p53 α-Helix mimetics antagonize p53/MDM2 interaction and activate p53

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Abstract

Overexpression or hyperactivation of MDM2 contributes to functional inactivation of wild-type p53 in nearly 50% of tumors. Inhibition of p53 by MDM2 depends on binding between an NH_2-terminal (residues 16–28) p53 α-helical peptide and a hydrophobic pocket on MDM2, presenting an attractive target for development of inhibitors against tumors expressing wild-type p53. Here we report that novel p53 α-helical peptide mimics based on a terphenyl scaffold can inhibit MDM2-p53 binding *in vitro* and activate p53 *in vivo*. Several active compounds have been identified that inhibit MDM2-p53 binding in an ELISA assay with IC_{50} of 10 to 20 μmol/L and induce p53 accumulation and activation in cell culture at 15 to 40 μmol/L. These results suggest that p53 α-helical mimetics based on the terphenyl scaffold may be developed into potent p53 activators. [Mol Cancer Ther 2005;4(6): 1019–25]

Introduction

p53 is a transcription factor mutated in ~50% of human tumors (1). Activation of wild-type p53 induces a large number of downstream genes that lead to cell cycle arrest or apoptosis. In unstressed normal cells, p53 is present at very low levels due to rapid degradation through the ubiquitin-dependent proteasome pathway. MDM2 is an important regulator of p53 turnover by binding p53 and promote its ubiquitination by acting as an ubiquitin E3 ligase (2). In normal cells, stress signals such as DNA damage induce p53 accumulation by phosphorylation of p53 and MDM2 (3, 4). Mitogenic signals activate p53 by induction of the ADP ribosylation factor (ARF) tumor suppressor encoded by an alternative open reading frame in the *p16INK4a* locus, which inhibits the ability of MDM2 to ubiquitinate p53 (2). The intricate signaling network converging on MDM2 is critical for stress response and tumor suppression by p53. Overexpression of MDM2 abrogates the ability of p53 to induce cell cycle arrest and apoptosis (5). In about 30% of human osteogenic sarcomas and soft tissue sarcomas, MDM2 is overexpressed due to gene amplification, implicating its role in the development of these tumors (6, 7). In tumors without MDM2 amplification, hyperactivation of MDM2 due to silencing of ARF expression also leads to p53 inactivation (8). Therefore, MDM2 is a key factor in the tolerance of wild-type p53, making it an attractive target for the development of antitumor agents.

Initial validation of MDM2 as a drug target was achieved by two approaches. Inhibition of MDM2 expression using antisense oligonucleotides resulted in strong activation of p53, leading to growth arrest or apoptosis (9, 10). Second, inhibition of MDM2-p53 binding by microinjecting a monoclonal antibody to MDM2 also activated p53 in cells and induced growth arrest (11). These studies provided a proof of concept for activating p53 by targeting MDM2. The crystal structure of MDM2 in complex with the NH_2-terminal peptide of p53 suggests that the binding is a pocket-ligand type interaction in which the p53 peptide forms an amphipathic α-helix and interacts with a hydrophobic groove on the globular MDM2 domain (12). This mode of interaction suggests that small molecules may be able to compete for MDM2 binding and activate p53.

Several approaches have been pursued to develop MDM2 inhibitors. Phage display has been used to identify peptides that can inhibit MDM2/p53 binding with IC_{50} at the 100 nmol/L level *in vitro* (13). Recently, a modified peptide that can inhibit MDM2-p53 binding *in vitro* (IC_{50} ~10 nmol/L) and activate p53 in cultured cells (100 μmol/L) have been synthesized (13). A high-throughput screen of chemical libraries resulted in the identification of Nutlins (14), which are *cis*-imidazoline analogues that can inhibit p53-MDM2 binding with IC_{50} of 100 to 300 nmol/L in a surface plasmon resonance–based assay using recombinant MDM2 and a p53 fragment. Nutlin-3 showed the ability to activate p53 in cell culture at 5 to 10 μmol/L and inhibit tumor growth when given orally at 200 mg/kg (14). This study showed the exciting possibility of using small molecules to target p53-MDM2 interaction. Because experimental compounds often have deficiencies in areas such as bioavailability and toxicity, development of compounds based on diverse chemical motifs will be necessary to obtain MDM2 inhibitors suitable for therapeutic application.

