

Allele-Specific p53 Mutant Reactivation

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SUMMARY

Rescuing the function of mutant p53 protein is an attractive cancer therapeutic strategy. Using the National Cancer Institute's anticancer drug screen data, we identified two compounds from the thiosemicarbazone family that manifest increased growth inhibitory activity in mutant p53 cells, particularly for the p53^{R175} mutant. Mechanistic studies reveal that NSC319726 restores WT structure and function to the p53^{R175} mutant. This compound kills p53^{R172H} knockin mice with extensive apoptosis and inhibits xenograft tumor growth in a 175-allele-specific mutant p53-dependent manner. This activity depends upon the zinc ion chelating properties of the compound as well as redox changes. These data identify NSC319726 as a p53^{R175} mutant reactivator and as a lead compound for p53-targeted drug development.

INTRODUCTION

TP53 is the most frequently mutated gene in human cancer with mutation frequencies ranging from 38%–50% in some reports to as high as 75% and 96% in pancreatic adenocarcinoma and high-grade serous ovarian carcinomas, respectively (Hingorani et al., 2005; Cancer Genome Atlas Research Network, 2011; Petitjean et al., 2007). The majority of mutations are mis-sense mutations that occur most frequently in six “hotspot” codons within the DNA binding domain (Olivier et al., 2010). These mutant proteins are classified as either DNA contact mutants (e.g., p53^{R273H}) when the mutation occurs in a DNA binding residue or conformational mutants (e.g., p53^{R175H}) when a conformational change causes a loss of WT p53 DNA binding.

Mutant p53 proteins are found at high concentrations in tumor cells relative to WT p53, mostly because of a loss of WT p53 transcription of the *MDM2* gene that negatively regulates p53, as well as other tumor-specific alterations, such as loss of *p16^{INK4a}* (Haupt et al., 1997; Midgley and Lane, 1997; Terzian et al., 2008). The concept that these mutant proteins are functional

and regulate important processes relevant to tumor biology is referred to as the mutant p53 gain-of-function (GOF) phenotype (Sigal and Rotter, 2000). Properties attributed to mutant p53 GOF include enhanced tumorigenesis, invasion, and metastasis (Adorno et al., 2009; Dittmer et al., 1993; Liu et al., 2000; Muller et al., 2009). Taken together, these properties make mutant p53 an attractive target for drug development.

The National Cancer Institute's (NCI) anticancer drug screen has reported growth inhibition half maximal inhibitory concentration (IC₅₀) on 48,129 compounds tested on a panel of sixty human tumor cell lines (NCI60 screen). Given that the p53 status (WT, null, or mutant) of these cell lines is known (Ikediobi et al., 2006; Shoemaker, 2006), we hypothesized that this screen could be used to uncover drugs targeting p53 mutant tumors. Thus, we developed a methodology to identify compounds with increased activity in a panel of tumor cell lines with p53 mutations, relative to p53 WT controls. In this study, we attempted to validate this methodology using two compounds that belong to the thiosemicarbazone family of metal ion chelators. We further investigated one of the compounds with particular toxicity to cell lines

Significance

The next generation of anticancer drugs will be defined by compounds that selectively kill cancer cells while leaving normal cells undisturbed. We developed an in silico screening methodology that identified such a compound that selectively kills cancer cells with a p53^{R175} mutation. The mechanism of action restores WT structure and function to the p53^{R175} protein using its zinc-chelating and redox properties. This unique mechanism may allow medicinal chemistry to design other compounds that renature other p53 mutants that fail to coordinate zinc. *TP53* is the most commonly mutated gene in human cancer, and the p53^{R175} mutant is the third most frequently found mis-sense mutant. The potential pool of patients in the United States for this drug would approximate 32,000 annually.

containing a p53^{R175} mis-sense mutation, identifying the mechanism of this toxicity and the properties of the compound that are relevant to this mechanism.

RESULTS

Identification of Thiosemicarbazones with Activity in Cell Lines Expressing Mutant p53

Our methodology deals with the intrinsic heterogeneities of the NCI60 screen (Figure 1A; Supplemental Experimental Procedures available online). This takes as input IC₅₀ data, applies data normalization to obtain a working definition of a good response, and then ranks compounds manifesting a good response using a scoring function. This score function identifies those compounds with an enrichment of good responders in the case group (mutant p53), while simultaneously having a depletion of good responders in the control group (wild-type p53). To reduce the heterogeneity in the mutant p53 group, we focused on mutations in hotspot codons 175, 248, and 273 (11 cell lines), whereas the control group was composed of sixteen p53 wild-type cell lines. Applying this methodology, we observed that three of the highest scoring compounds belonged to the thiosemicarbazone family (NSC319725, NSC319726, and NSC328784), which preferentially inhibited p53 mutant cell lines (Figure 1B). When comparing the IC₅₀s of these compounds by p53 mutational status, we see that the low IC₅₀s are enriched of cells with a p53 mutation (Figure 1B, red), whereas the majority of p53 wild-type cells exhibit high IC₅₀s (Figure 1B, blue). In contrast, two reported mutant p53 reactivators (PRIMA-1 and MIRA-1), as well as an additional thiosemicarbazone currently in clinical trials (Triapine), scored poorly using this methodology in comparison to NSC319726 (Figures S1A–S1D).

We validated two of our screened compounds (NSC319725 and NSC319726) using a mouse fibroblast cell line containing no functional *TP53* gene (10(3)), from which several stable human cytomegalovirus-driven mutant p53 transfectants (175, 248, and 273) were derived (Dittmer et al., 1993). Balb/c 3T3 fibroblasts were used as a p53 WT control because this is the same background as the 10(3) and its derived cell lines. Both compounds exhibited growth inhibition at markedly lower concentrations in cells expressing mutant p53 as compared to the WT control, particularly in the 175 allele (Figure 1C). The IC₅₀ for the NSC319725 treated 175 mutant was ≥ 100 -fold lower than the WT. For NSC319726, the effect was even greater as the IC₅₀ for the 175 mutant was 8 nM, whereas the IC₅₀ of the WT was not reached. Similar to the Balb/c 3T3, these two compounds were remarkably nontoxic to WI38 human fibroblasts (p53 WT), as an IC₅₀ for both compounds was not obtained (Figure 1D). Furthermore, NSC319726 did not induce WT p53 protein levels or transcriptional activity as common cytotoxic agents, such as etoposide, do in vitro (Figure S1E).

We further validated NSC319726 by employing additional p53 mutant cell line systems. In a set of isogenic mouse embryonic fibroblast (MEF) cell lines from p53^{+/+}, p53^{-/-}, and p53^{R172H/R172H} mice, we found that NSC319726 exhibited a much higher sensitivity for the MEF-p53^{R172H/R172H} cell line as compared to the p53^{+/+} and p53^{-/-} controls (Figure 1E). We compared the sensitivities across human tumor cell lines with

different p53 “hot spot” mutations (175, 248, and 273). With the exception of one p53^{R175} cell line (RXF393), the 175 cells exhibited similar IC₅₀'s that were approximately 10-fold and in some instances 100-fold lower than the other hotspot mutants, indicating a 175 allele preference of growth inhibition by NSC319726 (Figure 1F). It is important to emphasize that the results of these assays depend to a significant degree on a number of factors, including the method of assaying cell viability, the proliferation rates of the cells, and the confluency at the time of drug exposure, as well as differences in redox in tumor cells. To diminish intra-assay variability, we used the Guava-ViaCount assay to compare the effects of NSC319726 across tumor cell lines that varied by p53 status given the heterogeneity of these cell lines. In both the MTS and Guava-ViaCount assay, we found it to be important to have the cells at 50%–60% confluency at the time of drug exposure. Cell growth inhibition assays carried out under different conditions can and do give different IC₅₀s.

