Reactivation of Mutant p53 and Induction of Apoptosis in Human Tumor Cells by Maleimide Analogs*

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Reactivation of mutant p53 is likely to provide important benefits for treatment of chemotherapy- and radiotherapy-resistant tumors. We demonstrate here that the maleimide-derived molecule MIRA-1 can reactivate DNA binding and preserve the active conformation of mutant p53 protein in vitro and restore transcriptional transactivation to mutant p53 in living cells. MIRA-1 induced mutant p53-dependent cell death in different human tumor cells carrying tetracycline-regulated mutant p53. The structural analog MIRA-9 showed antitumor activity in vivo against human mutant p53-carrying tumor xenografts in SCID mice. The MIRA scaffold is a novel lead for the development of anticancer drugs specifically targeting mutant p53.

The p53 tumor suppressor responds to various types of cellular stress, e.g. DNA damage, hypoxia, and oncogenic signaling, and triggers cell cycle arrest, apoptosis, or senescence (1). This is achieved through transcriptional transactivation of specific target genes carrying p53 DNA binding motifs (1, 2). p53 is frequently mutated in tumors, indicating a strong selection for inactivation of the p53 tumor suppressor pathway during tumor development (3). Most p53 mutations are missense point mutations clustering in the core domain (residues 94–292) that harbors the p53-specific DNA binding activity (4). Amino acid substitutions in the core domain result in loss of specific DNA binding, either because of substitution of residues that contact DNA (so-called DNA contact mutants) or substitution of residues that are critical for the structural integrity of the DNA binding interface of the core (so-called structural mutants) (5).

As a result, mutant p53 proteins fail to activate transcription of p53 target genes and, hence, do not trigger a p53-dependent biological response. Inability to activate expression of the target genes appears to have consequences for mutant p53 expression levels in tumor cells. Several negative regulators of p53, including the ubiquitin ligase MDM2, which targets p53 for impairment, and triggers cell cycle arrest, apoptosis, or senescence (1).

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Reactivation of Mutant p53 by MIRA

His273/Ser309 mutant p53. CaspaTag TM Pan fluorescein caspase (VAD) activity kit was from Intergen (Oxford, UK), carboxyfluorescein (FAM)-labeled caspase inhibitor FAM-DEVD-FMK was obtained from Immunochemistry Technologies (Bloomington, MN), polyclonal rabbit anti-p53 and anti-Bax antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and fluorescein isothiocyanate-conjugated anti-rabbit Ig was from Vector Laboratories (Burlingame, CA).

Plasmids—The plasmids encoding the GST-human wild-type p53 fusion protein and the GST-human mutant p53 protein His175 were described earlier (36).

Chemical Library—A library of low molecular weight compounds was obtained from the NCI, National Institutes of Health, Bethesda, MD. For more information, see web site dtp.nci.nih.gov.

Chemical Library Screening and Growth Suppression Assays—Saos-2 cells carrying tetracycline-regulated His273 mutant p53 were used for screening of the Diversity Set from NCI, National Institutes of Health (15). Mutant p53 expression was shut off by incubation of cells with doxycycline for 24 h, trypsinized, and plated at 3,000 cells per well with or without doxycycline. After 48 h of incubation, the reagent WST-1 (Roche Applied Science) was added. The degree of WST-1 reduction, which reflects cell viability, was measured in a microplate reader at λ490 nm according to the manufacturer’s instructions (Roche Applied Science).

FACS Analysis—Cells were placed on 12-well plate and treated with compounds. After 48 h of incubation, cells were harvested by trypsinization, fixed with 70% ethanol, treated with RNase A (0.25 mg/ml), and stained with propidium iodide (0.02 mg/ml). Cells were analyzed by the BD FACScan. Data were analyzed by the CellQuest software, version 3.2.1.

Colony Formation Assays—Cells were treated with MIRA-1 (mutant p53-dependent induction of rapid apoptosis) for 24 h, trypsinized, and seeded in plates at 500 cells per plate. Colonies were stained with Giemsa and counted 14 days after seeding.