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Here, we report the design and biological characterization of nonpeptidic synthetic molecules based on the terphenyl scaffold that mimic the NH_2-terminal α-helix of p53. We show that terphenyl derivatives block p53/MDM2 interaction *in vitro* and activate p53 in cell culture. The results identify a novel α-helical mimetics scaffold for the development of MDM2 inhibitors.

Materials and Methods

Syntheses of Terphenyl Derivatives

A modular synthetic route to all of the terphenyl derivatives was developed involving sequential Suzuki-coupling of the corresponding methoxyphenylboronate and phenyltriflate derivatives (15, 16). Flanking carboxylic acid functionalities are attached on both ends of the terphenyl backbone in order to increase the polarity of the inhibitors and improve their solubility in aqueous solution.

ELISA

Glutathione *S*-transferase-MDM2 fusion protein containing full-length human MDM2, and His6-tagged human p53 (a kind gift from Dr. Hua Lu) were expressed in *E. coli* and affinity purified by binding to glutathione-agarose and Ni^{2+}-NTA beads under nondenaturing conditions using standard protocols. ELISA plates were incubated with 2.5 μg/mL His6-p53 in PBS for 16 hours. After washing with PBS + 0.1% Tween 20 (PBST), the plates were blocked with PBS + 5% nonfat dry milk + 0.1% Tween 20 (PBSMT) for 0.5 hours. Compounds were dissolved in DMSO. Glutathione *S*-transferase-HDM2 (5 μg/mL) was mixed with test compounds in PBSMT + 10% glycerol + 10 mmol/L DTT and added to the wells. The plates were washed with PBST after incubating for 1 hour at room temperature, incubated with MDM2-specific monoclonal antibody 5B10 hybridoma supernatant diluted 1:10 in PBSMT for 1 hour, followed by washing and incubation with horseradish peroxidase-rabbit anti-mouse immunoglobulin antibody for 1 hour. The 5B10 antibody recognizes a COOH-terminal epitope on MDM2 (17), thus ensuring that the assay detects full-length MDM2 binding to p53. The plates were developed by incubation with tetramethyl benzidine peroxidase substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) and measured by absorbance at 450 nm.

Western Blot

HCT116+/+ (with wild-type p53) and HCT116−/− cells (p53-null) were kindly provided by Dr. Bert Vogelstein (Sidney Kimmel Cancer Center, Johns Hopkins University, Baltimore, MD; ref. 18). Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride], centrifuged for 5 minutes at 10,000 × *g*, and the insoluble debris were discarded. Cell lysate (10–50 μg protein) was fractionated by SDS-PAGE and transferred to Immobilon P filters (Millipore, Bedford, MA). The filter was blocked for 1 hour with PBS containing 5% nonfat dry milk, 0.1% Tween 20. The following antibodies were used: 3G9 for MDM2 (17); DO-1 for p53 (PharMingen, San Diego, CA); p21 antibody (Oncogene Research Products, Uniondale, NY); p53 phospho-Ser^{15} and phospho-Ser^{46} antibodies (Cell Signaling Technology, Beverly, MA). The filter was developed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence-plus reagent (Amersham, Piscataway, NJ).

Luciferase Reporter and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays

HCT116 cells were stably transfected with p53-responsive BP100-luciferase reporter plasmid (containing the p53 binding site from the MDM2 P2 promoter) and selected for 2 weeks with 700 μg/mL G418 (19). Pooled stable colonies were cultured in 24-well plates and treated with compounds for 24 hours. Luciferase activity was determined using identical amounts of cell lysate.

Cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using the Cell Titer kit (Promega, Madison, WI). Cells were cultured in 24-well plates and treated with compounds for 48 hours. Culture medium was replaced with fresh medium containing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent and the cells were cultured for 15 to 30 minutes. Conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent into color-absorbing product by metabolically active cells were measured by determining absorbance at 490 nm.