Induction of p53^{R175}-Dependent Apoptosis by NSC319726

To determine if the inhibition of cell growth was mediated by apoptosis, we performed Annexin-V staining of different cell lines treated with NSC319726. We observed an increase in the number of Annexin-V-stained cells with a maximum increase in the 175 mutant (Figure 2A). Similarly, treatment of three different ovarian carcinoma cell lines (TOV112D [p53^{R175H}], OVCAR3 [p53^{R248W}], and SKOV3 [p53^{-/-}]) with NSC319726 resulted in an induction of apoptosis in the p53^{R175} mutant more than 2-fold higher than the other two mutant cell lines (Figure 2B). When we silenced expression of the p53^{R175} mutant protein by siRNA, we observed a marked reduction in sensitivity to cell growth inhibition, demonstrating that the NSC319726 mechanism is at least partially dependent on the p53^{R175} mutant protein (Figure 2C).

NSC319726 Induction of a “WT-like” Conformational Change in the p53^{R175} Mutant Protein

Because NSC319726 induced a p53 apoptotic function in p53^{R175} cells, we investigated if NSC319726 restored WT conformation to the mutant p53^{R175} protein. Using conformation-specific antibodies by immunofluorescence, we observed that NSC319726 induced a conformation change in the p53^{R175} mutant to a structure that was recognized by the WT-specific antibody (PAB1620) and was no longer recognized by the mutant-specific antibody (PAB240; Figure 3A). Quantification of the fluorescence intensity for PAB240 was reduced by 5-fold, whereas that of PAB1620 was increased by 2-fold (Figure 3B). We confirmed this conformation change by PAB240 immunoprecipitation of NSC319726-treated TOV112D lysates, indicating a more than 85% decrease in PAB240 immunoreactivity (Figure 3C). Next, we sought to confirm this conformation change in an additional cell line. NSC319726 treatment of a MEF cell line derived from p53^{R172H/R172H} mice resulted in a loss of PAB240 immunofluorescence staining (Figure 3D). The PAB1620 antibody staining is not shown because this antibody is human-specific. This demonstrates that the conformation change induced by NSC319726 occurs in both human (p53^{R175}) and mouse (p53^{R172}) proteins.

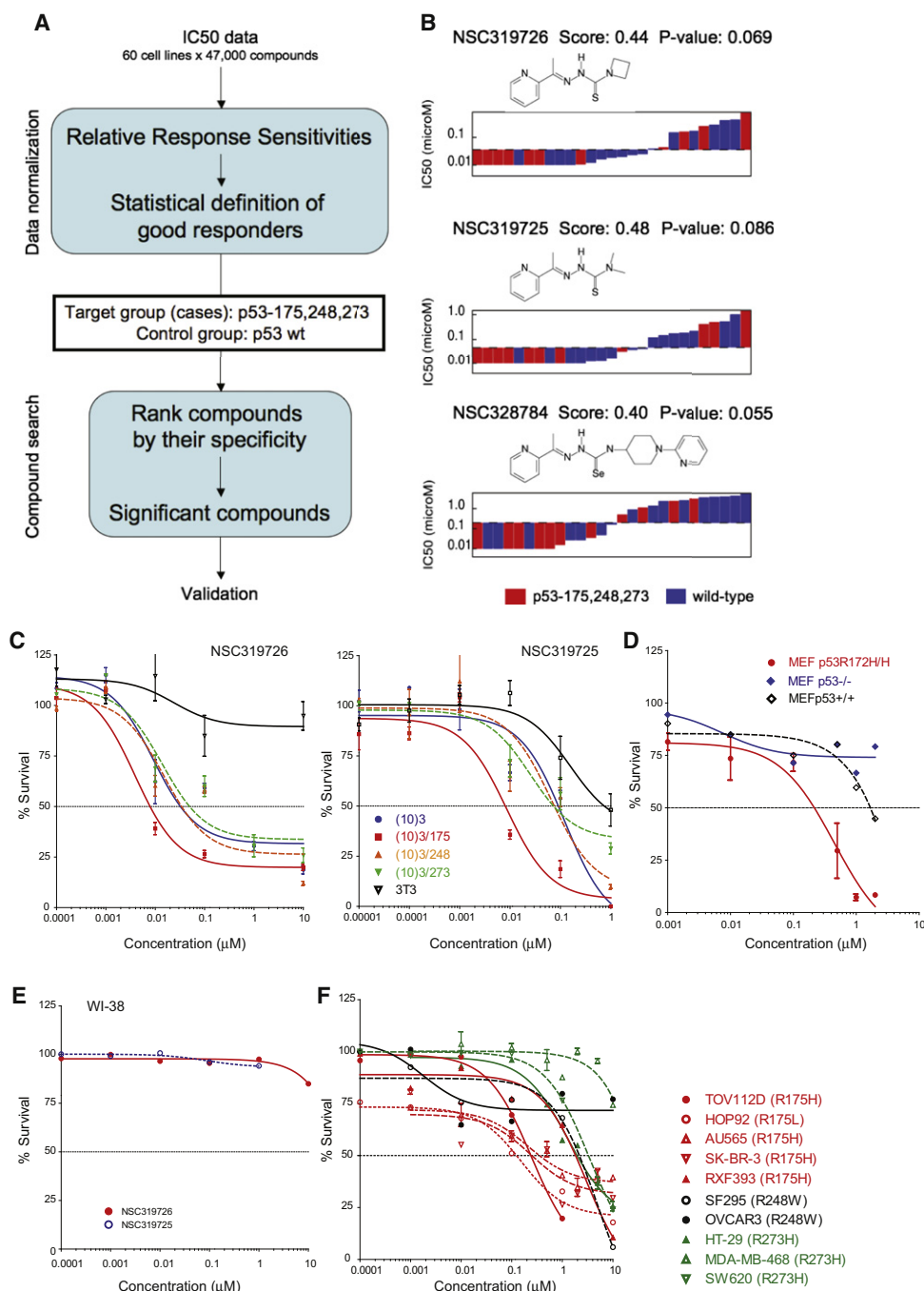


Figure 1. Identification of Thiosemicarbazones with Activity in Cell Lines Expressing Mutant p53

(A) Flow diagram of the in silico screen methodology.

(B) IC₅₀ distribution and chemical structure of the thiosemicarbazones aligning lower IC₅₀s to higher IC₅₀s in cells with mutant p53 (red) and WT p53 (blue), analyzed with the NCI60 data set. In the NCI60 data set, the human tumor cell lines are treated with five serial dilutions of each compound for 48 hr at 37°C.

(C) Validation of the sensitivity of the compounds in the cell lines expressing mutant p53. Cell growth inhibition assays using a mouse embryonic fibroblast (MEF) cell line system consisting of (10)3 (p53^{-/-}), (10)3/175, (10)3/248, (10)3/273, and 3T3 (p53^{+/+}). (Left panel) Growth inhibition with NSC319726. (Right panel) Growth inhibition with NSC319725.