DNA Binding Assays—The GST-p53 proteins were prepared as described (15). Band shift assays were performed in binding buffer containing 100 mM HEPES pH 7.5, 50 mM KCl, 1 mg/ml bovine serum albumin, 0.1% Triton X-100, 2 mM MgCl₂, and 1 mM dithiothreitol essentially as in Ref. 36.

ELISA—20 ng of GST-wtp53, GST-mtp53-175, and GST-mtp53-248 were heated at 37 °C for 30 min or kept on ice. The procedure was performed with or without tested compounds. Preparation for ELISA analysis was done as described by Ref. 17. Briefly, the treatment samples were diluted with coating buffer (150 mM KCl, 25 mM HEPES) supplemented with 10 mM dithiothreitol. The whole mixture was applied to ELISA plates (MaxiSorp, Nunc) and incubated at +4 °C for 35 min. The wells were washed with coating buffer and blocked by 5% skim milk in PBS at +4 °C for 1 h. Wells were rinsed twice with PBS followed by addition of mouse primary antibodies (Pab 1620 or Pab 240) diluted 1:250 in coating buffer. Samples were incubated at +4 °C for 30 min. Wells were rinsed twice with PBS. After that, a secondary antibody (anti-mouse, conjugated with horseradish peroxidase) was incubated with samples at +4 °C for 30 min. Then plates were washed five times with PBS, and a peroxidase substrate was added. An absorbance at λ405 nm was monitored by the ELISA reader.

In Vivo Studies—All in vivo studies were approved by the local animal ethical committee in Stockholm, Sweden, and animal care was in accordance with institutional guidelines. For treatment with MIRA-3, 15 SCID mice were divided into 3 groups. Each mouse was inoculated with H1299-His175 cells (5 × 10⁶) in one flank. Xenografts were allowed to grow for 5 days up to the formation of palpable tumors. Control animals received intraperitoneal injections of PBS once daily for 10 days; two groups received intraperitoneal injections of 10 mg/kg or 1 mg/kg of MIRA-3 once daily for 5 or 10 days, respectively.

RESULTS

Mutant p53-dependent Growth Suppression by MIRA-1—We previously screened the Diversity set of low molecular weight compounds from NCI, National Institutes of Health using p53-null Saos-2 osteosarcoma cells carrying tetracycline-regulated His273 mutant p53 (Tet-off) and the WST-1 cell proliferation assay. This led to the identification of PRIMA-1, a quinuclidinone that inhibits growth of human tumor cells in a mutant p53-dependent manner in vitro and in vivo (18). We also identified another low molecular weight compound in this screen. This compound (Fig. 1A) suppressed growth of Saos-2-His273 cells expressing mutant p53 but did not significantly affect
growth of the same cells cultured in the presence of doxycycline that shuts off mutant p53 expression (Fig. 1B). It was designated MIRA-1.

Several structural analogs of MIRA-1 from the NCI repository were tested for mutant p53-dependent growth inhibition. Two of these showed mutant p53-dependent activity similar to that of MIRA-1. The structures of these substances, named MIRA-2 and MIRA-3, are shown in Fig. 1B. Most other analogs, including MIRA-A, MIRA-B, and MIRA-C (Fig. 1C) did not have any detectable effect on the growth of Saos-2-His273 cells at concentrations up to 25 μM. Interestingly, the active analogs share a saturated 3–4 double bond in the maleimide group of the MIRA scaffold, which is absent in the inactive molecules.

Next we tested whether MIRA-1 could induce mutant p53-dependent growth inhibition in human SKOV ovarian carcinoma or H1299 lung adenocarcinoma cells carrying His175 or His273 mutant p53 under control of the tetracycline promoter. The results are presented in Fig. 2 and summarized in Table I. In all three cell lines expressing mutant p53 under control of the tetracycline-dependent promoter we observed mutant p53-dependent growth suppression similar to that seen in Saos-2-His273 cells. The difference between the mutant p53-expressing and the corresponding p53-null cells was statistically significant (p = 0.001). The MIRA-1 analogs MIRA-2 and MIRA-3 showed similar activity as MIRA-1 (Fig. 2). Survival of normal human fibroblasts after treatment with 25 μM MIRA-1 was around 70%.

