Phe-176 mutant p53 supports the data from our bandshift assays.

We further confirmed the reactivation of DNA binding by PRIMA-1 in living cells using EGFP and LacZ reporter-based assays with both endogenous and ectopically expressed mutant p53. Moreover, PRIMA-1 induced the endogenous p53 target genes p21 and MDM2 exclusively in the presence of mutant p53 expression. Interestingly, we noticed a reduction in the total levels of p53 protein and a cytoplasmic relocalization of p53 24 h after treatment of cells with PRIMA-1, as shown by immunofluorescence staining and western-blotting (Fig. 3*c* and data not shown). This would be expected if PRIMA-1-mediated reactivation of mutant p53 induces expression of MDM2 that targets p53 for degradation in the cytoplasm. However, the amount of mutant p53 reactivated by PRIMA-1 was apparently sufficient to trigger apoptosis before its degradation by MDM2.

The 22/23 double mutation in the N-terminal transactivation domain abolishes transcriptional transactivation by wild-type p53 (ref. 20). We found that cells expressing His-175 mutant p53 with the same double mutation were substantially less sensitive

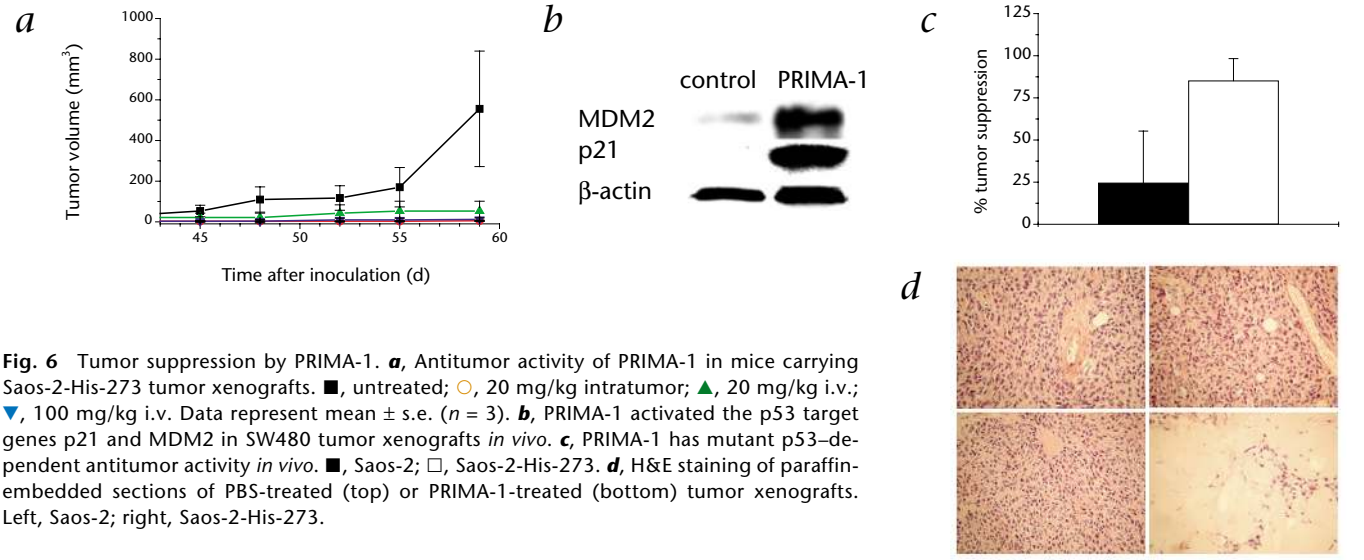


Fig. 6 Tumor suppression by PRIMA-1. ***a***, Antitumor activity of PRIMA-1 in mice carrying Saos-2-His-273 tumor xenografts. ■, untreated; ○, 20 mg/kg intratumor; ▲, 20 mg/kg i.v.; ▼, 100 mg/kg i.v. Data represent mean ± s.e. (n = 3). ***b***, PRIMA-1 activated the p53 target genes p21 and MDM2 in SW480 tumor xenografts *in vivo*. ***c***, PRIMA-1 has mutant p53–dependent antitumor activity *in vivo*. ■, Saos-2; □, Saos-2-His-273. ***d***, H&E staining of paraffin-embedded sections of PBS-treated (top) or PRIMA-1-treated (bottom) tumor xenografts. Left, Saos-2; right, Saos-2-His-273.

to PRIMA-1-induced growth suppression. In addition, pretreatment with cycloheximide abrogated PRIMA-1-induced growth suppression, indicating the requirement for *de novo* protein synthesis. We therefore conclude that restoration of specific DNA binding and transcriptional transactivation function to mutant p53 is critical for induction of apoptosis by PRIMA-1.

