Small-Molecule NSC59984 Restores p53 Pathway Signaling and Antitumor Effects against Colorectal Cancer via p73 Activation and Degradation of Mutant p53

Shengliang Zhang¹,², Lanlan Zhou¹,², Bo Hong¹, A. Pieter J. van den Heuvel¹, Varun V. Prabhu¹,², Noel A. Warfel³, Christina Leah B. Kline¹,², David T. Dicker¹,², Levy Kopelovich³, and Wafik S. El-Deiry¹,²

Abstract

The tumor-suppressor p53 prevents cancer development via initiating cell-cycle arrest, cell death, repair, or antiangiogenesis processes. Over 50% of human cancers harbor cancer-causing mutant p53. p53 mutations not only abrogate its tumor-suppressor function, but also endow mutant p53 with a gain of function (GOF), creating a proto-oncogene that contributes to tumorigenesis, tumor progression, and chem- or radiotherapy resistance. Thus, targeting mutant p53 to restore a wild-type p53 signaling pathway provides an attractive strategy for cancer therapy. We demonstrate that small-molecule NSC59984 not only restores wild-type p53 signaling, but also depletes mutant p53 GOF. NSC59984 induces mutant p53 protein degradation via MDM2 and the ubiquitin–proteasome pathway. NSC59984 restores wild-type p53 signaling via p73 activation, specifically in mutant p53-expressing colorectal cancer cells. At therapeutic doses, NSC59984 induces p73-dependent cell death in cancer cells with minimal genotoxicity and without evident toxicity toward normal cells. NSC59984 synergizes with CPT11 to induce cell death in mutant p53-expressing colorectal cancer cells and inhibits mutant p53-associated colon tumor xenograft growth in a p73-dependent manner in vivo. We hypothesize that specific targeting of mutant p53 may be essential for anticancer strategies that involve the stimulation of p73 in order to efficiently restore tumor suppression. Taken together, our data identify NSC59984 as a promising lead compound for anticancer therapy that acts by targeting GOF-mutant p53 and stimulates p73 to restore the p53 pathway signaling. Cancer Res; 75(18): 1–11. © 2015 AACR.

Introduction

Tumor-suppressor p53 protects cells from oncogenesis and promotes sensitivity to anticancer therapy. Over 50% of human cancers harbor mutant p53, which inactivates p53 pathway signaling and its tumor-suppressor function (1). p53 DNA mutations not only abrogate the p53 tumor-suppressor function, but can also endow mutant p53 with a gain of function (GOF), rendering it a proto-oncogene (2, 3). One property of mutant p53 GOF is to form aberrant protein complexes with numerous interacting protein factors, including a subset of transcription factors such as SP1, NF-Y, p53, and p63/p73, to perturb their activities (4). The GOF of mutant p53 contributes to tumorigenesis, tumor progression, and resistance to therapy (3). Therefore, targeting mutant p53 is an attractive strategy to overcome drug resistance and to sensitize tumors to cancer therapy. This concept is particularly further developed and mechanistically explored in this study with NSC59984.

Some small-molecule compounds targeting mutant p53 have been selected on the basis of putative conformational changes within mutant p53 to restore wild-type p53. For example, CP31398 (5), PRIMA-1 (6), and NSC319726 (7) have been proposed to cause a conformational shift from mutant to wild-type p53, reactivating p53 function in tumor suppression. Although several small molecules can restore the p53 pathway, the GOF of mutant p53 can remain in the tumor cell and can represent an obstacle to tumor suppression as well as therapeutic efficacy. Eliminating mutant p53 is an approach that we decided to pursue in an attempt to abolish the GOF properties of mutant p53 in tumor cells, and with the idea that mutant p53 may represent a challenge for the general approach to stimulate p73, given the ability of mutant p53 to quench the tumor-suppressive activity of p73. Few compounds have been reported to destabilize mutant p53, including 17AAG, Saha, gambogenic acid, and arsenic (8–11). However, those compounds are incapable of restoring the p53 pathway of mutant p53 in tumor cells, and they have many other targets and mechanisms, making them nonspecific. Thus, small molecules with the dual capability to

¹Penn State Hershey Cancer Institute, Hershey, Pennsylvania. ²Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Department of Medical Oncology and Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania. ³Division of Cancer Prevention, National Cancer Institute, Bethesda, Maryland.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Prior presentation: The work was presented, in part, at the AACR 104th Annual Meeting in Washington, DC, April 2013.