Immunofluorescence Staining

HCT116 cells cultured on chamber slides were treated with compounds for 16 hours. Cells were fixed with acetone-methanol (1:1) for 3 minutes at room temperature, blocked with PBS + 10% normal goat serum for 20 minutes, and incubated with anti-p53 monoclonal antibody Pab1801 hybridoma supernatant at 1:10 dilution in PBS + 10% normal goat serum for 2 hours. The slides were washed with PBS + 0.1% Triton X-100, incubated with FITC-goat anti-mouse IgG in PBS + 10% normal goat serum for 1 hour, washed with PBS + 0.1% Triton X-100 and mounted.

Results and Discussions

Inhibition of p53-MDM2 Binding by Terphenyl Derivatives *In vitro*

We recently developed a group of α-helical mimetics of the NH_2-terminal p53 α-helix (amino acids 16–28, Fig. 1A; ref. 12). The design is based on a terphenyl scaffold containing alkyl or aryl substituents on the three ortho positions in order to mimic the i, i+4, i+7 substituents on the helical exterior (Fig. 1C; refs. 15, 16). The terphenyl derivatives project functionality in a geometry similar to that of α-helices, whereas the helical backbone was reduced to a relatively simple synthetic scaffold (Fig. 1B). The terphenyl compounds were screened in an ELISA assay using full-length recombinant p53 and MDM2 proteins purified from *E. coli*. The assay measures the binding of glutathione *S*-transferase-MDM2 to His6-p53 immobilized on the surface of ELISA plates. An MDM2-inhibitory peptide optimized by phage display selection (MPR**F**MDY-**W**EG**L**N, IC_{50} ~0.5 μmol/L; critical hydrophobic residues

