Small-molecule modulators of p53 family signaling and antitumor effects in p53-deficient human colon tumor xenografts

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p53 deficiency is common in almost all human tumors and contributes to an aggressive chemo- or radiotherapy-resistant phenotype, therefore providing a target for drug development. Molecular targeting to restore wild-type p53 activity has been attempted in drug development and has led to the identification of CP-31398, PRIMA1, and the Nutlins. However, strategies targeting p53-activated transcriptional responses or p53 family member expression in p53-deficient tumors have yet to be explored. Here we demonstrate the use of noninvasive bioluminescence imaging in a high-throughput cell-based screen of small molecules that activate p53 responses and cell death in human tumor cells carrying a mutant p53. We isolated a number of small molecules that activate p53 reporter activity, increase expression of p53 target genes such as p21(WAF1) or death receptor 5 (KILLER/DR5) of TNF-related apoptosis-inducing ligand (TRAIL), and induce apoptosis in p53-deficient cells. Some of the compounds activate a p53 response by increasing p73 expression, and knockdown of transactivating isoforms of p73 by small interfering RNA reduces their induction of p53-responsive transcriptional activity. Some compounds do not induce significant p73 expression but induce a high p53-responsive transcriptional activity in the absence of p53. *In vivo* experiments demonstrate potent antitumor effects of selected compounds, using either HCT116/p53(−/−) or DLD1 human colon tumor xenografts. The results establish the feasibility of a cell-based drug screening strategy targeting the p53 transcription factor family of importance in human cancer and provide lead compounds for further development in cancer therapy.

apoptosis | cancer | drug development | imaging

The p53 protein represents an important target for drug development, because it provides a key difference between normal cells and tumor cells. p53 is mutated in over half of all human tumors and, among almost all of the remaining tumors, the pathway of p53-induced cell cycle arrest and apoptosis is deficient due to mouse double minute 2 (MDM2) overexpression or alternative reading frame (ARF) deficiency (1). Furthermore, deficiency of p53 activity in tumor cells promotes resistance to chemo- and radiotherapies and a more malignant phenotype (2–4). p53 also plays an important role in receptor-mediated extrinsic cell death, e.g., TNF-related apoptosis-inducing ligand (TRAIL)-resistant bax-null cells can be sensitized to TRAIL by activation of p53 by chemotherapeutics (5). Efforts have been made to target p53 with an attempt to restore p53 function in tumor cells (1, 6). These strategies include introduction of wild-type p53 into tumor cells and rescue of mutant p53 in a wild-type conformation, which led to the discovery of potent death-inducing small molecules such as CP-31398 (7) or PRIMA1 (8). Efforts have also been directed at liberating wild-type p53 from blockade by MDM2 by using small molecules such as the Nutlins (9). However, strategies targeting p53-activated transcriptional responses or p53 family member expression in p53-deficient tumors have yet to be explored or described. In the absence of p53 or the presence of mutant p53, p53 family members, e.g., p73, may function instead of p53 in the pathway of tumor suppression (10). It has been shown that p73 can be activated by some chemotherapeutics and plays a role in DNA damage-induced cell cycle arrest and apoptosis (11).

With the development of real-time noninvasive bioluminescence imaging of p53 transcriptional activity *in vitro* and *in vivo* (12), we performed a high-throughput cell-based functional screen for small molecules that trigger a p53-like transcriptional response in p53-deficient tumor cells. We exposed SW480 human colon adenocarcinoma cells that expressed a p53-responsive firefly luciferase reporter to the diversity set of small molecules collected by the National Cancer Institute (NCI). Screening led to the identification of some structurally related, as well as structurally dissimilar, molecules that activate p53-responsive transcriptional activity in p53-deficient tumor cells. *In vivo* experiments demonstrated potent antitumor effects of selected compounds using HCT116/p53(−/−) or DLD1 human tumor xenografts. The results establish the feasibility of a cell-based drug screening strategy using bioluminescence to target the p53 transcription factor family in human cancers and provide lead compounds for further development in cancer therapy.

Results

p53 Family Transcriptional Activators Identified from Screening the Diversity Set of the NCI Developmental Therapeutics Program by Bioluminescence Imaging of Human Colon Cancer Cells Expressing Mutant p53 and a p53-Responsive Reporter. We stably expressed a human p53 reporter, PG-13-luc, which carries the firefly luciferase gene under the control of 13 p53-responsive elements, in the human colon adenocarcinoma cell line SW480 that bears a mutant p53 (R273H, P309S). With the firefly luciferase-expressing cell line, and by the method of noninvasive real-time imaging (12), we screened the NCI Developmental Therapeutics Program's diversity set of ≈2,000 chemical agents accumulated over a 30-year period to identify small molecules that can reactivate p53 signaling in the tumor cells with mutant p53 and cause cell death. The diversity set was initially screened at two doses (10 and 50 μM) to discover candidates that can modulate mutant p53, stimulate p73, or induce reporter expression in a manner independent of the p53 family.