Corresponding Author: Wafik S. El-Deiry, Fox Chase Cancer Center, 333 Cottman Avenue, Room P2035, Philadelphia, PA 19111. Phone: 215-214-4233; Fax: 215-214-1590; E-mail: wafik.eldeiry@gmail.com
doi: 10.1158/0008-5472.CAN-13-1079
©2015 American Association for Cancer Research.
restore the p53 pathway and deplete mutant p53 GOF proteins represent a novel strategy for cancer therapy and appear desirable to pursue for further therapeutic development.

p73, a member of the p53 family, is a transcription factor with high structural and sequence homology with p53. p73 has been found to have similar functions as wild-type p53 (12). p73 can transactivate the vast majority of p53 transcriptional target genes by binding to their regulatory regions in the same manner as p53, thereby affecting cell growth and cell death pathways (13). Unlike p53, p73 is rarely deleted or mutated in human cancer. p73 is activated by complex signaling pathways in mammalian cells under stress (14). Activated p73 induces apoptosis and enhances chemosensitivity. A large variety of chemotherapeutic agents, such as camptothecin, etoposide, and cisplatin, can upregulate p73 expression (15). However, mutant p53 inhibits p73 activation by binding with p73 to form an inhibited complex with respect to the transactivation of target genes (4). Therefore, p73 provides a legitimate and bona fide attractive target to restore the p53 pathway in cancer therapy. A peptide, 37AA, has been found to cause p73-dependent cancer cell apoptosis (16). A small-molecule, RETRA, was shown to release p73 by disturbing interaction of p73 and mutant p53 (17). These studies support the strategy of bypassing dysfunctional p53 signaling in cancer therapy through stimulation of p73-dependent signaling, while at the same time attempting to eliminate mutant p53, as is reported here.

In this article, we use a functional cell-based high-throughput screening strategy to identify small-molecule compounds that both destabilize mutant p53 and restore wild-type p53 pathway via the activation of p73 in cancer cells. One such compound, NSC59984, appears to have a favorable therapeutic index, is nongenotoxic at effective doses that preferentially kill tumor cells, and displays antitumor effects in vivo in a p73-dependent manner.

Materials and Methods

High-throughput screening

Functional cell-based screening for small molecules that can increase p53-transcriptional activity was performed using noninvasive bioluminescence imaging in human colorectal cancer cells that stably express a p53-regulated reporter (Supplementary Materials and Methods), as previously described (18).

Cell lines

SW480, DLD-1, HCT116, and p53-null HCT116 cells that stably express a p53-regulated luciferase reporter were generated in our laboratory in 2003 (18, 19). MRC5, Wi38, Hop92, and RXF393 were obtained from the ATCC and cultured as recommended. Cells were regularly authenticated by bioluminescence, growth, and morphologic observation.

CellTiter-Glo luminescent cell viability assay

Cells were seeded at 5,000 cells per well on 96-well plates. Cells were mixed with an equal volume of CellTiter-Glo reagents (Promega), following the manufacturer's protocol, and bioluminescence imaging was measured using the IVIS imager.

Knocking down p73 expression by retroviral shRNA

Cells were infected with retrovirus containing the pSIREN-RetroQ retroviral vector recombinant with Tap73 RNAi. Cells were cultured with blasticidin for several weeks, and blasticidin-resistant clones were selected. Knockdown of p73 was detected by measuring p73 protein levels by Western blot analysis (Supplementary Materials and Methods) with anti-p73 antibody (Bethyl laboratories Inc.).

Overexpression of p73 by adenovirus infection

Cells were infected with an adenovirus that expresses p73-beta (Ad-p73) or wild-type p53 (Ad-p53) and cultured for 24 hours, as previously described (20). Then, the infected cells were cultured in fresh medium and subjected to different treatments.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). Reverse transcription used SuperScript II First-Strand Synthesis System (Invitrogen) with random primers. qRT-PCR reactions used SYBR Green Master Mix with the Real-Time PCR Detection systems (Bio-Rad). Primers for quantitative RT-PCR (qRT-PCR) are in Supplementary Materials and Methods.

Colony formation assays

Five hundred cells per well on 6-well plate were treated with NSC59984 for 3 days, then, cells were cultured with drug-free complete medium for 2 weeks with fresh medium changed every 3 days. Cells were fixed with 10% formalin and stained with 0.05% crystal violet at the end of 2 weeks period of cell culture (21).

In vivo antitumor assays

All animal experiments were approved by the Institutional Animal Care and Use Committee at Penn State University. Five million DLD-1 and p73 knockdown DLD-1 cells were implanted subcutaneously in the opposite flanks in each CRL nude mouse (female, 4–6 weeks old). Treatment with NSC59984 (i.p. injection) was initiated when the tumor masses reached a size of 3 to 5 mm. NSC59984 (45 mg/kg) was injected by i.p. route every 5 days. Fifteen days after treatment, the mice were euthanized.