The ability of MIRA-1 to suppress growth in a mutant p53-dependent manner was further evaluated using a colony formation assay. Saos-2 or Saos-2-His273 cells were treated with different doses of MIRA-1 and seeded in plates. The cells were stained with Giemsa and scored for the appearance of colonies after 14 days. Treatment with 5 μM MIRA-1 dramatically reduced the number of colonies formed by His273-expressing Saos-2 cells (14.9 ± 1.1% of untreated control), but was much less efficient in inhibiting p53-null Saos-2 cells (48.3 ± 1.2% of untreated control).

MIRA Restores the Apoptosis-inducing Function to Mutant p53—To investigate if MIRA-1 was able to induce mutant p53-dependent apoptosis, we performed propidium iodide staining and FACS analysis. Fig. 3A shows DNA content profiles of Saos-2 and Saos-2-His273 cells treated with 10 μM MIRA-1 for 48 h. MIRA-1 caused a substantial increase in the fraction of cells with a sub-G₁ DNA content in the presence of mutant p53, indicating DNA fragmentation and cell death. Fig. 3B shows quantification of the fraction of sub-G₁ cells after 48 h of treatment of Saos-2 and Saos-2-His273 cells with MIRA-3. We also tested whether MIRA-3 treatment had any effect on caspase activity using the CaspaTag reagent. As shown in Fig. 3C, MIRA-3 induced significant caspase activation in the mutant p53-expressing Saos-2 cells but not in the corresponding p53-null cells, indicating induction of mutant p53-dependent apoptosis.

To confirm that MIRA-1 could restore the apoptosis-inducing function to mutant p53, we tested the effect of caspase inhibitors on MIRA-1-induced growth suppression. Saos-2-His273 cells were treated with MIRA-1 in the presence or absence of the caspase inhibitors Z-DEVD-FMK and BOC-D-FMK. Induction of cell death was determined by propidium iodide staining and FACS analysis and expressed as percentage of cells with sub-G₁ DNA content. Z-DEVD-FMK suppressed cell death induced by MIRA-1 by -3.5-fold and BOC-D-FMK suppressed cell death induced by MIRA-1 by 7-fold (data not shown). Thus, we conclude that MIRA-1 and its active analogs induce mutant p53-dependent cell death by apoptosis.

MIRA-1-induced Apoptosis Depends on the Transactivation Function of p53—To examine if MIRA-1 exerts its effect through p53-mediated transcriptional transactivation and de novo protein synthesis, we tested the effect of cycloheximide on MIRA-1-induced growth inhibition/apoptosis. Treatment of SKOV-His175 cells with cycloheximide prior to addition of MIRA-1 resulted in a 4-fold increase in cell survival (data not shown). Moreover, the viability of SKOV cells carrying the His175–22/23 p53 mutant, which has an inactivated transactivation domain because of additional mutations at positions 22 and 23, was at least three times higher than that of SKOV-His175 cells after MIRA-1 treatment (data not shown). Taken together, these results strongly support the notion that tran-
scriptional transactivation by p53 is critical for MIRA-1-induced cell death.

Modulation of p53 Conformation by MIRA—To get insight into the molecular mechanism of MIRA-1-mediated reactivation of mutant p53, we tested the effect of the compound on p53 conformation. A majority of the amino acid substitutions in p53 that occur in tumors destabilize the native conformation of the core domain, resulting in loss of the conformation-dependent epitope for the monoclonal antibody PAb1620 and appearance of an epitope recognized by the monoclonal antibody PAb240 (19). Heat denaturation of the wild-type p53 has a similar effect. Therefore, we examined if MIRA-1 could stabilize the native (wild type) conformation of p53 upon heating. We found that 25 μM MIRA-1 preserved the PAb1620 epitope in recombinant wild-type and mutant p53 proteins heated for 30 min at 37 °C (Fig. 4A). MIRA-1 partially prevented unfolding of p53 protein as shown by reduced appearance of PAb240 reactivity upon heating at 37 °C. The non-conformational N-terminal epitope recognized by monoclonal antibody DO-1 was not affected by incubation with MIRA-1 (data not shown).