The initial screen (Fig. 1*A*) manifested two classes of compounds, those that activated p53-responsive reporter expression without apparent induction of cell death (red color due to high levels of bioluminescence) and those that appeared to cause toxicity and elimination of the baseline reporter signal indicative of cell death (black color due to loss of cell viability), during a

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Abbreviations: TRAIL, TNF-related apoptosis-inducing ligand; DR5, death receptor 5; NCI, National Cancer Institute; NSC, National Service Center.

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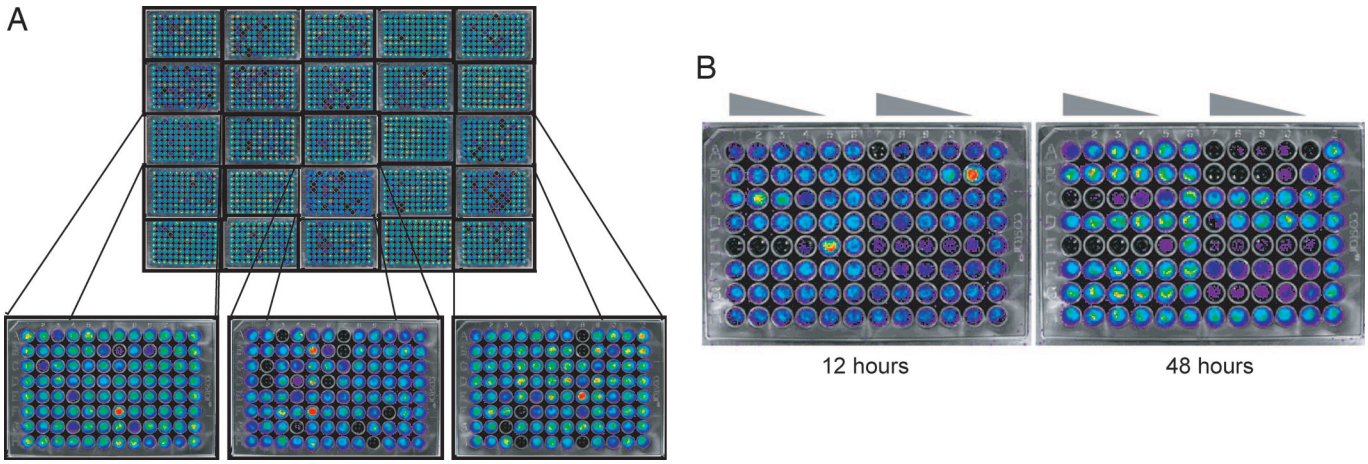


Fig. 1. Functional screening of the NCI Developmental Therapeutics Program diversity set for p53-family transcriptional activators in SW480 mutant p53-expressing human colon cancer cells. (*A*) SW480 cells, stably expressing the p53-responsive reporter PG13-luc, were seeded in 96-well plates at a density of 5×10^4 cells per well. p53-responsive transcriptional activity was imaged by the IVIS imaging system after exposure to the diversity set. (*B*) Secondary screening with selected compounds at 2-fold increasing concentrations (range of 10–200 μM) and time points (as indicated).

time course of 12–48 h. The two classes of compounds comprised ≈10% of the total number of compounds tested. It is possible that some compounds leading to apparent loss of cell viability may have inhibited luciferase activity without causing cell death; these were excluded in secondary screening and not further pursued. We sought to identify small molecules that activated a p53-responsive transcriptional activity and subsequently led to cell death. In secondary screening, we varied drug doses over a wider range (up to 200 μM) and performed time courses to evaluate the fate of cells that showed increased bioluminescence intensity at early time points (within 12 h) and then loss of viability during a time course of up to 72 h. Using this secondary screening procedure, we identified 33 compounds (Table 1, which is published as supporting information on the PNAS web site) that appeared to induce p53-responsive reporter activation at low drug doses but that, at later time points or at higher drug doses, induced cell death (Fig. 1*B* and data not shown).