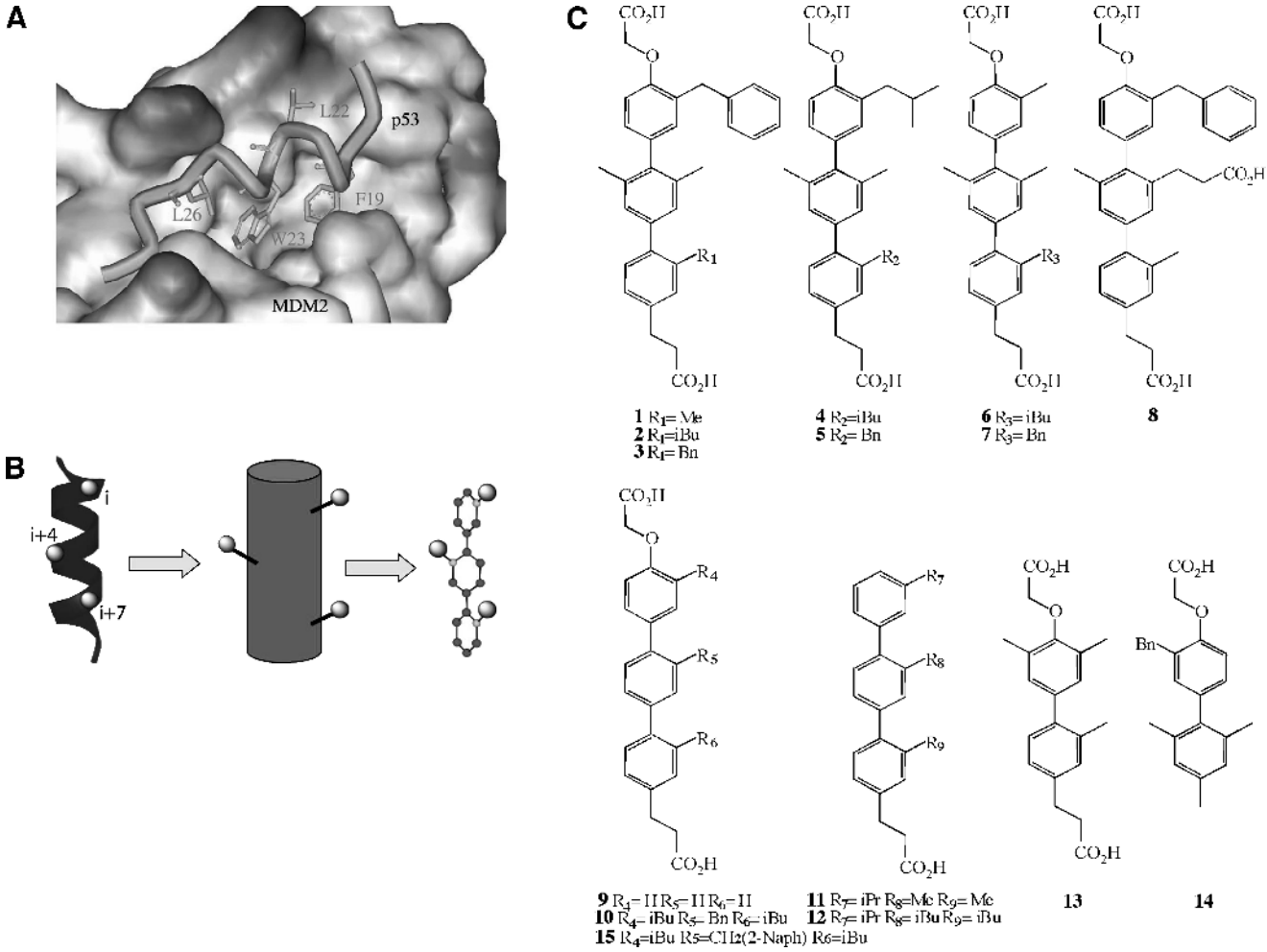

Figure 1. Inhibitor design of terphenyl derivatives to mimic the α-helical secondary structure of p53 NH_2-terminal peptide. **A,** X-ray crystal structure of the MDM2/p53 complex. The p53 peptide is shown as the helix. The key side chains of F19, W23, and L26 are shown in stick representation. **B,** α-helical mimicry design based on a terphenyl scaffold. **C,** terphenyl compounds screened in the ELISA assay.

in boldface) and a mutant version (MPR**A**MDY**A**EG**A**N, with alanine mutations on key residues) were used as positive and negative controls to verify the specificity of the ELISA assay (Fig. 2A; ref. 13). An example of ELISA results for two active terphenyl compounds #**1** and #**6** is shown in Fig. 2B. The IC_{50} of terphenyl derivatives required to disrupt p53/MDM2 interaction are summarized in Table 1. The results showed that of 15 terphenyl compounds tested, 9 (#**1**–**7**, #**12**, #**15**) showed significant activity with IC_{50} ranging from 10 to 20 μmol/L.

By screening compounds with a range of side chains on the 2-, 2′-, 6′- and 2′-positions of the three phenyl rings, we found that the isobutyl or benzyl group as 2-substituent on the upper phenyl ring in general, provided the best inhibition results (#**2**–**5**, and #**15**). These compounds showed the strongest activity in disrupting p53/MDM2 interaction with IC_{50} of 10 to 15 μmol/L (Table 1). The importance of hydrophobic side chains was further confirmed by the weak binding of #**9**, which lacks the hydrophobic substituents. It was also shown that the terphenyl backbone is critical for the inhibition. The 1- terminal carboxyl group seemed to be necessary for inhibition of MDM2 *in vivo* as seen in #**1**, #**2**, #**4**, and #**6** compared with #**11** and #**12**, presumably due to their elevated polarity and better solubility in aqueous solution. On the other hand, the extra acid functionality on the middle phenyl ring decreased the binding affinity of #**8**. Biphenyls #**13** and #**14**, which include part of the terphenyl lead #**1**, were nearly inactive in ELISA (Table 1).

Activation of p53 Transcriptional Function

To test whether the terphenyl derivatives can stimulate p53 activity, HCT116 colon cancer cells with wild-type endogenous p53 were stably transfected with p53-inducible BP100-luciferase reporter containing the p53-responsive element from the MDM2 P2 promoter (19). Pooled