We also tested whether MIRA was capable of increasing the fraction of p53 protein with the PAb1620 epitope during incubation at 0 °C. Treatment of recombinant GST-wild-type p53 protein with 25 μM MIRA-1 for 2 h at 0 °C resulted in an increase of the PAb1620+ fraction (Fig. 4B). MIRA-2 and MIRA-3 also induced the PAb1620 epitope. Three MIRA analogs that were inactive in our WST-1 cellular proliferation assay were also unable to induce the PAb1620 epitope. Thus, MIRA cannot only prevent unfolding of wild-type and mutant p53 at 37 °C but also restore native conformation to wild-type p53 at 0 °C.

Restoration of Sequence-specific DNA Binding of Mutant p53 Proteins—Because specific DNA binding is critical for p53 tumor suppressor function, we examined whether MIRA could stimulate this activity using a band shift assay. Incubation of GST-wild-type p53 at 37 °C for 30 min completely abolished sequence-specific DNA binding in the presence of PAb421 antibody. However, incubation at this temperature in the presence of MIRA-1 preserved DNA binding of GST-wild-type p53 in a concentration-dependent manner (Fig. 5A). We also tested whether MIRA could enhance the specific DNA binding activity of mutant p53 proteins in extracts from human tumor cells. MIRA-1 enhanced DNA binding of the Trp282 mutant p53 in extracts from BL60 cells (Fig. 5B) and His175 mutant p53 in extracts from CW678 cells (Fig. 5C). PAb421 or PAb1801 monoclonal antibodies were added to the
reaction mixture to supershift p53-DNA complexes. Our further analyses of a wide range of mutant p53 proteins using extracts of human tumor cell lines carrying different p53 mutations revealed that MIRA-1 stimulated DNA binding of 3 of 13 tested mutant p53 proteins (Table II).

**Restoration of the Transcriptional Transactivation Function of Mutant p53 in Living Cells**—Having established that MIRA-1 can stimulate the specific DNA binding of mutant p53 in vitro, we asked whether MIRA-1 could also restore transcriptional transactivation to mutant p53 in living cells. We treated SKOV-His175 cells transiently transfected with a p53-responsive EGFP reporter with MIRA-1. Fig. 6A shows that treatment with 5 μM MIRA-1 for 24 h induced EGFP expression in these cells. Furthermore, immunostaining revealed induction of both MDM2 and Bax in SKOV-His175 cells treated with 10 μM MIRA-1 for 24 h (Fig. 6B). Interestingly, p53 levels appeared lower after treatment with MIRA-1 (Fig. 6C).

As further confirmation of the ability of MIRA-1 to rescue transcriptional transactivation of mutant p53, we examined whether MIRA-1 was able to induce p53 target genes in a mutant p53-dependent manner. Treatment of H1299-His175 cells expressing mutant p53 with MIRA-1 resulted in a significant induction of both MDM2 and p21 (Fig. 6D). Importantly, treatment of the same cells in the absence of mutant p53 expression and treatment of parental H1299 cells did not cause any induction of MDM2, nor p21. In addition, MIRA-1 induced MDM2 and p21 in SW480 colon carcinoma cells carrying endogenous His273/Ser309 mutant p53 (Fig. 6E), but not in HCT116 colon carcinoma cells that carry wild-type p53. We did not observe any stabiliza-