(D) Sensitivity to NSC319726 in a set of isogenic MEF cell lines from p53^{+/+}, p53^{-/-}, and p53^{R172H/R172H} mice.

(E) NSC319725 and NSC319726 do not inhibit cell growth of WI38 (p53^{+/+}) human fibroblasts.

(F) The sensitivity of NSC319726 in human tumor cell lines with hotspot p53 mutations. Cell growth inhibition was analyzed by MTS assay (C) or by Guava ViaCount assay (D–F). In both the MTS and Guava ViaCount assays, cells were treated with five to seven serial dilutions of the compounds (0.00001 to 10 μM) for 3 days. The error bars are ± SD.

See also Figure S1.

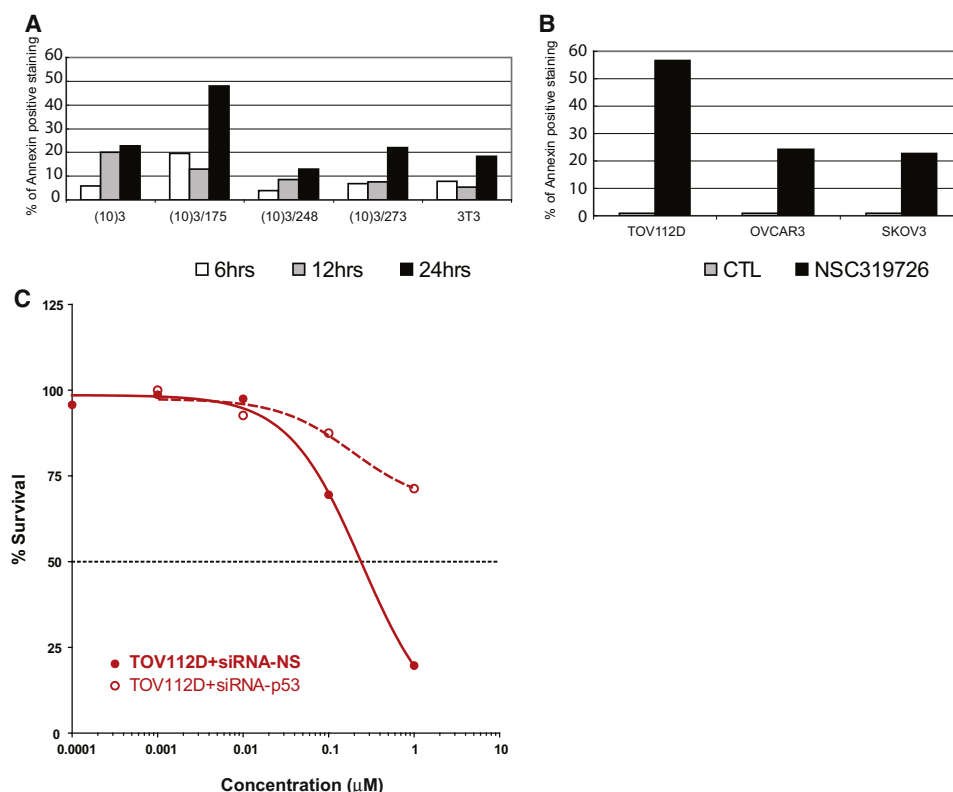


Figure 2. The p53-175-Mutant-Dependent Apoptosis Induced by NSC319726

The apoptosis is measured with Annexin-V staining using Guava Nexin reagent and Guava PCA instrument.

(A) Apoptosis is measured in the (10)3-derived MEF cells.

(B) Apoptosis is measured in a panel of ovarian cancer cell lines, TOV112D (p53^{R175H}), OVCAR3 (p53^{R248W}), and SKOV3 (p53^{-/-}). The treatment is 1 μM NSC319726 (as in the other figures, unless described differently) for 24 hr.

(C) Growth inhibition by NSC319726 in TOV112D cells with siRNA knockdown of p53^{R175H} mutant protein, measured as in Figure 1E.

Restoration of p53 Transactivational Function through the “WT-like” Conformational Change Induced by NSC319726

To determine if the conformation change observed with the p53^{R175} mutant resulted in restoration of p53 transcriptional function, we examined p21 protein levels after NSC319726 treatment in TOV112D and SKOV3 cells. We found that NSC319726 induced p21 in the TOV112D cell line but not in the SKOV3 (Figure 4A). Treatment of the TOV112D cells with the DNA damaging agent etoposide failed to induce p21 (Figure 4A). This suggests that the p21 induction in NSC319726-treated TOV112D cells was p53^{R175}-mutant-dependent.

We note NSC319726 caused a reduction in the levels of the mutant protein (Figure 4A). We examined this further by measuring the mutant protein levels at different time points over a 24 hr period. Treatment of TOV112D cells with NSC319726 decreased the stabilization of the mutant protein with the lowest levels seen at 6 hr with a return to pretreatment levels by 24 hr. This p53 mutant protein destabilization was not seen when we treated two cell lines containing the p53 DNA contact mutants R248 (OVCAR3) and R273 (SW620) with NSC319726. This indicates allele specificity to the NSC319726-mediated effect on p53 mutant protein levels. We hypothesized that the decrease in p53^{R175} protein stability was due to restora-

tion of Mdm2 negative regulation of the “WT-like” p53^{R175} mutant. To test this, we performed this experiment in the presence of Nutlin-3 (small-molecule antagonist of Mdm2) and found that Nutlin-3 abrogated the decrease in stability of the p53-175 mutant induced by NSC319726 (Figure 4B). This restoration of Mdm2-negative regulation is the result of transactivation of the *MDM2* promoter by the “WT”-like p53^{R175} mutant (Figures 4C and 4D).

We confirmed the ability of NSC319726 to restore DNA binding properties to the p53^{R175} mutant protein by chromatin immunoprecipitation (ChIP). Using the p53 antibody, DO-1, ChIP analysis of NSC319726-treated TOV112D cells revealed the restoration of site-specific DNA binding of p53^{R175} mutant to the promoters of *p21*, *PUMA*, and *MDM2* (Figure 4C). HCT116 cells treated with etoposide, as well as RNA polymerase II binding of the *GAPDH* promoter, were used as controls (Figure 4C). We compared the mRNA levels of several p53 targets (*p21*, *PUMA*, and *MDM2*) in the TOV112D (p53^{R175H}), OVCAR3 (p53^{R248W}), and SKOV3 (p53^{-/-}) cells upon treatment and found that NSC319726 increased the levels of all three p53 target genes in TOV112D cells, particularly the apoptotic gene *PUMA* (Figure 4D). To provide further evidence that NSC319726 restored site-specific p53 transactivational function, we transfected the TOV112D cells with a luciferase reporter plasmid