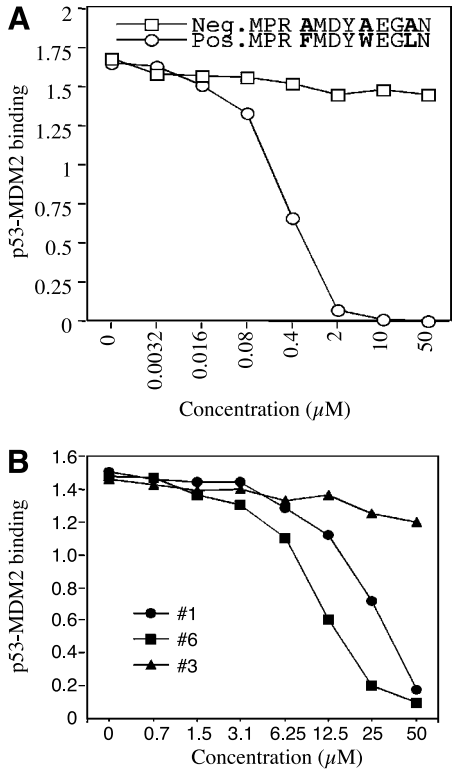

Figure 2. Inhibition of p53-MDM2 binding by terphenyl derivatives. **A,** validation of ELISA assay using control peptides. The positive control is an optimized peptide identified by phage display as a potent inhibitor of MDM2-p53 binding. The negative control peptide has three key bulky hydrophobic residues mutated to alanine (in *boldface*). His6-p53 was immobilized on the surface of 96-well ELISA plates and glutathione *S*-transferase-MDM2 was presented as a ligand in solution. MDM2 captured on the plate through binding to p53 was detected by incubation with MDM2 monoclonal antibody 5B10 and protein A-horseradish peroxidase. Inhibition of MDM2-p53 interaction led to reduction in signal. **B,** terphenyl compounds were analyzed by ELISA at indicated concentrations and the effects on p53-MDM2 binding are shown for terphenyl #**1** and #**6**. Compound #**9** is a scaffold negative control.

transfectants were treated with the synthetic inhibitors. Luciferase expression level was measured after 24 hours. Activation of p53 transcriptional function was observed after treatment with terphenyl #**1**, #**2**, #**4**, and #**6**. An example of the dose dependent p53 activation by terphenyl #**1** and #**6** is shown in Fig. 3A. The maximal level of p53 activation after treatment with terphenyl #**1** (10-fold) was higher than that achieved by the positive control DNA-damaging agent camptothecin in this cell line (5-fold at 0.2 μmol/L; Fig. 3B). p53 activation was lost at high concentrations of terphenyl #**1** and #**6**, most likely due to nonspecific toxicity from the compounds. The results of luciferase reporter assay are also summarized in Table 1. Disruption of MDM2-p53 interaction *in vivo* is expected to result in p53 stabilization. This was confirmed by immunofluorescence staining of HCT116 cells treated with active compound #**6** and #**1**, revealing nuclear accumulation of p53 (Fig. 3C).

The terphenyl derivatives with 6′-methyl side chain on the middle phenyl ring induced activation of p53, suggesting that more rigid conformation caused by the increased steric hindrance was favored. The terminal carboxylic acids were also essential for activating p53 *in vivo*, although it may not be needed *in vitro* (as in #**12**). It is important to note that although the SAR study was limited, we found that compounds that did not significantly inhibit MDM2-p53 binding *in vitro* also did not activate p53 *in vivo*. This suggested that p53 activation by the active compounds were most likely due to their ability to inhibit MDM2 binding rather than by indirect mechanisms.

p53-Dependent Growth Inhibition by Terphenyl Derivatives

To further test the biological effects of p53 activation by terphenyl derivatives, HCT116 cells were treated with #**1**, #**6** and control compound #**9** for 48 hours and cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 3D). To measure the p53-mediated effect, HCT116 cells with wild-type p53 or with targeted deletion of p53 were used (20). Compound #**6** induced a significant reduction in viable cell number in wild-type HCT116 cells but not in the p53-null derivative (Fig. 3D), suggesting that it inhibited cell proliferation in a p53-dependent manner. The control backbone compound #**9** did not affect growth of both cell lines. Therefore, the growth inhibitory activity of #**6** is likely due to its ability to activate p53. Weaker but p53-dependent inhibition of proliferation was also observed using compound #**1** in the same assay (data not shown). These results suggested that activation of p53 by terphenyl derivatives has moderate but specific antiproliferative effects.