### Table II: MIRA-1 stimulates the DNA binding of mutant p53

| Cell line | Mutation | MIRA-1-induced DNA binding |
|-----------|----------|---------------------------|
| ScabER    | Leu110   | −a                        |
| HT3       | Val245   | −                         |
| Namalva   | Gln248   | +a,b                      |
| BL49      | Tyr176/Trp248 | +a,b                  |
| HS 578T   | Phe157   | −                         |
| BL41      | Gln248   | −                         |
| SW 480    | His273/Ser309 | −                   |
| T47D      | Phe194   | −                         |
| Raji      | Gln215/His234 | −                   |
| CW 678    | His175   | +a,b                      |
| MDA-MB-231| Lys280   | +a/b                      |
| BL60      | Trp282   | +b                        |
| C33A      | Cys273   | −                         |
| KRC/Y     | Phe176   | −                         |

a No DNA binding.
b +a, +b, ++, +++, relative efficiency of DNA binding.

**FIG. 4. Effect of MIRA-1 on p53 conformation.** A, left panel, MIRA-1 preserves the conformation-dependent PAb1620 epitope upon heating of the p53 protein for 30 min at 37 °C. Percentage of epitope compared with the control untreated proteins maintained on ice is shown. A, right panel, MIRA-1 prevents unfolding of p53 measured as the percentage of the PAb240 epitope in p53 upon heating at 37 °C. B, effect of MIRA-1, MIRA-2, and MIRA-3, and inactive analogs MIRA-A, MIRA-B, and MIRA-C, on the PAb1620 epitope in p53.
We examined the effect of MIRA-3 treatment on the pro-apoptotic p53 target gene PUMA in Saos-2-His273 cells. As shown in Fig. 6, MIRA-3 induced PUMA in Saos-2-His273 cells in a dose-dependent manner. No effect on PUMA expression was observed in the parental p53-null Saos-2 cells. These results provide solid evidence for restoration of p53-dependent transcriptional transactivation by MIRA.

Analysis of Information in the NCI Data Base—Information in the NCI data base allows in silico analysis of the activity of many compounds in the NCI Diversity Set on a panel of human tumor cell lines. In particular, the available data allows estimation of how sensitivity of tumor cells to a certain compound correlates with mutant p53 levels and whether a compound shows any preferential growth suppression of tumor cells expressing mutant p53 or wild-type p53. Our analysis of the activity of MIRA-1 on the panel of human tumor cell lines revealed a statistically significant correlation between IC50 values for MIRA-1 and mutant p53 levels among the subset of cell lines carrying mutant p53 according to sequencing. However, we did not find any significant difference between tumor cell lines expressing wild type and those expressing mutant p53.

Antitumor Activity of MIRA-3 in Vivo—To study the antitumor effect of the MIRA type of compounds in vivo, we inoculated SCID mice with H1299-His175 cells and treated the mice with intraperitoneal injections of MIRA-3 when palpable tumors had formed. Control mice treated with PBS developed tumors with an average volume of 450 ± 234 mm3 after 34 days. Treatment with MIRA-3 at a dose of 1 mg/kg resulted in tumor size of 298 ± 122 mm3. Treatment with MIRA-3 at this dose did not result in any weight loss or other obvious toxic effects during 2 weeks of treatment. However, treatment with 5 and 10 mg/kg caused acute toxicity, such as diarrhea and weight loss, that prevented continuation of the experiment. Thus, MIRA-3 shows antitumor effect in vivo upon systemic administration but also has toxic effects at higher doses.

DISCUSSION

The remarkably high frequency of p53 mutations in a wide range of human tumors (www.iarc.fr/p53) and the well-documented association between mutant p53 status and poor response to conventional radiotherapy and chemotherapy in many clinical studies emphasizes the need to develop novel efficient strategies for targeting mutant p53 in tumors. We previously screened the Diversity Set of low molecular weight compounds from the NCI, National Institutes of Health and identified two low molecular weight compounds that showed preferential killing of mutant p53-expressing tumor cells. One
of these molecules, PRIMA-1, has been described (18). Here we present data on MIRA-1 and its active analogs, a structurally distinct group of compounds that also targets mutant p53.