Induction of p53 Target Gene Expression, Cell Cycle Arrest, and Apoptosis in p53-Deficient Cells. The chemical library screening was directed at restoring "p53 responses" in p53-deficient cells. The small molecules identified by the cell-based screening procedure appeared to be able to restore p53 responses in p53-deficient colon tumors and to eliminate viable cells. We further tested their function on wild-type p53-expressing and p53-knockout HCT116 colon adenocarcinoma cell lines. A number of candidate modulators of signaling by the p53 family appeared to induce expression of p53 target genes such as p21 or DR5 (13) either with or without stabilizing p53 protein in HCT116 cells (Fig. 2*A*). Compounds nos. 1 [National Service Center (NSC)5159], 14 (NSC143491), 23 (NSC254681), and 33 (NSC639174) appeared to increase p53 expression in parental HCT116 cells, and this was accompanied by increased expression of DR5 and p21 proteins (Fig. 2*A*) in a manner similar to doxorubicin (adriamycin). Nos. 11 (NSC123111) and 15 (NSC146109) also increased p53 expression, but their induction of the p53 targets DR5 and p21 was more modest (Fig. 2*A*). A number of other compounds, including nos. 3 (NSC28992), 5 (NSC49692), 12 (NSC127133), 16 (NSC150412), and 17 (NSC162908), appeared to increase p53 target gene expression with a slight or no significant effect on p53 protein expression in HCT116 cells (Fig. 2*A*).

We further tested a number of selected compounds on HCT116/p53(−/−) cells to verify the possibility of induction of p53 target gene expression in the absence of p53. Fig. 2*B* shows that the selected compounds appeared to significantly induce DR5 and p21 expression in p53-null HCT116 cells, whereas

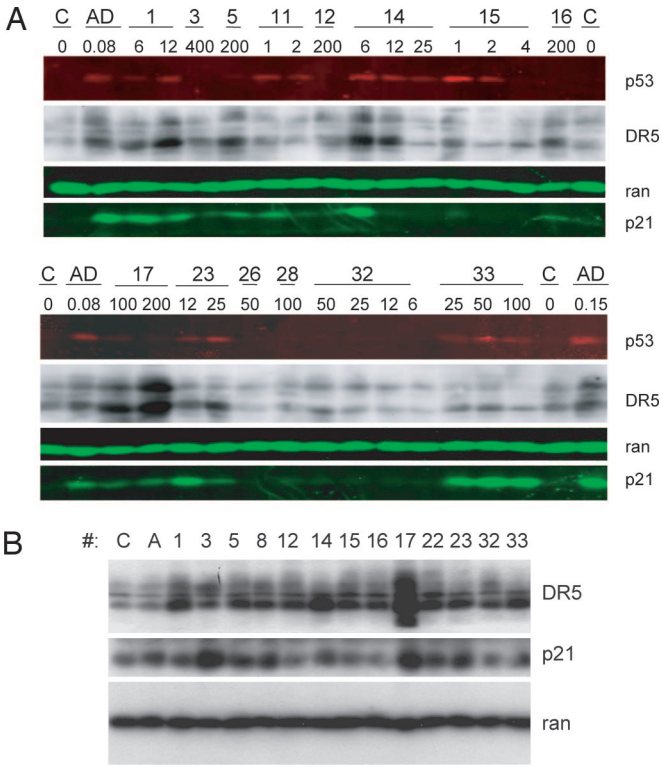
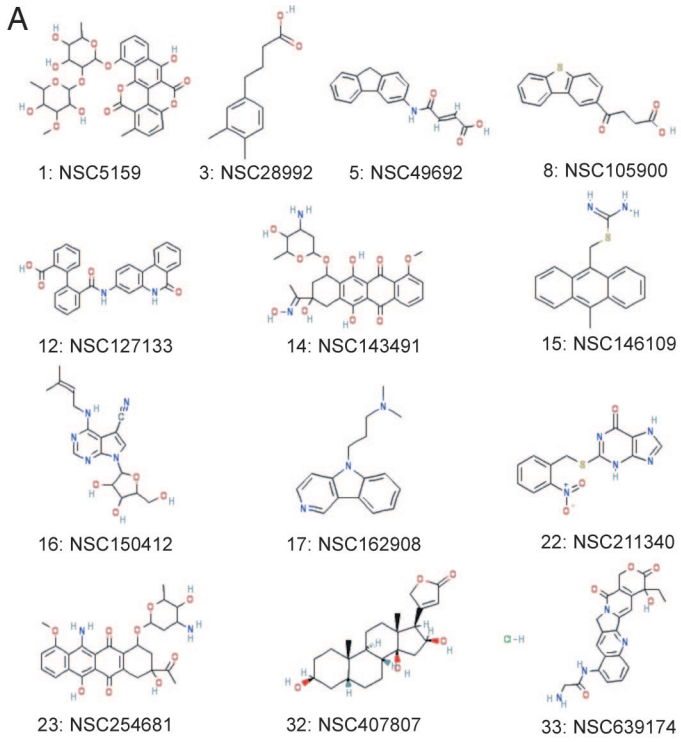
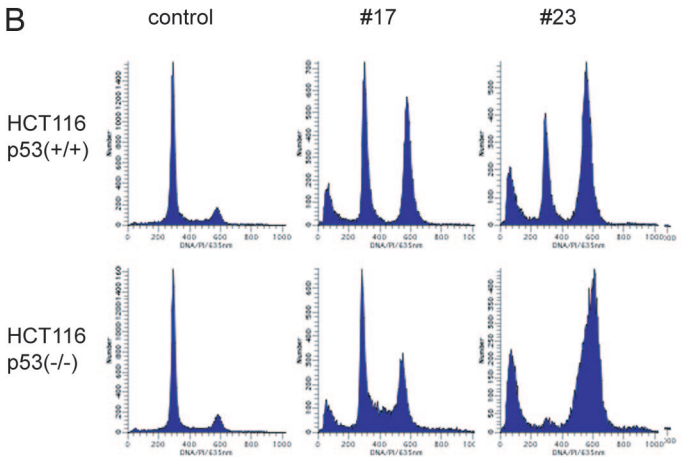