The molecular mechanism of mutant p53 reactivation is largely unknown. Structural studies of the mutant p53 core domain predicted that Ser-245 mutant p53 is the most promising with regard to pharmacological rescue whereas globally denatured β-sandwich and Zn-region mutants should be refractory to reactivation[21]. However, experimental data suggest that different types of p53 mutants can be functionally restored[9,22]. It is conceivable that structural analysis of the isolated core domain provides insufficient information because sequences outside the core domain may significantly affect p53 activity[23–28]. This suggests that screening for molecules that only interact with the p53 core domain would be inadequate for the identification of mutant p53–reactivating drugs. Our screening approach should permit the selection of compounds that may have effects on the domains adjacent to the core as well.

Our results demonstrating that PRIMA-1 can restore wild-type conformation to recombinant mutant p53 and preserve the DNA binding of wild-type p53 in the absence of cellular proteins suggest that PRIMA-1 interacts directly with p53. However, it is also possible that PRIMA-1 reactivates p53 by other mechanisms. Further studies—including nuclear magnetic resonance imaging and/or X-ray crystallography analysis—will be required to address this question.

The mechanism behind PRIMA-1-mediated restoration of mutant p53 function, albeit incompletely understood, is fundamentally different from the stabilization of the wild type–specific PAb1620 epitope achieved by CP31398 (ref. 29). Addition of PRIMA-1 to cellular extracts restored the DNA binding to mutant p53 in bandshift assays and PAb1620$^+$ conformation in ELISA, suggesting that PRIMA-1 acts on previously unfolded protein. Whereas CP31398 confers protection from thermal denaturation, PRIMA-1 converts the mutant form of the protein into a properly folded active form. Therefore, these two compounds may have entirely different effects in living cells. Whereas PRIMA-1 will force already accumulated mutant p53 to adopt an active conformation, the effect of CP31398 will be restricted to newly synthesized p53.

Comparison of tumor volumes and histological sections of Saos-2 and Saos-2-His-273 xenografts treated with PRIMA-1 demonstrated that the antitumor effect of PRIMA-1 was dependent on mutant p53 expression. Furthermore, PRIMA-1 caused induction of p53 target genes in SW480 xenograft tumors. Taken together, these results support the idea that PRIMA-1 is capable of functional reactivation of mutant p53 *in vivo*. We observed significant tumor suppression following both intratumoral and i.v. administration of PRIMA-1. The effect of i.v. administration suggests that systemic treatment with new drugs based on PRIMA-1 will be feasible. This would be of great importance for treatment of patients with disseminated disease. In order to overcome the potential problem of selection for resistance, it may be necessary to combine PRIMA-1 treatment with other agents to simultaneously attack multiple lesions in tumor cells. Alternatively, PRIMA-1 may act synergistically with conventional chemotherapy or radiotherapy, allowing lower doses of drugs or irradiation for efficient tumor-cell killing.

The identification of a small molecule able to restore biochemical and biological function to mutant p53, resulting in significant tumor suppression *in vivo*, opens exciting prospects for future cancer therapy. A more detailed investigation of the molecular mechanism behind PRIMA-1-mediated reactivation of mutant p53 may provide a basis for the design of new potent and tumor-specific drugs.