Statistical analysis

All results were obtained from triplicate experiments, unless otherwise indicated. Statistical analyses were performed using Prism4 Software (GraphPad Software, Inc.), ANOVA, and the Student t test. Statistical significances were determined by P < 0.05. Combination indices were calculated using the Chou–Talalay method with CalcuSyn software (Biosoft).

Results

NSC59984 specifically restores p53 pathway signaling in mutant p53-expressing human colorectal cancer cells

To identify small molecules that could restore p53 pathway signaling, we screened approximately 1990 small molecules from the National Cancer Institute (NCI; Bethesda, MD) chemical diversity library II using a functional cell-based assay. A small molecular weight compound, NSC59984 (IUPA name is (E)-1-(4-methylpiperazin-1-yl)-3-(5-nitrofuran-2yl)prop-2-en-1-one; Fig. 1A) was found to increase p53-responsive reporter activity in both mutant p53-expressing human colorectal cancer cells and normal colorectal cells. To determine the appropriate dose of NSC59984 for subsequent experiments, we treated SW480 (mutant p53 R273H, P309S) and DLD-1 (mutant p53 S241F) cells in a dose-dependent manner (1/slope ¼ 31.37 in SW480, and 29.75 in DLD-1, P0.01 compared with cells lacking mutant p53; Fig. 1B and C). Consistent with p53 activation, endogenous protein levels of p21, Puma, Noxa, and DR5, target genes of p53, were significantly upregulated in SW480 cells and
NSC59984 restores p53-responsive transcriptional activity in mutant p53-expressing tumor cells. A, structure of NSC59984. B, imaging bioluminescence assay of p53-responsive transcriptional activity in cells at 24 hours after NSC59984 treatment. Data are representative of triplicate wells. C, the fold increase of p53-responsive bioluminescence in cells at 8 hours after NSC59984 treatment. E, protein levels of p53 target genes in cells at 8 hours after NSC59984 treatment. HCT116 cells (slope = 328.4; Fig. 1B and C). Puma and p21 were significantly increased in response to 25 μmol/L of NSC59984 in HCT116 cells and p53-null HCT116 cells (Fig. 1E), the mRNA level of p21 was not significantly increased in response to 25 μmol/L of NSC59984 (Fig. 1F). Furthermore, mRNA levels of p21, Noxa, and Puma were not upregulated at the mRNA level in these two cell lines, which lack mutant p53, in response to NSC59984 treatment. Noxa mRNA was slightly increased in response to 25 μmol/L of NSC59984 in HCT116 and 12 μmol/L of NSC59984 in p53-null HCT116 cells. However, Noxa mRNA was increased much less in these two cells than in mutant p53-expressing cancer cells DLD-1 and SW480 (Fig. 1D). Consistent with results showing lack of increase in mRNA levels of p53 target genes, protein levels of Puma, DR5, and Noxa were not upregulated in HCT116 and p53-null HCT116 cells treated with NSC59984. Although p21 protein was upregulated in HCT116 cells and p53-null HCT116 cells (Fig. 1E), the mRNA level of p21 was not significantly increased in response to NSC59984 treatment (Fig. 1D), suggesting that NSC59984-mediated upregulation of p21 protein occurs at a posttranslational level in HCT116 and p53-null HCT116 cells. Taken together, these results indicate that NSC59984 restores p53 pathway signaling specifically in mutant p53-expressing human cancer cells.

NSC59984 induces cell death in tumor cells but not normal cells with little or no genotoxicity

We investigated the effect of NSC59984 on cell death in tumor cells because NSC59984 restores the p53 pathway in mutant p53-expressing cancer cells. We first determined EC50 values for NSC59984 using a panel of cancer cell lines bearing different p53 mutations. The EC50 of NSC59984 varied among different cancer cell lines tested, which harbor different p53 mutations. The EC50 of NSC59984 in most cancer cells was found to be significantly lower than those of normal cells (Fig. 2A). FACS analysis showed that 25 μmol/L of NSC59984 increased the sub-G1 DNA content (26%–56%) in cancer cells, but not in normal cells at 72 hours after treatment (Fig. 2B). The high dose of NSC59984...
Zhang et al.

Figure 2.
NSC59984 induces cell death in cancer cells with no genotoxicity. A, cell viability of cells treated with NSC59984 for 72 hours. Cell viability data were normalized to those of DMSO treatment as control in each cell line and data analyses were performed using PRISM4 software (left). EC50 data are expressed as mean ± SD in normal fibroblast cells (normal; n = 3), p53-mutant cancer cells (MTP53; n = 9), and cancer cells with wild-type p53 (WTp53; n = 3; right). B, cell-cycle profiles of cells at 72 hours after NSC59984 treatment. C, colony formation of cancer cells. A percentage of colonies was obtained for each respective cell line treated with DMSO. Data represent mean ± SD; *, P < 0.05. D, cleaved PARP protein level in cells treated with NSC59984 for 30 hours. E, genotoxicity of NSC59984 in SW480 cells at 8 and 16 hours after NSC59984 treatment. F, genotoxicity of NSC59984 in DLD-1 and HCT116 cells treated with NSC59984 for 8 hours. G, SW480 cells were treated with NSC59984 and DNA damage agent etoposide (EPT) for 8 hours. Genotoxicity was measured by γH2AX.