Fig. 2. Protein levels of p53 target genes p21 and DR5 were induced by selected compounds in HCT/p53(+/+) cells (*A*) or HCT/p53(−/−) cells (*B*). In *A*, doses of compounds (μM) are listed above each lane. Cells treated with compounds were harvested and lysed for SDS/PAGE and immunoblotted with p21 or DR5 antibodies. Ran was used as a protein loading control. Doses of compounds in *B* were as follows: 2 μM for no. 15; 12 μM for nos. 1 and 23; 20 μM for nos. 20 and 32; 100 μM for no. 33; 200 μM for nos. 5, 8, 12, 16, 17, and 22; and 400 μM for no. 3. The dose for adriamycin was 0.2 μg/ml. Cells were incubated for 16 h at 37°C with the various drugs before cell harvest. In *A* and *B*, "C" refers to control untreated cells, whereas "AD" in *A* and "A" in *B* refer to doxorubicin (adriamycin) treatment.



	NSC#	p53 level	p73 level	p53 targets in HCT/p53(-/-) cells: p21	p53 targets in HCT/p53(-/-) cells: DR5
1	5159	+++	+/-	+	+
3	28992	-	+/-	++	+
5	49692	+	-	+	+
8	105900	ND	+	+	+
12	127133	-	+	-	+
14	143491	+++	++	+	++
15	146109	+++	-	-	+
16	150412	+/-	-	-	+
17	162908	+	+/-	+++	++++
22	211340	ND	-	+	+
23	254681	+++	++	+	+
32	407807	-	-	-	+/-
33	639174	+++	ND	+	++



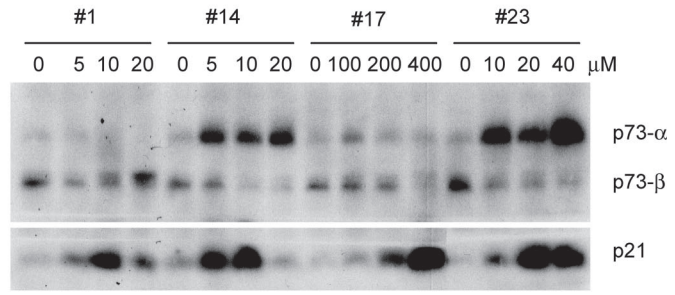


Fig. 3. Selected small molecules and their effects on p53 family signaling and tumor cell growth suppression. (*A*) Structures of isolated compounds and summary of their effects on the p53 family and transcriptional targets. (*B*) Cell cycle profiles of HCT/p53(+/+) and HCT/p53(−/−) in response to treatment by selected compounds. The dose of no. 17 was 200 μM, and for no. 23, it was 10 μM. (*C*) p73 protein levels were elevated in HCT116/p53(−/−) cells in response to treatment by selected compounds at various concentrations as indicated.