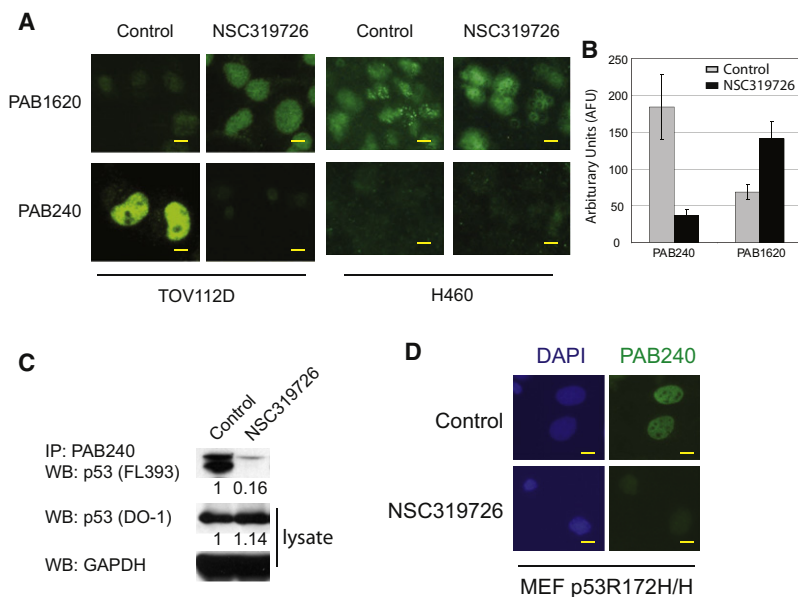


Figure 3. A “WT-like” Conformational Change in the p53-175 Mutant Protein Induced by NSC319726

(A) Immunofluorescence of TOV112D (p53^{R175H}) cells using p53-conformation-specific antibodies (PAB1620 for WT conformation and PAB240 for mutant conformation). The H460 cell line is used as a control to show that the WT p53 conformation is not changed by NSC319726. All scale bars represent a size of 25 μ m.

(B) Quantification of PAB240 and PAB1620 staining indicates significant differences upon treatment with NSC319726 ($p < 0.0001$, t test). The error bars are \pm SD.

(C) Immunoprecipitation of mutant p53 protein in TOV112D cells using PAB240 and detected by p53 (FL393) by western blot.

(D) Immunofluorescence of MEF p53^{R172H/H} cells using the PAB240-mutant-specific antibody.

In all experiments (A, C, and D), cells are treated with NSC319726 (1 μ M) for 6 hr before harvesting for immunostaining or immunoprecipitation.

containing 20 base pairs of the p53 response element in the *p21* promoter. NSC319726 treatment induced a 2.5-fold increase in luciferase activity that was not seen in MEF cells expressing the 248 and 273 alleles, indicating that the restoration of transcriptional function is allele-specific (Figure 4E). We next utilized gene expression microarrays to examine the transcriptional activity of a larger cohort of p53 targets in TOV112D cells. NSC319726 treatment produced a p53 target expression signature remarkably different from the untreated controls, confirming that the conformation change of the p53^{R175} mutant results in a transcriptionally active protein (Figure 4F). Table S1 reports the average expression log₂-ratios between cells treated with NSC319726 and untreated controls, focusing on probes that map to p53 target genes. The list of p53 targets were selected from a literature review, providing a comprehensive list of experimentally verified targets of p53 (Riley et al., 2008).

In Vivo Allele-Specific p53^{R175H} Mutant Reactivation by NSC319726

To investigate the p53 mutant's reactivation in vivo, we performed toxicity assays in p53^{+/+}, p53^{-/-}, and p53^{R172H} knockin mice (Lang et al., 2004). Mice were housed and treated according to guidelines and all the mouse experiments are done with the approval of the Institutional Animal Care and Use Committee (IACUC) of UMDNJ-Robert Wood Johnson Medical School. We hypothesized that p53^{R172H} mice would experience greater toxicity for a given dose of NSC319726 as compared to p53^{+/+} mice. By day 3 of treatment (10 mg/kg/day), all seven of the p53^{R172H/R172H} mice had died, whereas only one of nine p53^{+/+} mice had died. By day 4, the survival of the p53^{+/+} mice was 70%, whereas the survival of p53^{+/R172H} was 30%, suggesting a clear dosage effect that is dependent on the *TP53*^{R175} genotype (Figure 5A). Next, we lowered the dose to 5 mg/kg and found that by day 7, the p53^{+/+} and p53^{-/-} mice exhibited a 100% survival compared to only 30% of the p53^{R172H/R172H} mice (Figure 5A).

We examined the tissues of p53^{+/+} and p53^{R172H/R172H} mice after treatment for evidence of apoptosis as well as gene expression of a panel of p53 targets. We detected abundantly more apoptotic cells in the sections of the spleen and thymus of p53^{R172H/R172H} mice as compared with the p53^{+/+} controls (Figure 5B). We also detected elevated mRNA levels of a number of p53 targets in a tissue specific fashion in the p53^{R172H/R172H} mice as compared with the p53^{+/+} controls, most notably in the lung, spleen, thymus, and small intestine (Figure 5C).

We tested the ability of NSC319726 to inhibit the growth of xenograft mouse tumors derived from human tumor cell lines carrying different p53 alleles. At a dose of 1 mg/kg, tumor growth of the H460 (p53^{+/+}) and MDAMB468 (p53^{R273W}) xenografts was not inhibited relative to the vehicle control, whereas tumor growth was significantly inhibited in the TOV112D (p53^{R175H}) xenografts (Figure 5D). When we lowered the dose 10-fold to 0.1 mg/kg in the TOV112D mice, we observed only a small difference in tumor growth inhibition, demonstrating both a dosage effect of the drug and a larger therapeutic window. Taken together, these findings provide in vivo evidence for allele-specific p53 mutant reactivation.

Zinc Ion Chelation and Redox Changes Are Important for the NSC319726-Mediated p53^{R175} Reactivation Mechanism

Thiosemicarbazones are metal ion chelators with a strong affinity for iron, copper, and zinc (Yu et al., 2009). They have been investigated as anticancer agents and have been shown to inhibit DNA synthesis by inhibiting the iron-dependent enzyme ribonucleotide reductase (RR) but at much higher concentrations than are employed to inhibit the growth of p53^{R175} mutant cells. To determine if the metal ion chelating property of NSC319726 is important to its p53^{R175} mutant activity we added NSC319726 to TOV112D cells in the presence of various concentrations of FeSO₄. We found that FeSO₄ at concentrations above 15 μ M completely abrogated the activity

of NSC319726. At concentrations below 15 μM , the activity of NSC319726 was inhibited in a dose-dependent manner (Figure 6A).

We suspected iron was not the relevant metal ion, because iron is not associated with the p53 protein, and the treatment of the TOV112D cells with another iron chelator, desferrioxamine (DFO), had no apoptotic effect (Figure S2A). However, zinc is required for proper folding of WT p53, and the 175 mutant is classified as a non-zinc-binding mutant because it fails to coordinate zinc (Butler and Loh, 2003; Joerger and Fersht, 2007). When we added NSC319726 to TOV112D cells in the presence of different concentrations of ZnCl_2 , we found that there was an optimal zinc concentration range (5–15 μM) in which the activity of NSC319726 increased 2-fold (Figure 6B). We did not test higher concentrations over 100 μM of ZnCl_2 in combination with NSC319726 because we found that concentrations above 100 μM of ZnCl_2 (by itself) were toxic to cells. This toxicity was independent of p53 status (Figures S2B and S2C).