(50 μmol/L) led to a 55% to 93% cancer cells to have sub-G1 content but only 12% and 30% of DNA content was found to be in sub-G1 in Wi38 and MRC-5 normal human fibroblast cells (Fig. 2B). Taken together, these data suggest a favorable therapeutic index between normal and cancer cells. Colony formation assays further confirmed that NSC59984 was toxic toward cancer cells. Thus, NSC59984 significantly reduced colony numbers in cancer cells (Fig. 2C). We also examined cleaved PARP as a hallmark of caspase-3–dependent cell death in cells treated with NSC59984 for 30 hours. As shown in Fig. 2D, NSC59984 increased PARP cleavage in cancer cells in a dose-dependent manner, but PARP cleavage was not observed in normal cells at the same doses. On the basis of these findings, we conclude that NSC59984 induces cell death in cancer cells but displays little or no cytotoxicity toward normal cells at the doses tested.

To determine whether NSC59984 has chemical genotoxicity that may be involved in cell death and p53 pathway restoration, we examined the level of γH2AX, a marker of genotoxicity due to DNA double-strand breaks (22). No γH2AX was found in SW480 and DLD-1 cancer cells treated with NSC59984 within 16 hours, even at high concentrations of 50 μmol/L (Fig. 2E–G). These data suggest that NSC59984 has little or no genotoxicity at the doses that effectively kill mutant p53-expressing cancer cells. We observed an increase of γH2AX in HCT116 cells at 8 hours after 12 μmol/L of NSC59984 treatment.

NSC59984 induces mutant p53 protein degradation through MDM2-mediated ubiquitination in cancer cells

Given that NSC59984 restores p53 pathway signaling specifically in mutant p53-expressing cancer cells, we hypothesized that mutant p53 was the molecular target in NSC59984-treated cancer cells. NSC59984 treatment downregulated various mutant p53 proteins in a dose-dependent manner in SW480, DLD-1, Hop92 (mutant p53 R175L), and RFX393 cells (mutant p53 R175H; Fig. 3A). In contrast, wild-type p53 protein was upregulated in MRC-5 normal cells treated with 50 μmol/L of NSC59984.
p53 protein. Neither cleaved PARP (Fig. 3), nor sub-G1 DNA content to 8.7% and 8.2% from 4.5% and 5.8% of DMSO control (Fig. S1). At this dose, 25% to 50% of total mutant p53 protein was degraded in DLD-1 and RXF393 cells treated with NSC59984 (Supplementary Fig. S1) in the context of reduced mutant p53 protein expression. Increasing dose of NSC59984 (50 μmol/L) slightly increased sub-G1 DNA content to 8.7% and 8.2% from 4.5% and 5.8% of DMSO treatment in RXF393 and DLD-1 cells, respectively (Supplementary Fig. S1). At this dose, 25% to 50% of total mutant p53 protein was degraded in DLD-1 and RXF393 cells treated with NSC59984 (Fig. 3A). Taken together, these results suggest that cell death is not a significant mechanism by which NSC59984 reduces mutant p53. Thus, these results exclude the possibility of cell death as a major mechanism for the NSC59984-mediated depletion of mutant p53 protein.

Ubiquitination is a major mechanism by which p53 protein stability is regulated (23). We treated mutant p53-expressing cancer cells with MG132, a proteasome inhibitor. MG132 treatment rescued the NSC59984-mediated downregulation of mutant p53 (Fig. 4A). Moreover, we detected increased ubiquitination of mutant p53 in cancer cells treated with NSC59984 and MG132 (Fig. 4C). Taken together, our results suggest that NSC59984 causes mutant p53 protein ubiquitination and proteasomal degradation. To further determine whether p53 transcription contributes to the NSC59984-mediated decrease of mutant p53, we examined the mRNA level of mutant p53 in SW480 cancer cells. We found that p53 mRNA was not decreased in SW480 cells at 3 hours or at 16 hours of continuous NSC59984 treatment as compared with the DMSO control (Fig. 4D). These results taken together suggest that the effect of NSC59984 on decrease of mutant p53 protein occurs mostly at the posttranslational level.