adriamycin had no obvious effect on DR5 and little effect on p21 expression in HCT116/p53(−/−) cells. The corresponding elevation of mRNA levels of DR5 and p21 (Fig. 6, which is published as supporting information on the PNAS web site) indicates that some of these compounds activated p53 target gene transcription in both p53(+/+) and p53(−/−) cells. Of particular interest, no. 17 induced the highest p53 transcriptional activity and DR5 levels in both HCT116/p53(+/+) and HCT116(−/−) cells (Fig. 2; Figs. 7*B* and 8, which are published as supporting information on the PNAS web site), but modestly induced p53 levels (Fig. 7*B*) and did not increase p73 expression (Fig. 3*C*). Moreover, a number of additional compounds tested, including nos. 8 (NSC105900), 22 (NSC211340), and 32 (NSC407807), were found to increase DR5 and p21 expression in the p53-null HCT116 cells (Fig. 2*B*). The importance of this observation is in establishing that it is possible to identify small molecules with the potential to induce p53 target gene expression in p53-deficient cells.

We further evaluated the ability of selected compounds from the chemical library screen to induce apoptosis of human colon tumor cells and the dependence of their effects on endogenous p53 status. Compound nos. 1, 14, 17, and 23 were chosen because they gave stronger responses in the reporter assays in p53-null HCT116 cells (Fig. 8) in addition to increasing the expression of the p53 target genes DR5 and p21 (Fig. 2). We found that these four compounds induced a subG1 peak characteristic of apoptosis in either HCT116/p53(+/+) or HCT116/p53(−/−) cells (Fig. 3*B* and data not shown). Interestingly, compound no. 17 induced apoptosis in the p53-null cells without suppressing the S-phase population as observed in the wild-type p53-expressing HCT116 cells. Compound no. 23 also induced apoptosis in p53-null HCT116 with a greatly reduced G1 arrest as observed

in wild-type p53-expressing HCT116 cells (Fig. 3*B*). These results suggest that the cell cycle arrest responses after exposure to either no. 17 or 23 depended on p53, whereas the apoptotic responses might be independent of p53.

In addition, we observed the induction of a p53-responsive reporter after exposure of mutant p53(S241F)-expressing human DLD1 colon cancer cells or p53-null SKOV3 ovarian cancer cells (Fig. 9, which is published as supporting information on the PNAS web site) to selected compounds.

DNA Damage Signaling and p73 Are Involved in the Mechanism of Action of Selected Compounds. We then questioned whether the p53 family member p73 is involved in the p53-responsive transcriptional activity induced by the compounds we identified. p63, the other p53 family member, was not tested, because the transactivating (TA) form of p63 is rarely expressed in malignant and normal tissues except for germ cells of the ovary and testis (14). As shown in Fig. 3*C*, nos. 14 and 23 were strong inducers of p73, whereas the DNA-damaging agent, adriamycin, increased p73 only slightly (data not shown). Additional compounds, including nos. 8, 12, and 16, were shown to induce p73 protein expression (data not shown). Knockdown of TAp73 by retrovirus-mediated si-p73 in HCT116/p53(−/−) cells reduced baseline expression of the p53 reporter and suppressed p53-responsive transcriptional activity-induced by compounds nos. 1, 14, and 23, whereas the activity induced by no. 17 was not hindered (Fig. 5*D*). This indicates that no. 17 may induce p53 transcriptional activity in p53(−/−) cells through an alternative pathway that may not involve p73. Knockdown of p73 was demonstrated by Western blot (Fig. 10, which is published as supporting information on the PNAS web site).

To determine whether DNA damage signaling is involved in the mechanism of action of selected compounds, we tested by Western blot the status of phosphorylation and acetylation of p53, which are sensitive indicators of DNA damage. We found that nos. 14 and 23 were strong inducers of p53 phosphorylation at ser20 (Fig. 7 *B* and *C*) and acetylation at lys382 (Fig. 7*A*). We also tested γH2AX, which was positive after treatment with nos. 14 and 23, but not with nos. 1 and 17 (data not shown). These data indicate that DNA damage signaling is involved in nos. 14 and 23-induced cell death, but not for nos. 1 and 17, which may act by a novel mechanism that requires further investigation.