Another property of thiosemicarbazones is their effect on the redox state of the cell. Thiosemicarbazone:Fe complexes cause oxidative stress by the creation of hydroxyl radicals through Fenton chemistry (Kalinowski and Richardson, 2007; Richardson et al., 2006, 2009). This is relevant to the mechanism of NSC319726 as redox changes have been reported to influence WT p53 folding (Hainaut and Milner, 1993a). In support of this, we observed a statistically significant decrease in the levels of the cellular reductant glutathione upon NSC319726 treatment of TOV112D cells at 1, 3, and 24 hr (Figure 6C). To determine the importance of these redox changes to the activity of NSC319726 we treated TOV112D cells in the presence of the reducing agent N-acteyl-cysteine (NAC) and the oxidizing agent diamide. We found that 5 mM NAC inhibited the apoptotic activity of NSC319726, whereas diamide (100 μM) enhanced it (Figure 6D). These data suggest that ROS changes are important for the apoptotic mechanism of NSC319726 on p53^{R175} mutant cells.

DISCUSSION

The reactivation of p53 in mouse tumor models has been shown to be a highly effective therapeutic strategy (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). Several small molecules have been claimed to reactivate mutant p53, including CP-31398, WR-1065, PRIMA-1, and MIRA-1 (Bykov et al., 2002, 2005; Foster et al., 1999; North et al., 2002). With the exception of one compound, WR1065, all have been identified using traditional chemical screens (Bykov et al., 2002, 2005). Traditional chemical screens favor the use of matched case/control cell lines derived from the same parental cell line, engineered such that the case cell line carries the molecular alteration under consideration. This can be a fundamental caveat, because cancers are known to be heterogeneous in nature. Here, we demonstrate our methodology to screen for compounds manifesting increased sensitivity in a panel of cell lines carrying p53 mutations independently of their diverse genetic backgrounds and cell type specificity, which is a more realistic model of what is observed in the clinic. Applying this methodology to the NCI60 screen we identified three compounds from the thiosemicarbazone family. Follow up experiments with two of these

compounds (NSC319725 and NSC319726) corroborated the predicted p53-mutant-specific growth inhibitory properties. It is possible that this methodology could be used to identify compounds with increased sensitivity in tumor cell lines carrying mutations in other major oncogene/tumor suppressor pathways.

It is important to note that cell viability assays in Figure 1C and the apoptosis assays in Figure 2A indicate that there is an apoptotic mechanism that is independent of p53 mutational status (null, mis-sense mutant). What initiates this apoptosis is unclear but may be related to either an increase in ROS levels or ribonucleotide reductase (RR) inhibition, two reported mechanisms of action for thiosemicarbazones. Nontumor cell lines with a WT p53 gene (Balb/c 3T3, WI38) showed relatively little to no growth inhibition by NSC319726 at these same doses, which would argue against the inhibition of RR as the explanation. If increased ROS levels are the reason, we speculate that this apoptosis may be due to the inability to compensate for these oxidative changes in a cell lacking a functional p53 transcription factor.

In distinction, the mechanism of apoptosis in a p53^{R175} mutant cell is dependent on the mutant p53^{R175} mutant protein. NSC319726 treatment induces a WT-like conformational change in the p53^{R175} mutant protein that restores sequence-specific p53 transcription. This is best observed in the *in vivo* experiments employing both knockin p53 mutant mice and xenografts.

We have demonstrated that the metal ion chelating properties of NSC319726 are required for the mutant-p53-mediated apoptotic activity. Interestingly, metal ion chelation has been shown to induce p53 conformational changes (Hainaut and Milner, 1993b; Yu et al., 2009). The fact that the 175 mutant fails to bind zinc and that zinc chloride at low concentrations (5–15 μM) enhances the activity leads us to hypothesize that NSC319726 may serve as a source of zinc to allow the 175 mutant to refold. Such a metallochaperone function was demonstrated for another zinc chelator, Nitriloacetate, which facilitated refolding of the p53 WT DNA binding domain (that was previously unfolded by removing the zinc; Butler and Loh, 2007). Further biophysical studies are needed to confirm this.

Structural studies of the p53 DNA binding domain indicate that the zinc ion is coordinated by four amino acids (C176, H179, C238, and C242; Cho et al., 1994; Joerger et al., 2005; Wong et al., 1999). Mutations in any of these residues result in the inability to coordinate zinc. In contrast, the R175H mutant is not directly involved in zinc binding. It is generally believed that a histidine residue at this location induces structural distortions in the protein that prevent it from binding zinc (Joerger and Fersht, 2007). If the metallochaperone hypothesis is correct then it is plausible that NSC319726 may reactivate other zinc binding mutants.

The question of what activates mutant p53 to become a better transcription factor and induce an apoptotic mechanism after a WT conformational change is an important one. Most likely this is due to the elevated oxidative state in the mutant p53 cell. This oxidative state is the result of the combination of NSC319726 treatment and elevated ROS levels in p53 mutant cells (due to loss of p53-mediated regulation of the redox state; Sablina et al., 2005). In support of this is the observation that an oxidizing agent, diamide, enhances the apoptotic activity of NSC319726, whereas NAC inhibits it.