Methods

Cells and plasmids. The human Saos-2-His-273 osteosarcoma, H1299-His-175 lung adenocarcinoma, and SKOV-His-175, His-273 and His-175-22/23 ovarian carcinoma-cell lines carry the indicated tetracycline-regulated mutant p53 constructs. The His-175-22/23 mutant p53 has substitution of Leu 22/Trp 23 for Gln/Ser in the transactivation domain. The human HCT-116 colon carcinoma-cell line carries wild-type p53 and the A431 colon carcinoma-cell line carries His-273 mutant p53 and a p53-responsive LacZ reporter. Other cell lines used are indicated in Supplementary Table A. The plasmids encoding the GST-p53 fusion proteins have been described[31]. The p53-EGFP plasmid contains 13 synthetic p53 consensus DNA binding sites in front of the EGFP coding sequence. Transient transfection experiments were performed with Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen Life Technologies, Groningen, the Netherlands).

Screening of a chemical library and growth suppression assays. A library of low-molecular-weight compounds was obtained from the National Cancer Institute (NCI), Bethesda, Maryland. For more information, see http://dtp.nci.nih.gov. Saos-2-His-273 cells grown in the presence or absence of doxycycline were treated with compounds from the library (Diversity Set) at a concentration of 25 μM in 96-well plates. Growth suppression was assessed by the WST-1-cell proliferation reagent (Roche Diagnostics, Bromma, Sweden) after 48 h incubation. Absorbance of samples was measured at 490 nm.

***In vitro* assays.** For FACS analysis, cells were stained with propidium iodide and analyzed on a Becton Dickinson FACScan (Mountain View, California) according to standard procedures. TUNEL staining, immunostaining, LacZ staining, preparation of cell extracts, ELISA and western-blotting were performed according to standard procedures. GST–p53 proteins were prepared and analyzed for specific DNA binding as described[13,31]. For PAb1620 staining, cells were fixed with 4% formaldehyde. The anti-p53 monoclonal antibodies PAb1620, PAb240 and PAb1801 were obtained from Calbiochem (Darmstadt, Germany). The anti-p53 rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, California), the anti-MDM2 monoclonal antibody was from Neo Markers (Fremont, California) and the anti-p21 monoclonal antibody was from Transduction Laboratories (Lexington, Kentucky). Secondary antibodies (FITC-conjugated horse anti-mouse immunoglobulin, Texas Red-conjugated goat anti-rabbit Immunoglobulin) were from Vector (Burlingame, California). All other reagents were from Sigma–Aldrich Sweden AB (Stockholm, Sweden).

Animal studies. All animal studies were approved by the local animal ethical committee in Stockholm, Sweden, and animal care was in accordance with institutional guidelines. For toxicity assessment, 12 SCID mice (average weight, 20 g) were divided into 4 groups. 3 groups received daily i.v. injections of 1, 10 and 100 mg/kg of PRIMA-1 in PBS for 5 d. Control animals were injected with PBS. Changes in body weight were monitored for 1 mo after the last injection. For assessment of the antitumor activity of PRIMA-1, 12 SCID mice were inoculated with 1×10^6 Saos-2-His-273 cells in 90% Matrigel (Becton Dickinson, Le Pont-De-Claix, France) subcutaneously and unilaterally into the right flanks. After 3 d, mice were divided into 4 groups. 2 groups received i.v. injections of PRIMA-1 at a dose of either 20 or 100 mg/kg, 1 group received intratumor injections of PRIMA-1 at a dose of 20 mg/kg, and the last group was used as a control. Injections were performed twice daily for 3 d. Tumor volume was measured at 2 mo.

PRIMA-1 mediated activation of p53 target genes *in vivo* was assessed by western-blot analysis using extracts from SW480 xenografts excised from mice 16 h after intratumor injection. To compare the effect of PRIMA-1 on p53-null and mutant p53-expressing xenograft tumors, 10 SCID mice were inoculated with 1×10^6 Saos-2 and Saos-2-His-273 in 90% Matrigel in the right and left flanks, respectively. After 5 d, mice were treated twice a day with i.v. injections of PRIMA-1 (100 mg/kg) or PBS for 10 d. Paraffin-embedded tumor sections were stained with H&E. In all experiments, mean tumor volumes in treated and control animals were compared at a given time point using the Wilcoxon matched-pairs test.

Note: Supplementary information is available on the Nature Medicine *website (http://medicine.nature.com/supplementary_info/).*

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Competing interests statement

The authors declare that they have no competing financial interests.

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