To investigate the role of MDM2 in NSC59984-mediated mutant p53 protein degradation, we treated mutant p53-expressing cancer cells with nutlin-3, an MDM2 inhibitor. Nutlin-3 treatment partially rescued the NSC59984-induced decrease in mutant p53 (Fig. 4B). We further found that mutant p53 was phosphorylated at Thr55 and MDM2 was phosphorylated at Ser166 in response to NSC59984 treatment in SW480 cells (Fig. 4A and B). Both phosphorylation of p53 at Thr55 and phosphorylation of MDM2 at Ser166 are important protein modifications that allow p53 degradation via MDM2 (24, 25). Taken together, these results suggest that NSC59984 induces mutant p53 protein degradation, in part, through an MDM2-mediated proteasomal mechanism.

To determine whether there is a wild-type conformational shift in p53 after NSC59984 treatment of mutant p53-expressing cancer cells, we examined the wild-type p53 protein in RXF393 (p53 R175L) cells using the Pab1620 antibody. p53 R175L is a mutant that has been previously examined after exposure of p53-mutant cancer cells, we examined the wild-type p53 protein in RXF393 (p53 R175L) cells using the Pab1620 antibody.

Our results suggest that NSC59984 specifically induces mutant p53 protein degradation, at least in part, via the MDM2-mediated proteasomal pathway.
NSC59984 restores p53 pathway signaling through activation of p73

It is possible that mutant p53 degradation leads to the release of p73 from their mutual complex. Therefore, we hypothesized that p73 function may be stimulated in NSC59984-treated cancer cells as a mechanism to restore p53 pathway signaling. To address this issue, we examined the effect of NSC59984 on the p53 pathway in mutant p53-expressing tumor cells in which p73 was either overexpressed by adenovirus infection or constitutively knocked down by shRNA. NSC59984 treatment significantly increased p53-responsive bioluminescence interestingly to much higher levels in the p73-overexpressing SW480 cells than in the wild-type p53-overexpressing SW480 cells (Fig. 5A). The NSC59984-induced p53-responsive bioluminescence was abrogated by knockdown of p73 (Fig. 5B). We consistently observed that NSC59984-mediated upregulation of endogenous of p21, Puma, DR5, and Noxa proteins was reduced by p73 knockdown (Fig. 5). However, a small amount of p21 protein induction was still observed in p73 knockdown DLD-1 cells treated with 25 μmol/L of NSC59984 (Fig. 5C). NSC59984 was found to upregulate p21 expression at the protein level in HCT116 cells (Fig. 1). It is possible that p21 expression is regulated at transcriptional and protein levels in DLD-1 cells in which mRNA and protein levels of p21 were increased in response to NSC59984, although we believe only the transcriptional effects are p53-dependent (Fig. 1). Thus, knockdown of p73 only partially inhibited the NSC59984-mediated increase of p21 protein in DLD-1 cells (Fig. 5C). Taken together, these results indicate that p73 appears to be required for NSC59984 to restore the p53 pathway in mutant p53-expressing cancer cells.

NSC59984 induces p73-dependent cell apoptosis in cancer

Given the role of p73 in the NSC59984 restoration of the p53 pathway, we next investigated the impact of p73 on NSC59984-induced cell death. NSC59984 treatment synergized with exogenous p73 to reduce cell viability in DLD-1 cancer cells (Fig. 6A and Supplementary Table S1). In contrast, cell viability in p73 knockdown DLD-1 cells was found to be higher than those in DLD-1 cells after NSC59984 treatment (Fig. 6B). FACS assays revealed that the percentage of cells with sub-G1 content was increased by NSC59984; however, the effect of NSC59984 was significantly reduced by knockdown of p73 (Fig. 6C), suggesting that deficiency of p73 rescues cells from NSC59984-induced apoptosis. Consistent with these observations, NSC59984-induced PARP cleavage was partially abrogated by knockdown of p73 in DLD-1 cells at 24 hours of NSC59984 treatment (Fig. 6D). Taken together, these results suggest that NSC59984 induces p73-dependent cell death in mutant p53-expressing cancer cells.

CPT11 is a DNA-damaging agent used as cancer therapy in the clinic to treat colorectal cancer. CPT11 treatment has been reported to increase p73 protein levels in cancer cells (15). To test whether NSC59984 mediates the cellular sensitivity to conventional chemotherapy, the combination of NSC59984 and CPT11 was applied to cancer cells and normal human fibroblast cells. There was synergic activity of combinational treatment with NSC59984 and CPT11 in SW480 colon cancer cells as well as in normal fibroblast MRC5 and Wi38 cells (Supplementary Table S2). Cell viability assay showed that combinational treatment with NSC59984 and CPT11 significantly reduced cell viability in SW480 cancer cells as compared with those in MRC5 and Wi38 normal cells (Fig. 6E).