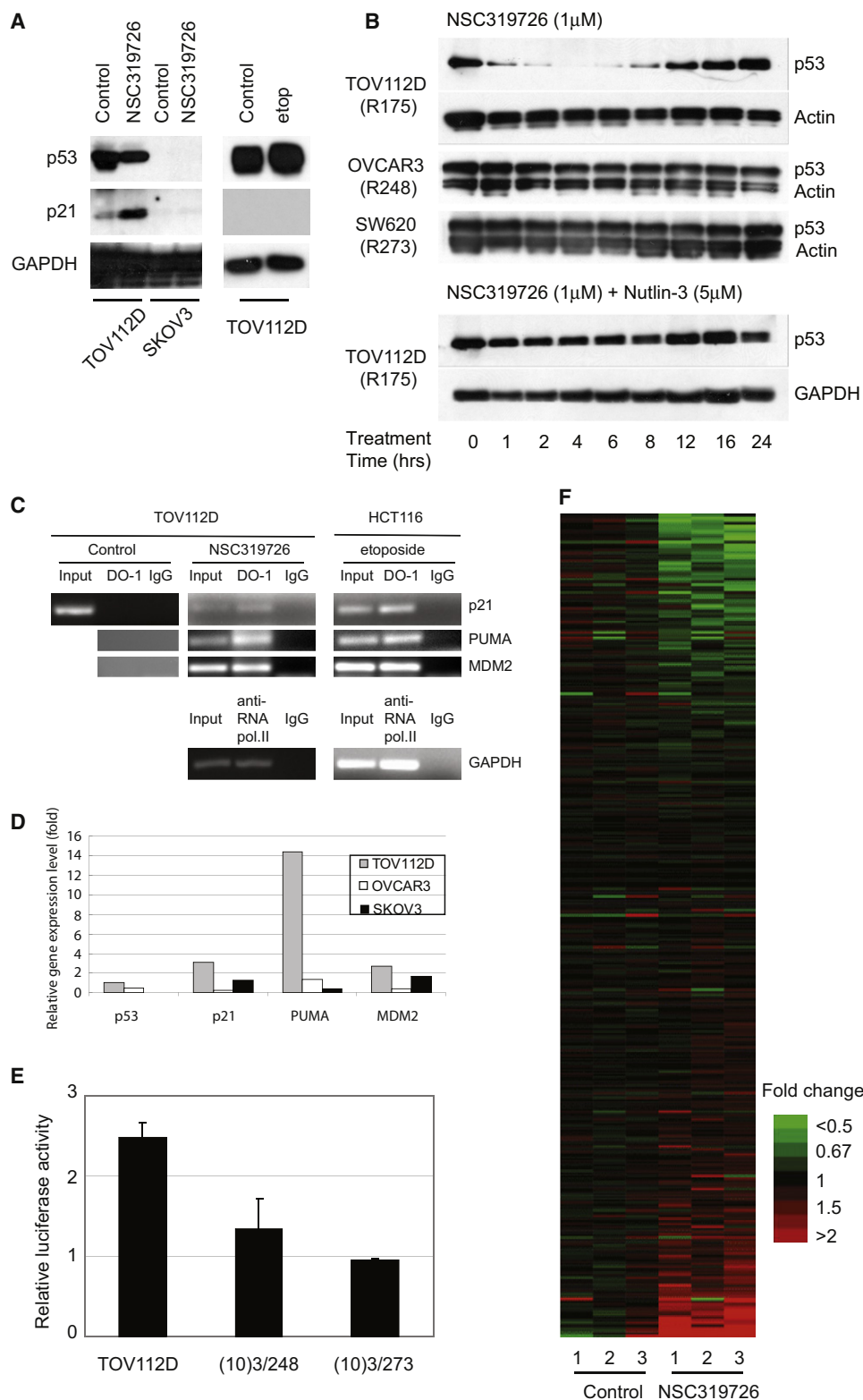


Figure 4. Restoration of Site-Specific p53-175 Mutant Protein Transactivational Function by NSC319726

(A) TOV112D and SKOV3 cells are treated with NSC319726 or etoposide and protein extracts are analyzed by western blot with antibodies directed against p21, p53. GAPDH is used as the internal (loading) control.
(B) Stability of p53 mutant protein in TOV112D (p53^{R175}), OVCAR3 (p53^{R248}), and SW620 (p53^{R273}) cells treated with NSC319726 (1 μ M) with/without Nutlin-3 (5 μ M) over a 24 hr time course. Protein levels are shown by western blot using antibodies to p53 (DO-1) or either actin or GAPDH.

Whereas inhibition of RR activity is a known mechanism of action of thiosemicarbazones, we feel this is unlikely involved in the p53^{R175} reactivation mechanism because (1) the drug is completely nontoxic to human fibroblasts at the IC₉₀ for p53^{R175} mutant cells and (2) the dose that inhibited p53^{R175} mutant xenograft tumor growth was completely nontoxic to mice. However, the doses we used in our mouse toxicity experiments were considerably higher, and RR inhibition may explain some of the toxicity observed in WT mice.

NSC319726 is an attractive lead compound for drug development for three reasons:

- (1) In vivo p53^{R175} mutant reactivation can be observed at doses that are nontoxic to WT animals;
- (2) The compound exhibits a wide therapeutic window when given intravenously; and
- (3) The target (p53 mutant protein) is found at high levels in cells.

The pool of potential patients for such a drug would be fairly large given the fact that the 175 mutant is the third most commonly found p53 mis-sense mutant, making up an estimated 5.5% of all mis-sense mutants (Olivier et al., 2010). Using the IARC TP53 database (<http://www-p53.iarc.fr/>), we estimate the annual incidence in the United States of cancer patients carrying the TP53^{R175} allele to be more than 32,000. Our findings support the growing trend in developmental therapeutics in which the efficacy of future cancer drugs will depend upon the knowledge of the patient's tumor genotype.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions

The mouse embryonic fibroblasts (MEFs) (10)3 and its derived cell lines with various human p53 mutations (R175, R248, and R273) were previously derived (Dittmer et al., 1993). 3T3 is derived from Balb/c and has p53^{+/+} alleles. MEF p53^{R172H/R172H}, MEF p53^{+/+} and MEF p53^{-/-} cells are derived from C57BL/6 mice. All MEF cells are cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. TOV112D and WI38 are cultured in DMEM with 10% FBS. SKOV3 is cultured in McCoy's 5A with 5% FBS. H460 and MCF7 are cultured in RPMI with 10% FBS. HCT116 is cultured in McCoy's 5A with 10% FBS. OVCAR3 is cultured in RPMI1640 with 20% FBS.

MTS Assay and Viability Assay

MTS assay is done in accordance with the manufacturer's instructions (Promega, Madison, WI, USA). In brief, 5,000 cells of TOV112D cells (5,000 cells/well, in 100 μ l culture) are cultured in 96-well plate to reach the 50%–60% confluence on the second day when treated with serial dilutions of the

compounds. The growth is measured by MTS reagent and Victor Plate reader instrument (PerkinElmer, Waltham, MA, USA) after incubation for 3 days. Viability assays are done in accordance with the manufacturer's instructions for Guava ViaCount (Millipore, Billerica, MA, USA). In brief, the cells (5×10^4 cells/well, in 1 ml culture) are cultured in a 12-well plate to reach the 50%–60% confluence on the second day when treated with serial dilutions of the compound. The growth is measured by Guava ViaCount reagent and Guava PCA instrument after incubation for 3 days.

Apoptosis Assay Using Annexin Staining

The Annexin staining is done in accordance with the manufacturer's instructions (Millipore). In brief, the cells are cultured in 12-well plate, followed by treatment with the compound for different time periods. The cells are stained with the Nexin staining reagent and Annexin-positive cells are detected with the Guava PCA instrument.

Transfection of p53 siRNA

The siRNA transfection is done with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The p53 siRNA is from SMARTpool (Thermo Scientific/Dharmacon, Lafayette, CO, USA).

Immunofluorescent Staining

The cells are grown on the coverslip, followed by various treatments. The coverslips are fixed with 4% paraformaldehyde for 10 min and then permeabilized with 0.5% Triton X-100 for 5 min. The conformation of the mutant and WT p53 proteins can be recognized specifically by the antibodies PAB1620 (1:50, recognizing WT conformation) and PAB240 (1:200, recognizing mutant conformation) stained overnight, respectively. The secondary antibody, goat anti-mouse IgG is incubated for 40 min. PAB1620 and PAB240 are from EMD Chemicals (Gibbstown, NJ, USA). Fluorescent staining intensity was quantified using Adobe Photoshop.

Immunoprecipitation

The cell lysates (500 μ g) with various treatments are subjected to immunoprecipitation with ExctaCruz matrix (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the antibody PAB240 (4 μ g). The pull-down is detected by western blot with p53 (FL393; Santa Cruz Biotechnology), which recognizes all formats of the p53 protein. The density of the image relative to the control is determined using Adobe Photoshop.

Immunohistochemistry Staining

The mouse tissues are harvested and are subjected to immunohistochemistry (IHC) staining with cleaved caspase-3 (CC3, 1:100; Cell Signaling, Danvers, MA, USA).

Western Blot

The lysates (or immunoprecipitated products) are run on SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The detection of the protein level is done with the manufacturer's instructions (ECL, GE Healthcare, Waukesha, WI, USA). The p21 antibody is from EMD Chemicals. The GAPDH, p53 (DO-1) and actin antibodies are from Santa Cruz Biotechnology. The density is determined using Adobe Photoshop and is expressed as the ratio to the loading control (GAPDH) relative to the control.

(C) Chromatin immunoprecipitation (ChIP) assay. Lysates from TOV112D cells treated with NSC319726 (1 μ M) and HCT116 cells treated with etoposide (20 μ M) for 6 hr are subject to immunoprecipitation using anti-p53 antibody (DO-1) followed by PCR of p53 recognition elements (p53REs) of *p21*, *PUMA*, and *MDM2*. HCT116 treated with etoposide is used as a positive control for functional p53 DNA-binding property. ChIP with anti-RNA polymerase II antibody and PCR for *GAPDH* is also used as a positive control.