***In Vivo* Antitumor Effects of Selected Compounds.** We tested nos. 1, 14, 17, and 23 in colon-tumor xenograft-bearing mice to evaluate their toxicities and potential antitumor effects (Fig. 4). These compounds were chosen for further testing based on their ability to strongly induce p53 target gene expression (DR5 and p21) in p53-null cells (Fig. 2*B*). We chose the initial doses below maximal tolerated doses based on the NCI Developmental Therapeutics Program toxicology databases for chemical compound testing *in vivo*, so that mice would survive drug administration and allow subsequent evaluation of antitumor effects. We first tested p53-null HCT116 xenografts to document antitumor effects in p53-deficient tumors and designed an experiment to simulate therapy of established tumors. A total of 2×10^6 p53-null HCT116 cells were implanted on opposite flanks s.c. in each of six nude mice in each group. When tumor masses grew to ≈3–5 mm in diameter, drugs were administered intraperitoneally (no. 1, 100 mg/kg; no. 14, 50 mg/kg; no. 23, 10 mg/kg), and on the next day, additional groups received i.v. TRAIL (ref. 15; 100 μg per mouse via the tail vein). Tumor weights were determined 7 days later. As shown in Fig. 4, antitumor effects were observed with compounds nos. 1, 14, and 23, and a modest additive effect was observed with the combination of no. 23 with TRAIL. No overt toxicities were observed in mice treated with compounds nos. 1, 14, or 23. Moreover, at doses just below the maximal tolerated dose, no. 17 had no apparent *in vivo* antitumor effect on established HCT116/p53(−/−) xenograft, and at higher doses, no. 17 was found to be toxic to mice. Nonetheless, in the future, it may be possible to modify the structure of no. 17 or identify doses where synergistic interactions with TRAIL may be observed.

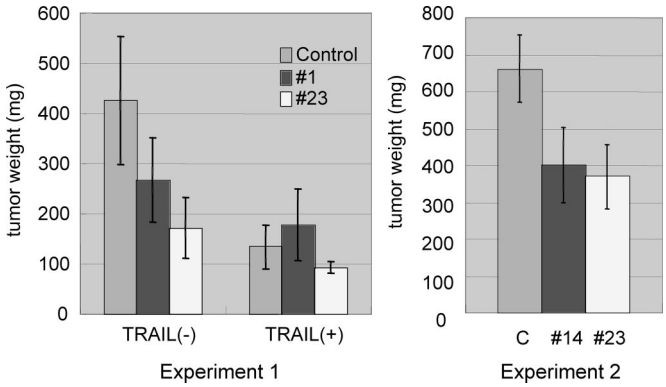


Fig. 4. *In vivo* antitumor effects of selected compounds. BALB/c nude mice were inoculated s.c. with 2 million HCT116/p53(−/−) cells in Matrigel on each flank. Six mice were used in each group, in each of the two experiments. When the tumor mass reached ≈3–5 mm, mice were treated with the compounds alone or after a single dose of TRAIL at 100 μg per mouse in experiment 1. At 7 days after treatment, mice were killed, and the tumor masses were weighed. The doses used were 100 mg/kg for no. 1, 50 mg/kg for no. 14, and 10 mg/kg for no. 23.

We further tested whether these compounds could stimulate a p53-responsive transcriptional activity in tumor xenografts. DLD1/PG13 cells were inoculated at both flanks at a dose of 5 million cells per site. Twenty-four hours after injection, compounds were delivered, and 16 h later, the intensity of bioluminescence of the tumor cells was imaged and recorded according to a protocol previously described (12). All four compounds stimulated a p53-responsive transcriptional activity in the tumor xenografts (Fig. 5 *A* and *B*). Consequently, treatment with the compounds hindered tumor growth (Fig. 5*C*).

Discussion

We performed a chemical library screen by a strategy using bioluminescence imaging to identify small molecules that can induce a p53-responsive transcriptional activity and subsequent apoptosis in tumor cells deficient in p53. The strategy of using bioluminescence imaging to screen for potential p53 activators has advantages over other conventional methods, because it is sensitive, noninvasive, and allows the recording of real-time kinetics of transcriptional change over a time period up to 2–3 days.

Based on this functional screening method, we obtained a number of small molecules from the NCI diversity set, which stimulated a strong p53 response not only in p53 wild-type tumor cells but also in p53-deficient cells, including p53-null and p53 mutant cells. The p53 transcriptional targets, p21 and DR5, were consequently induced. Subsequently tumor cells exposed to the compounds underwent cell cycle arrest and apoptosis. The p53-responsive transcriptional activity induced in HCT116/p53(−/−) cells may have been caused by p73, because a number of the compounds increased expression of transactivating isoforms of p73 in the cell line, including nos. 8, 12, 14, 16, and 23, whereas others, such as nos. 1 and 17, did not induce significant levels of p73. Of particular interest, compared to all other compounds isolated, no. 17 induced the highest transcriptional activity in both p53(+/+) and p53(−/−) HCT116 cells, but only a modest increase in p53 protein level in HCT116/p53(+/+)