NSC59984 represses xenograft tumor growth in vivo

We further tested the potential therapeutic effects of NSC59984 in nude mice bearing colon-tumor xenografts. NSC59984 did not cause an obvious change in mouse body weights (Fig. 7D), and no overt toxic effect of NSC59984 was observed in mice with the...
tested dose. NSC59984 treatment significantly repressed DLD-1 xenograft tumor growth as compared with the DMSO control (Fig. 7A). We further measured tumor weight at day 15. Tumor weight was reduced by 34% with NSC59984 treatment in DLD-1 xenograft tumors, suggesting the NSC59984 suppresses tumor growth ($P < 0.05$; Fig. 7C). In contrast, we did not observe tumor growth suppression in p73 knockdown DLD-1 xenograft tumors in response to NSC59984 at the same dose (Fig. 7B). NSC59984 treatment reduced tumor weight by 18% in p73 knockdown DLD-1 xenograft tumors (Fig. 7C). These results further confirm our observation in vitro that p73 is required for NSC59984 to induce tumor cell death in DLD-1 cells.

**Discussion**

Most small molecular weight compounds targeting mutant p53 in cancer therapy do so by either restoring the p53 pathway or by abolishing mutant p53 to remove GOF. Here, we report a small-molecule NSC59984 that not only specifically restores the p53 pathway through p73 but also depletes mutant p53 protein in mutant p53-expressing cancer cells.

There are several thousand mutations of p53 that have been reported in human cancer (26). Most small molecules restoring the p53 pathway have been identified and tested against a certain mutation of p53. For example, identification of Phalan083 was based on the Y222C mutation (27), SCH529074 was based on N268R mutation (28), PRIMA-1 was based on the mutations at residues 273 and 175 (6), and NSC-319726 was based on R175H mutation (7). Therefore, it has remained a challenge to develop universal p53-restoring drugs.

In our study, we demonstrate that NSC59984 causes degradation of multiple mutant p53 proteins in a variety of human cancer cell lines (Fig. 3A), suggesting the versatility of NSC59984 to target various mutants. Importantly, NSC59984 specifically targets mutant p53 and the restoration of the p53 pathway in mutant p53-expressing cancer cells, although it appears to have p53 pathway-independent effects in other tumor cell lines, that is, p21 upregulation, which may suppress their growth. We found that NSC59984 treatment leads to the degradation of mutant p53, but not wild-type p53 protein. Wild-type p53 protein was upregulated by NSC59984 at high doses in normal cells and cancer cells (Fig. 3B and C). We documented a corresponding effect of NSC59984 to specifically restore the p53 pathway in mutant p53-expressing colorectal cancer cells, but not reactivation of the p53 pathway in tumor cells with wild-type p53 or restoration of the p53 pathway in those that are p53-null. This conclusion is based on our findings that: (i) NSC59984 increased p53-Luc reporter bioluminescence only in mutant p53-expressing cancer cells SW480 and DLD-1; (ii) expression of the p53 target genes p21, Puma, DR5, and Noxa was significantly upregulated at the mRNA and the protein levels by NSC59984 in mutant p53-expressing cancer cells SW480 and DLD-1 as compared with those in wild-type p53-expressing HCT116 and p53-null HCT116.
(Fig. 1); (iii) NSC59984 significantly increased p53-responsive bioluminescence to much greater levels in p73-overexpressing SW480 cells than in p53-overexpressing SW480 cancer cells (Fig. 5A). These data suggest the specificity of NSC59984 for targeting p53 mutant in cancer. The specificity and versatility of NSC59984 for targeting mutant p53 indicates that NSC59984 is a promising small-molecule drug candidate for further development through targeting restoration of the p53 pathway, in part, through degradation of the mutant p53 protein.

The mutant p53 protein level is high in tumor cells due to its stabilization (4). Stabilization of mutant p53 is mostly due to the inability of mutant p53 to interact with MDM2, an E3 ubiquitin ligase (3). Our data demonstrate that NSC59984 induces mutant p53 protein degradation via MDM2-mediated ubiquitination and proteasomal degradation (Fig. 4). Another compound, NSC319726, provided a model for the wild-type conformational change from mutant p53 to be sequentially degraded through MDM2-mediated ubiquitination (7). However, we did not find a wild-type p53 conformational change in tumor cells treated with NSC59984 (Supplementary Fig. S2) or downregulation of wild-type p53 protein by NSC59984 treatment (Fig. 3B and C). These results suggest that NSC59984 mediates the downregulation of p53 due to mutant p53 degradation rather than restoration of a less stable wild-type p53 protein.