(D) qRT-PCR of *p21*, *MDM2*, and *PUMA* in TOV112D, OVCAR3, and SKOV3 cells with treatment of NSC319726 (1 μ M) for 24 hr. The RNA is extracted from the cells using a Qiagen RNeasy kit, and the gene expression level is measured by quantitative RT-PCR using TaqMan gene expression assays.

(E) TOV112D cells are transfected with a plasmid bearing the 20 bp p53 response element (p53RE) in *p21* promoter region and then treated with NSC319726 (1 μ M) for 24 hr, followed by luciferase reporter assay. The error bars are \pm SD.

(F) Microarray analysis of NSC319726 (1 μ M, 24 hr) treated and control TOV112D cells comparing gene expression levels of p53 targets. The list of the genes is presented in Table S1. The heatmap is shown with the color scale indicating fold change. The data from the microarrays have been deposited in the GEO database with accession number GSE35972.

See also Table S1.

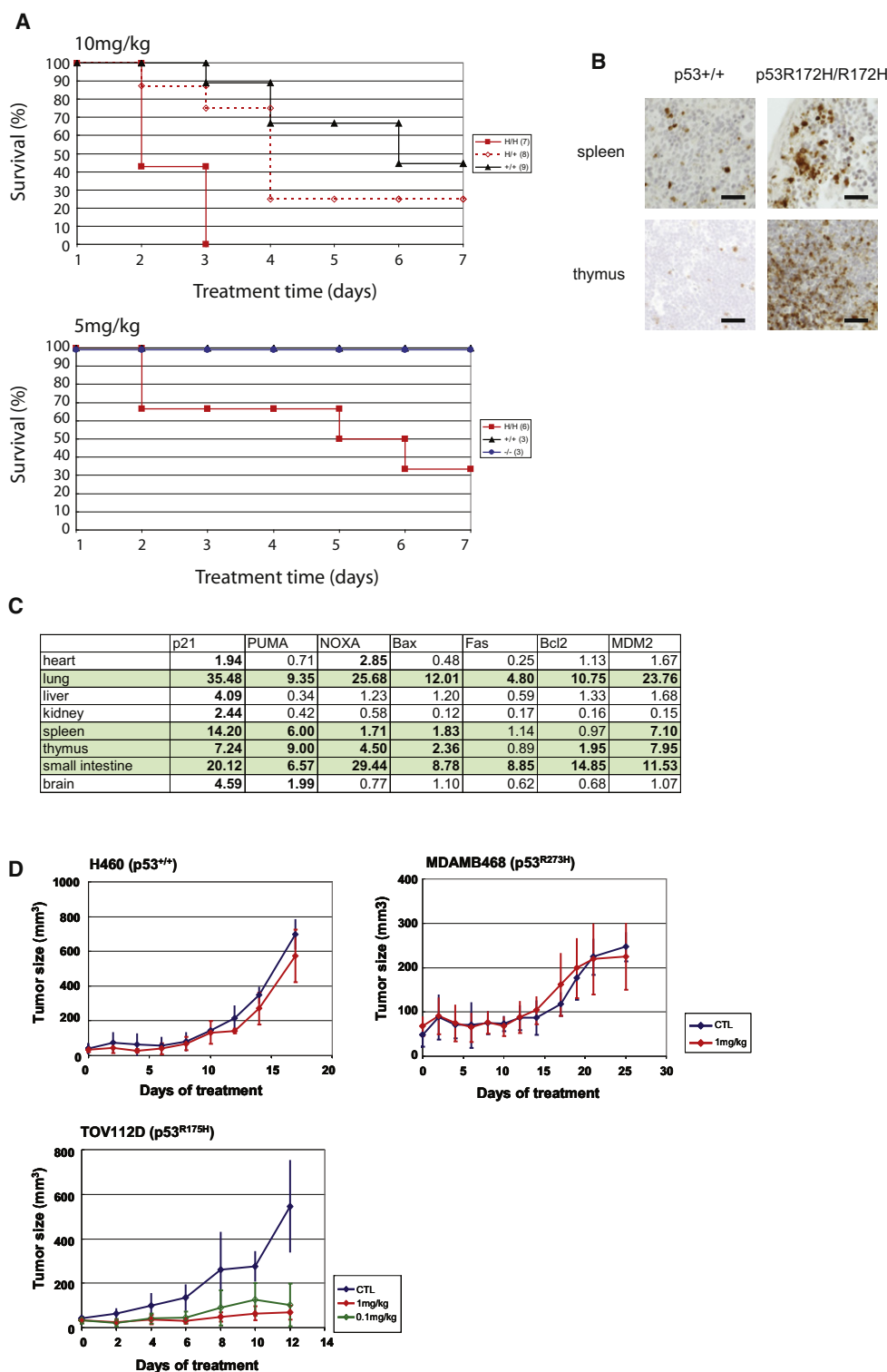


Figure 5. In Vivo Evidence of NSC319726-Mediated p53-175 Mutant Reactivation

(A) Toxicity assays of NSC319726 in p53^{R172H/R172H}, p53^{+/+}, and p53^{-/-} mice. Mice in the top panel are administered 10 mg/kg by daily intraperitoneal (i.p.) injection for up to 7 days. Mice in the bottom panel are administered 5 mg/kg daily (i.p.) for up to 7 days.

(B) Immunohistochemical staining with cleaved caspase-3 antibody of spleen and thymic tissues in p53^{+/+} and p53^{R172H/R172H} mice. All scale bars represent a size of 100 μ m.

(C) qRT-PCR of mRNA levels of several p53-regulated genes in p53^{+/+} and p53^{R172H/R172H} mouse tissues. The inductions of over 1.7 \times are highlighted in bold.