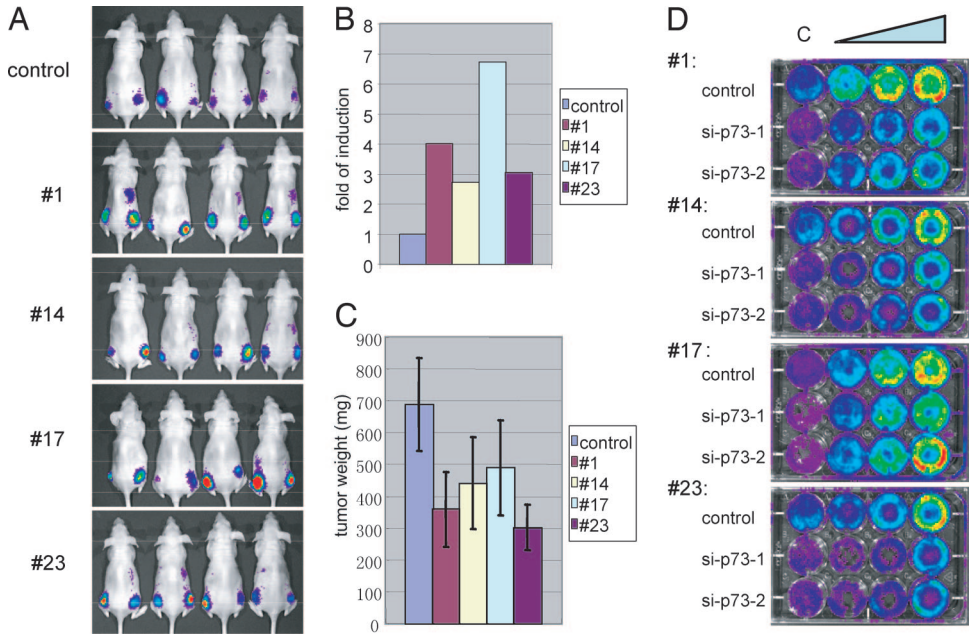


Fig. 5. p53 transcriptional activity is induced in DLD1 xenografts and effects of knockdown of p73 by siRNA on drug-induced transcriptional activity. DLD1/PG13 cells were inoculated s.c. with 5 million cells. At 24 h later, mice were treated with selected compounds (100 mg/kg for no. 1, 50 mg/kg for nos. 14 and 17, and 10 mg/kg for no. 23), and subsequently bioluminescence imaging was carried out after 16 h. Two weeks later, tumor masses were weighed. (*A*) Bioluminescence imaging of p53 transcriptional activity induced *in vivo*. (*B*) Calculated fold induction of p53 transcriptional activity. (*C*) Inhibition of tumor growth by selected compounds. (*D*) Effects of si-TAp73 on the transcriptional activity induced by selected compounds.

cells, and no significant increase in p73 was observed in HCT116/p53(−/−) cells. No. 17 may act through an unknown mechanism to enhance transcription of p53 targets, which requires further investigation. Apparently, DNA damage signaling is induced in the case of nos. 14 and 23, because sensitive indicators of DNA damage, such as p53 phosphorylation, acetylation, and γH2AX, were detected after drug exposure.

Some of the isolated chemical compounds (Fig. 3*A*) appear to have structural similarity. For example, nos. 5, 8, and 17 contain three ring structures with a side chain, whereas nos. 14 and 23 have four ring structures typical of anthracyclines with a substituted sugar residue. No. 1 is also known as chartreusin or lambdamycin and has been reported to inhibit cell cycle progression (16). Chartreusin binds to GC-rich tracts in DNA and can cause single-strand breaks (17). A synthetic derivative of chartreusin [6-O-(3-ethoxypropylonyl)-3′,4′-O-exo-benzylidene-chartreusin] has shown anticancer activity in a phase II study (17). No. 14 is Daunomycin 3-oxime hydrochloride, a derivative of daunorubicin, which is an anthracycline. This compound was found to inhibit the rad18 mutant yeast strain in the NCI yeast anticancer drug screen. No. 23 was previously identified in a high-throughput screen of inhibitors of hypoxia-inducible factor transcription but had minimal effects compared to other isolated compounds in that screen (18). The structure of no. 15 was recently described as the genotype (Small T antigen and RAS^{V12}) selective nonapoptotic cell death-inducing agent erastin (19). This substituted pseudourea structure (ChemBank identification no. 1070452) has also been found to affect nuclear localization of forkhead protein and to prevent iron uptake by mammalian cells. No. 32 is a derivative of digitoxigenin, which is predicted to have cardiac effects. No. 33 is a derivative of camptothecin also recovered in the screen of hypoxia-inducible factor inhibitors but did not show appreciable differences in inhibition of VEGF mRNA expression between normoxic and hypoxic cells (18). It is likely that nos. 14, 23, and 33 stabilize wild-type p53 in wild-type p53-expressing HCT116 colon tumor cells through an indirect mechanism due to their predicted effects as topoisomerase inhibitors and DNA-damaging agents. We found no other available information in the published literature or databases on the remaining structures.