MIRA-1 induces cell death in a mutant p53-dependent manner with a potency that is even higher than that of PRIMA-1. The IC$_{50}$ value for MIRA-1 in Saos-2 His273 cells was 10 μM whereas the IC$_{50}$ value for PRIMA-1 is over 15 μM under the same conditions (18). The selectivity of MIRA-1 for mutant p53-expressing human tumor cells carrying Tet-regulated mutant p53 is also similar to that of PRIMA-1. We observed a ratio of at least 2 between IC$_{50}$ values for the mutant p53-expressing versus the p53-null cells (Figs. 2 and 3). Thus, MIRA-1 shows significant mutant p53-dependent cell killing. Furthermore, like PRIMA-1, MIRA-1-induced cell death involves DNA fragmentation and caspase activation, indicating cell death by apoptosis. This is consistent with our results indicating that MIRA acts mainly via the p53 pathway. It is interesting to note, however, that MIRA-1 and PRIMA-1 differ considerably with respect to the kinetics of cell death induction. MIRA-1 induces cell death within 6–12 h (data not shown), whereas PRIMA-1 kills cells only after 24–48 h (18). This may reflect a different molecular mechanism of action or different kinetics of cellular uptake and/or degradation.

We have shown that MIRA-1 reactivates mutant p53 by several criteria. First, MIRA-1 was able to preserve native conformation of both wild-type and mutant p53 upon heating, as demonstrated by reactivity with the conformation-specific antibodies PAb1620 (wild type) and PAb240 (mutant). Second, treatment with MIRA-1 preserved sequence-specific DNA binding of wild-type p53 upon heating, and also enhanced the DNA binding of one globally denatured mutant form of p53, His175, and one DNA contact mutant, Gln248. Third, MIRA-1 treatment caused activation of a p53-dependent reporter in mutant p53-expressing cells and MIRA-1 and/or its analog MIRA-3 induced the p53 target genes p21, MDM2 and PUMA in a mutant p53-dependent manner. Moreover, a double mutant of p53 carrying substitution at residues 22 and 23 in the transactivation domain and the His175 substitution in the core domain was resistant to reactivation by MIRA-1. This confirms that MIRA-1 induces apoptosis via restoration of p53-dependent transcriptional transactivation. Finally, our finding that cycloheximide blocks the death-inducing effect of MIRA-1 demonstrates that de novo protein synthesis is required for MIRA-1-induced apoptosis. Taken together, these results indicate that MIRA-1 and some of its structural analogs act by shifting the equilibrium between the native and unfolded conformation of p53 toward the native conformation, leading to restoration of p53-mediated transactivation of target genes and induction of p53-dependent apoptosis.

Our DNA binding assays demonstrated that MIRA-1 stimulated DNA binding of some but not all mutant forms of p53. Moreover, MIRA-1 stimulated DNA binding of the Gln248 mutant in Namalva cells but not in BL41 cells, and similarly, the His273 mutant in SW80 cells was not affected by MIRA-1 in the DNA binding assay but conferred increased MIRA-1 sensitivity to Saos-2 or SKOV cells in our WST-1 assays. These findings are consistent with the involvement of cellular factors in the ability of MIRA-1 to reactivate mutant p53.

To determine whether MIRA compounds could inhibit tumor growth in vivo upon systemic administration, we treated SCID mice carrying human tumor xenografts with MIRA-3. This MIRA-1 analog was as potent in inhibiting tumor cell growth and inducing apoptosis as MIRA-1, as assessed by our WST-1 proliferation assay and FACS analysis. We observed a significant antitumor activity of MIRA-3 in vivo. However, the compound was toxic at the higher doses used, indicating a narrow therapeutic window. Elimination of such toxicity is a major goal in further lead optimization of MIRA type of compounds.