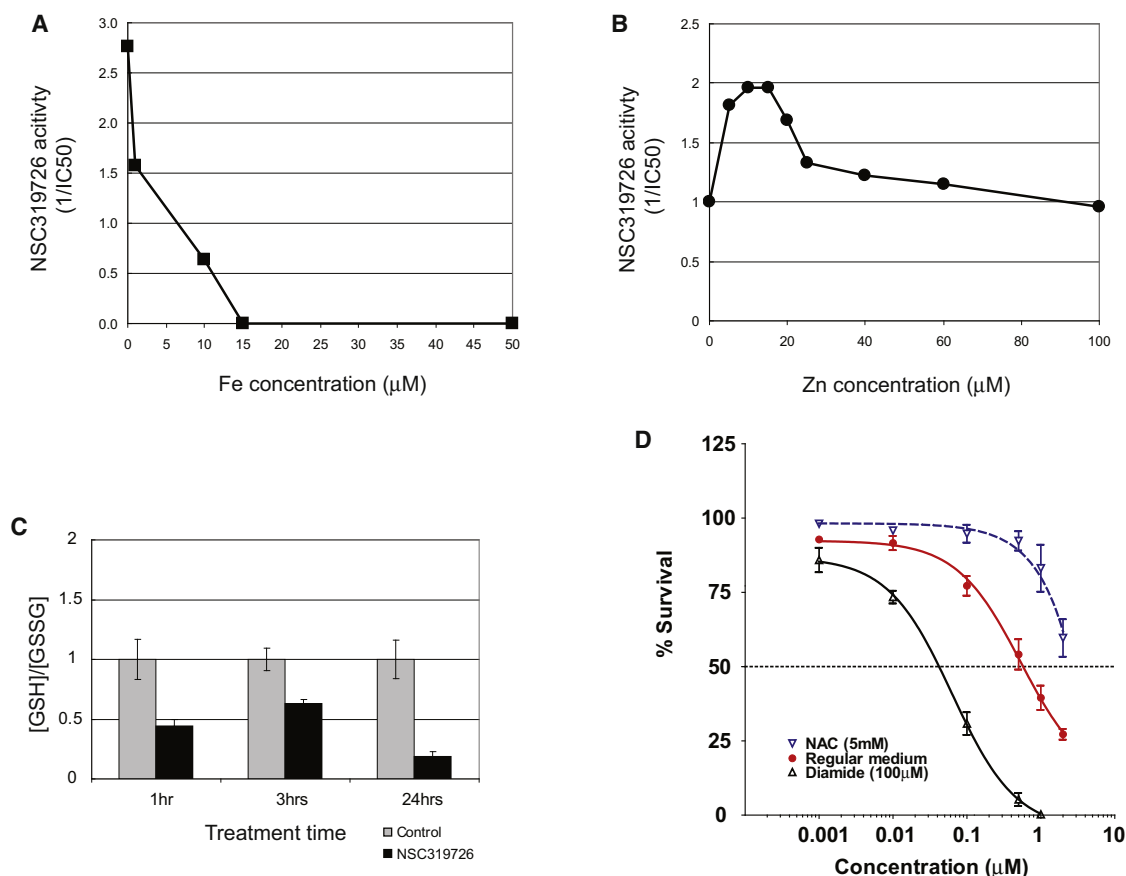


Figure 6. Zinc Ion Chelation and Redox Changes Are Important for the NSC319726-Mediated p53-175 Mutant Reactivation

(A) TOV112D cells are treated with NSC319726 with or without the presence of various concentrations of FeSO_4 , followed by measurement of growth inhibition. The NSC319726 activity is shown as $1/\text{IC}_{50}$ for cell growth inhibition.

(B) TOV112D cells are treated with NSC319726 with or without addition of various concentrations of ZnCl_2 , followed by measurement of growth inhibition. The NSC319726 activity is shown as $1/\text{IC}_{50}$ for cell growth inhibition.

(C) Ratio of reductant GSH and oxidative GSSG in the p53^{R175H} cells upon NSC319726 (1 μM) treatment is measured at several time points ($p = 0.0057$ at 1 hr, $p = 0.0027$ at 3 hr, and $p = 0.001$ at 24 hr, t test). The error bars are \pm SD.

(D) TOV112D cells are treated with NSC319726 using six serial dilutions (0.001 to 2 μM) with either N-acetyl cysteine (NAC, 5 mM) or diamide (100 μM) for 3 days. The growth inhibition was analyzed by MTS assay as in Figure 1C. The error bars are \pm SD.

See also Figure S2.

Luciferase Reporter Assay

The p53 recognition element (p53RE) in the p21 promoter region, constructed in pGL3 vector, is from Dr. Prives' laboratory (Columbia University, New York, NY, USA). It is transfected into the cells, followed by the treatment of NSC319726. The cell lysate is made and the luciferase reporter assay is done in accordance with the manufacturer's instructions (Promega).

Chromatin Immunoprecipitation and PCR

ChIP experiments are done using an EpiTect Chip One-Day Kit in accordance with the manufacturer's instructions (Qiagen, Venlo, The Netherlands). The recovered ChIP DNA is subject to PCR using primers flanking the p53 response elements (p53REs) in the p21, PUMA, and MDM2 genes. They are p21-F: 5'-GTGGCTCTGATTGGCTTTCTG, p21-R: 5'-CTGAAACAGGCAGC CCAAGG; PUMA-F: 5'-TCCTTGCCCTGGGCTAGGCC, PUMA-R: 5'-CGCGG ACAAGTCAGGACTTG; and MDM2-F: 5'-GGTTGACTCAGCTTTCTCTTG,

MDM2-R: 5'-GGAAAATGCATGGTTTAAATAGCC. The control primers are used for PCR of GAPDH provided by the kit.

RNA Extraction and Quantitative RT-PCR

RNA is extracted from the cells or mouse tissues using a Qiagen RNeasy Kit, and the gene expression level is measured by quantitative RT-PCR using TaqMan gene expression assays (Applied BioSciences, Carlsbad, CA, USA). The gene expression level is normalized with β -actin, and the average is presented with standard deviation from duplicates or triplicates of repeated experiments.

Microarray Assay

GeneChip Human Genome U133 Plus 2.0 array is from Affymetrix (Santa Clara, CA, USA). The RNA is extracted from the cells with or without treatment with NSC319726 and subject to microarray assay. The list of p53 targets were

(D) Efficacy assays of NSC319726. Xenograft tumors are generated using H460, MDAMB468, and TOV112D cells and are allowed to grow to 60 mm^3 prior to daily intravenous administration of NSC319726 at 1 or 0.1 mg/kg (TOV112D only). Tumor measurements are mean \pm SD. The following number of animals were used, H460 (Control-5, Treatment-5), MDAMB468 (Control-9, Treatment-8), TOV112D (Control-7, Treatment 1mg/kg-7, 0.1 mg/kg-7). CTL is control. The error bars are \pm SD.

selected from a literature review, providing a comprehensive list of experimentally verified targets of p53 (Riley et al., 2008).

Measurement of Level of Glutathione

The levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) are measured using the GSH-Glo Glutathione Assay in accordance with the manufacturer's instructions (Promega).

Mouse Experiments

Mice are housed and treated in accordance with guidelines and all the mouse experiments are done with the approval of Institutional Animal Care and Use Committee (IACUC) of the UMDNJ-Robert Wood Johnson Medical School. The mice with p53^{R172H/+} are a gift of Dr. Lozano (MD Anderson Cancer Center, Houston, TX, USA). The nude mice NCR nu/nu were purchased from Taconic. For the toxicity assays, 6- to 10-week-old mice were of the following genotypes, p53^{R172H/R172H} (n = 8), p53^{R172H/+} (n = 8), p53^{+/+} (n = 9), and p53^{-/-} (n = 3). Mice were treated with either NSC319726 at 10 or 5 mg/kg or vehicle control (dimethyl sulfoxide) by intraperitoneal injection daily for up to 7 days, followed by harvesting the mouse tissues. Xenograft tumor assays are derived from the human tumor cell lines, TOV112D, H460, and MDAMB468 (6 × 10⁶ cells/mouse). Tumor dimensions were measured every other day, and their volumes were calculated by length (L) and width (W) by using the formula: volume = L × W² × π/6. Tumors were allowed to grow to 60 mm³ prior to daily intravenous administration of NSC319726 at 1 or 0.1 mg/kg (TOV112D only). The following number of animals were used, H460 (Control-5, Treatment-5), MDAMB468 (Control-9, Treatment-8), and TOV112D (Control-7, Treatment 1 mg/kg-7, 0.1 mg/kg-7). The experiment was repeated two to three times with similar results.

ACCESSION NUMBERS

The data from the microarrays have been deposited in the GEO database with the accession number GSE35972.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2012.03.042.

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