In summary, our cell-based chemical library screening strategy, using molecular bioluminescence imaging to detect modulation of transcriptional responses and cell death, demonstrates that it is possible to restore p53 responses in p53-deficient cells, and that this may occur by targeting p53 family members such as p73. The induction of death receptor expression in tumor cells exposed to small-molecule activators of p53 family signaling supports the use of selected compounds in combination with TRAIL or death receptor agonists. Isolated lead compounds appear to have antitumor effects against p53-deficient human tumor xenografts without overt toxicity. Further efforts are required to determine whether p73 is the only target responsible for the observed antitumor effects in p53-null tumors, and additional larger-scale studies are necessary to evaluate selected activators of p53 family signaling against a broad range of human tumors harboring a wide array of mutant p53 proteins. Novel lead compounds may advance and/or undergo further structural and pharmaceutical refinements and additional preclinical testing before testing in clinical trials in cancer patients.

Methods

High-Throughput Screening. Cell-based screening for p53-family transcriptional activators was performed by using noninvasive bioluminescence imaging to evaluate drug effects. SW480 human colon cancer cells, stably expressing a p53 reporter, PG13-luc, were seeded in 96-well black plates with clear bottom (Corning) at a density of 5×10^4 cells per well. Compounds were added to the well at concentrations of 10 and 50 μM, respectively. p53 transcriptional activity was imaged by using an IVIS imaging system (Xenogen, Alameda, CA) during a time period ranging from 12 to 72 h.

Western Blotting. Cells were collected, and protein concentration was quantified by the Bio-Rad protein assay before SDS/PAGE.

Proteins were transferred to a PVDF membrane (Immobilon-P, Millipore) by a semidry transfer apparatus (Bio-Rad). The membranes with transferred proteins were blotted with 10% W/V nonfat dry milk and then incubated with the primary antibody and subsequently secondary antibodies, which were labeled by horseradish peroxidase, or near IR dyes. Signals were either visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) and exposed to an x-ray film or scanned by the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE). Anti-p53 DO-1 was obtained from Santa Cruz Biotechnology, and anti-p73 (AB-1) and anti-p21 (AB-1) were obtained from Calbiochem. Anti-ser20 of p53 was obtained from Cell Signaling Technology (Danvers, MA), and anti-DR5 antibody was obtained from Cayman Chemical (Ann Arbor, MI).

Flow Cytometry Assay. Adherent cells in a six-well plate were trypsinized and collected in 15-ml centrifuge tubes, to which were added the originally floating cells. The collected cells were ethanol-fixed and stained with propidium iodide (Sigma). The DNA content of the stained cells was then measured by using an Epics Elite flow cytometer (Beckman Coulter).

Si-TAp73 Retrovirus Construction. The pBS/U6 vector containing TAp73 RNAi was kindly provided by Leif W. Ellisen (ref. 20; Harvard Medical School, Boston), from which the expression cassette was removed and recombined to pSIREN-RetroQ retroviral vector (Clontech), which was reconstructed to express a blasticidin-resistant marker.

***In Vivo* Antitumor Assay.** BALB/c nude mice (Charles River Breeding Laboratories) were inoculated s.c. with 2 million HCT116/p53(−/−) cells in an equal volume of Matrigel. When tumor masses reached ≈3–5 mm in diameter, mice were treated with the compounds alone by i.p. injection or after a single i.v. dose of TRAIL at 100 μg per mouse. At 7 days after treatment, mice were euthanized by using an Institutional Animal Care and Use Committee-approved animal protocol, and the tumor masses were weighed. DLD1/PG13 cells were inoculated s.c. with 5 million cells. At 24 h later, mice were treated with selected compounds, and subsequently bioluminescence imaging was carried out after 16 h, as described (12).

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