MIRA-1 is structurally distinct from the previously described mutant p53-targeting compounds PRIMA-1 (18) and CP-31398 and CP-257042 (17), and therefore represents a novel class of mutant p53-reactivating molecules. Although we have shown that MIRA-1 restores native conformation and transcriptional transactivation to mutant p53 and induce tumor cell apoptosis in a mutant p53-dependent manner, its molecular mechanism of action remains to be elucidated. The MIRA compounds contain a maleimide group, which may react with thiol and amino groups in proteins (nucleophilic addition). Our examination of 23 structural analogs of MIRA-1 clearly demonstrated that only a subset of maleimide group-containing compounds were able to reactivate mutant p53. We found that the presence of the 3–4 double bond in the maleimide group is critical for the mutant p53-dependent activity (Fig. 1). Analogs lacking this double bond were inactive in our cell culture assays and failed to preserve native conformation of wild-type or mutant p53 proteins (Fig. 2). The presence of the reactive double bond is important for the ability of maleimide compounds to form adducts with thiol and amino groups. This raises the possibility that alkylation of cysteine and/or lysine residues in p53 by active MIRA compounds plays a role in stabilizing the native fold of the protein. Modification of cysteines or lysines is known to have an impact on p53 conformation and activity. The p53 core domain whose proper conformation is crucial for specific DNA binding contains 10 cysteine residues (19) that are potential targets for alkylation. Moreover, several studies have indicated that p53 activity is subject to redox regulation in cells (21–23). Certain reducing compounds, including the aminothiol WR1065 (24, 25) and selenomethionine (26), have been shown to stimulate the activity of p53. Oxidized and reduced forms of p53 have different oligomerization properties (27) and probably different biological activity. Oxidation/reduction of cysteines 121, 132, 138, and 272 could regulate p53 activity by structural modulation of the core domain (28). Alkylation of cysteine residues of wild-type p53 by N-ethylmaleimide results in decreased DNA binding (28). However, low micromolar concentrations of N-ethylmaleimide were shown to increase DNA binding (29). Similarly, we have observed that the effect of MIRA-1 and its active analogs on mutant p53 DNA binding in vitro is concentration-dependent with a maximum effect at a concentration around 250 μM to 1 mM, depending on the mutant form of p53, followed by reduced binding at higher concentrations. This suggests that the effect of MIRA-1 depends on the degree of overall p53 alkylation and/or alkylation of specific cysteine residues in the protein. Thus, it is conceivable that an optimal level of mutant p53 alkylation by MIRA-1 induces correct folding and enhances DNA binding of at least a fraction of p53 protein molecules. It is also possible that alkylation of p53 depends on the accessibility of thiol groups. Unfolded mutant p53 proteins are likely to expose a larger number of potential alkylation sites compared with correctly folded and more compact wild-type p53, and so mutant p53 may be more extensively modified by MIRA than wild-type p53 in cells.

The thiol-reducing amifostine derivative WR1065 has been shown to shift the Met272 mutant p53 toward wild-type conformation (24). Although the activity of WR1065 as a reducing agent appears different from that of MIRA compounds are possibly reducing compounds, the two types of molecules could nonetheless have similar effects on mutant p53 conformation, i.e., prevention of intramolecular or intermolecular disulfide bonds that might interfere with proper folding of the protein. Other compounds that modulate the redox status in cells have been shown to possess antitumor activity (30, 31). For example, N-ethylmale-
maleimide induces externalization of phosphatidylserines on the cellular membrane, leading to the increased phagocytosis of cancer cells by macrophages (32, 33). Other maleimide derivatives have also been shown to have anticancer activity in vitro and in vivo (34, 35).

Based on our data, we conclude that MIRA-1 and its active analogs are capable of reactivating mutant p53 and trigger mutant p53-dependent apoptosis. The MIRA compounds described here could serve as leads for the development of novel anticancer agents that rescue wild-type conformation and function of mutant p53. Further optimization of these compounds should aim at increasing their potency and reducing general toxicity, and also gaining further insights into their molecular mechanism of action. The targeting of mutant p53 by small molecules like MIRA will hopefully allow more efficient cancer therapy and significant clinical benefit in the future.

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