Hsp90 and Hsp70 are two molecular chaperones that stabilize mutant p53 protein by affecting the MDM2-mediated turnover of mutant p53 (29). Thus, mutant p53 escapes from MDM2-mediated degradation, and there are lower levels of MDM2 in mutant p53-expressing cells due to reduced transactivation of the MDM2 gene by p53 (3). Inhibition of Hsp90 has been shown to destabilize mutant p53 through MDM2 activity (9, 10). It remained unclear whether NSC59984 induces mutant p53 protein degradation through disturbing the MDM2–Hsp chaperone axis. A conformational change of mutant p53 is another possible mechanism of mutant p53 degradation by NSC59984. For example, CP31398 induces a wild-type conformational change in mutant p53 by modifying the unfolded mutant p53 (5). PRIMA-1 causes a conformational change by forming adducts with thiols in the mutant p53 core domain (30). Although NSC59984 did not restore a wild-type p53 conformation, it is possible that NSC59984 converts the mutant p53 structure to one more amenable to the MDM2-mediated ubiquitination to regulate mutant p53 degradation. We found phosphorylation of mutant p53 at Thr55 and phosphorylation of MDM2 at Ser166 in SW480 cancer cells treated with NSC59984 (Fig. 4). Phosphorylation of p53 at Thr55 and phosphorylation of MDM2 at Ser166 have been reported to contribute to p53 protein ubiquitination (24, 25). Our results suggest a possibility that NSC59984 induces mutant p53 and MDM2 protein modifications that contribute to mutant p53 protein degradation. It remains unclear how mutant p53 and MDM2 are phosphorylated by signaling pathways stimulated by NSC59984 in mutant p53-expressing cancer cells.

Mutant p53 protein degradation is an effective means to remove its GOF, resulting in release of p73 and other factors from inhibitory complexes with mutant p53. However, many mutant p53 protein targeting small molecules, such as HDACI and 17AAG, do not restore the p53 pathway (10, 31). Unlike these mutant p53-targeting agents, NSC59984 not only degrades mutant p53 protein to release p73, but also induces p73-dependent p53 restoration (Figs. 1 and 5). It is possible that NSC59984 converts the released p73 to an active form or stabilizes p73. Active p73 functions as a transcription factor to upregulate p53 target genes such as p21, puma, and DR5. We found that NSC59984 significantly induces p73-dependent p53 restoration only in mutant p53-expressing cancer cells (Figs. 1 and 5), but not in p53-null or wild-type p53-expressing HCT116 cells that...
contain wild-type p73 (Fig. 1). These results suggest that restoration of the p53 pathway occurs, at least in part, through the release of p73 in mutant p53-expressing tumor cells. The fraction of released and active p73 could be increased by mutant p53 degradation. We did not find increased p73 protein levels in the mutant p53-expressing tumor cells treated with NSC59984 at 8 hours by Western blot using anti-p73 antibody (Bethyl Laboratories Inc.). It is possible that posttranslational modifications of p73 protein are induced by NSC59984, and that such NSC59984-mediated modifications interrupt the anti-p73 antibody (Bethyl Laboratories Inc.) recognizing modified p73 protein. We found that NSC59984 significantly enhanced p73-dependent p53-responsive reporter bioluminescence in the p73-overexpressing cells (Fig. 5A), suggesting that active p73 is involved in NSC59984-mediated p53 restoration. p73 activity is regulated by complex posttranslational modifications and protein–protein interactions (14). The mechanism by which NSC59984 activates the released p73 remains to be further elucidated. Nevertheless, we demonstrate that p73 is required for NSC59984 to induce cell death in addition to restoring p53 pathway. We found that knockdown of p73 not only rescues cells from apoptosis induced by NSC59984 in vitro (Fig. 6), but also prevents the NSC59984-mediated suppression of xenograft tumor growth in vivo (Fig. 7). Although mutant p53 deletion has been reported to be sufficient to induce cell death (10, 31), our findings suggest that NSC59984-induced cell death is p73-dependent in mutant p53-expressing cancer cells. Activation of p73 is an important step for p73-induced cell death (14). How active p73 regulates cell death in response to NSC59984 treatment will be further investigated in the future. Because p73 is required for both NSC59984-mediated p53 restoration and cell death, it is possible that NSC59984 induces cell death via p73-dependent restoration of the p53 pathway. Therefore, NSC59984 offers a rational bypass mechanism of p53 restoration via the activation of p73 to kill cancer cells. On the basis of our findings, we provide a model for NSC59984 working in cancer cells (Fig. 7E). NSC59984 releases p73 from the inhibitory complex of mutant p53 by degrading mutant p53. The released p73 may be further converted to the active form by NSC59984. Active p73 then restores the p53 pathway. NSC59984-mediated p53 restoration and/or active p73 together with depletion of GOF may result in tumor suppression (Fig. 7E).

NSC59984 induces cell death in mutant p53-expressing cancer cells with minimal genotoxicity. Importantly, we did not find cytotoxicity of NSC59984 against normal cells at the doses tested (Fig. 2), suggesting the safety of NSC59984 administration for cancer therapy. Indeed, in vivo experiments demonstrate that i.p. injection of NSC59984 suppresses xenograft tumor growth (Fig. 7), but was not toxic toward animals, suggesting that NSC59984 may be further evaluated for clinical development. Mutant p53 protein degradation releases many factors, including p73, from the mutant p53 protein complex (4), which might result in p73 independent off-target effects. Because of the specificity of NSC59984 for mutant p53, the potential off-target effects may be limited in mutant p53-expressing cells and may not affect normal cells containing wild-type p53. However, the antitumor effect of NSC59984 on cancer cells is not limited in p53-mutant cancer cells (Fig. 2). We noted that NSC59984 induced cell apoptosis in wild-type p53-expressing as well as p53-null cancer cells, suggesting that the cell death induced by NSC59984 in these cell lines is p53-independent. It is possible there may be tumor-suppressive effects due to NSC59984-mediated upregulation of p21 protein in these two cell lines (Fig. 1). Our findings suggest that NSC59984 regulates p21 expression at the posttranslational level in HCT116 and p53-null HCT116 cancer cells. p21 regulation at the posttranslational level could be an off-target effect of NSC59984 in cancer cells, and
may involve effects of NSC59984 on MDM2 in a p53-independent manner that remains to be further unraveled.

*p73* is an important determinant of chemosensitivity. In response to cellular stresses and DNA damage, *p73* is activated through different signaling pathways and enhances chemosensitivity ([14, 15]). However, mutant *p53* inhibits *p73* activation, resulting in drug resistance. Our finding that NSC59984 rescues *p73* activity to restore the *p53* pathway provides a potential application of NSC59984 to reduce chemoresistance. Indeed, NSC59984 synergizes with CPT11 to suppress colorectal cancer cell growth (Fig. 6E and Supplementary Table S2). Therefore, NSC59984 warrants further evaluation in combination therapy to reduce the dose of CPT11 required for growth suppression in colorectal cancer. Combinatorial treatment with NSC59984 may minimize the side effects of CPT11 chemotherapy and increase its antitumor effects in colorectal cancer patients. Taken together, these results demonstrate that NSC59984 is a candidate therapeutic as both a single agent or in combination with conventional chemotherapy. On the basis of the findings of this study, we conclude that NSC59984 is a promising drug candidate that specifically targets mutant *p53* via a mechanism involving both mutant *p53* depletion and *p73*-dependent *p53* pathway restoration.

**Disclosure of Potential Conflicts of Interest**

W.S. El-Deiry has ownership interest (including patents) in p53-Therapeutics, Inc. W.S. El-Deiry is a founder of p53-Therapeutics, Inc., a biotech company focused on developing small-molecule anticancer therapies targeting mutant *p53*. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: S. Zhang, L. Kopelovich, W.S. El-Deiry

Development of methodology: S. Zhang, L. Zhou, B. Hong, V.V. Prabhu, N.A. Warfel, C.L.B. Kline, L. Kopelovich, W.S. El-Deiry

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Zhang, L. Zhou, A.P.J. van den Heuvel, D.T. Dicker, W.S. El-Deiry

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Zhang, L. Zhou, A.P.J. van den Heuvel, V.V. Prabhu, L. Kopelovich, W.S. El-Deiry

Writing, review, and/or revision of the manuscript: S. Zhang, A.P.J. van den Heuvel, N.A. Warfel, L. Kopelovich, W.S. El-Deiry

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Zhang, L. Zhou, D.T. Dicker, L. Kopelovich, W.S. El-Deiry

Study supervision: W.S. El-Deiry

Other (oversight of every aspect of the research): W.S. El-Deiry

**Grant Support**

The work was supported, in part, by NIH grants (N01-CN43302-WA17 and N01-CN43302-WA-27) to W.S. El-Deiry. W.S. El-Deiry is an American Cancer Society Research Professor.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 18, 2013; revised May 25, 2015; accepted June 14, 2015; published OnlineFirst August 20, 2015.

---

**References**


Small-Molecule NSC59984 Restores p53 Pathway Signaling and Antitumor Effects against Colorectal Cancer via p73 Activation and Degradation of Mutant p53

Shengliang Zhang, Lanlan Zhou, Bo Hong, et al.

Cancer Res Published OnlineFirst August 20, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-1079

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2015/08/20/0008-5472.CAN-13-1079.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.