Induction of p53 Accumulation and Activation *In vivo*

To further examine the effects of terphenyl derivatives on the p53 pathway, the accumulation of p53 was confirmed

Table 1. Summary of terphenyl compounds

Compound	ELISA IC_{50} (μmol/L)	p53 activation in HCT116
1	20	10-fold at 30 μmol/L
2	10	10-fold at 10 μmol/L
3	12	inactive
4	12	3-fold at 50 μmol/L
5	15	inactive
6	15	10-fold at 40 μmol/L
7	20	inactive
8	450	inactive
9	>1,000	inactive
10	130	inactive
11	150	inactive
12	10	inactive
13	450	inactive
14	300	inactive
15	10	inactive

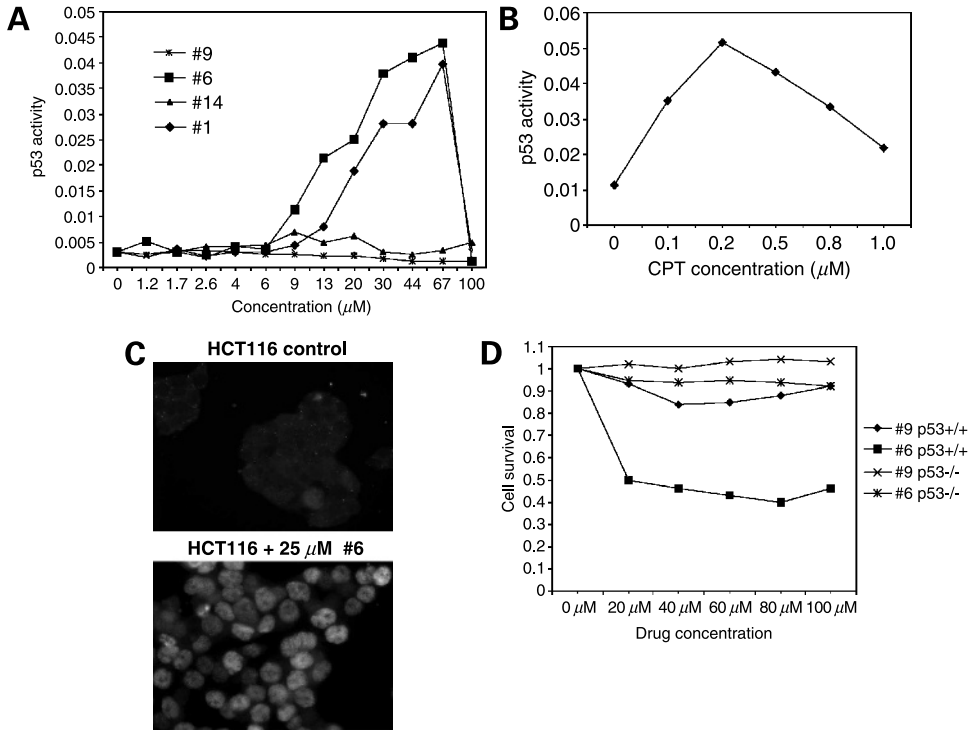

Figure 3. Activation of p53 by terphenyl derivatives. **A,** HCT116-BP100 cells were treated with compounds at indicated concentrations for 24 h and p53-induced luciferase expression was determined. **B,** HCT116-BP100 cells were treated with DNA-damaging agent camptothecin (CPT) at indicated concentrations for 24 h and p53 activity was determined. **C,** terphenyl #**6** induces nuclear p53 accumulation. HCT116 cells were treated with #**6** for 24 h, fixed and stained using anti-p53 monoclonal antibody Pab1801. **D,** p53-dependent growth inhibition by terphenyl #**6**. HCT116 cells with (+/+) and without (−/−) endogenous wild-type p53 were treated with indicated concentrations of compound #**6** and control #**9** for 48 h. Cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

by Western blot in HCT116 cells (Fig. 4A). Furthermore, two p53-responsive genes *p21WAF1* and *MDM2* were both induced in wild-type HCT116 cells but not in the p53-null derivative (Fig. 4A), indicating that the induction was dependent on p53 activation. These results were consistent with the luciferase reporter assay and suggested that increased MDM2 and p21 protein levels were due to activation of transcription. In the JAR tumor cell line with overexpressed MDM2 due to gene amplification (21), #**1** also induced p53 accumulation despite high MDM2 levels (Fig. 4B), indicating that the compound can overcome the effects of MDM2 overexpression. The combined results suggested that #**1** inhibits the ability of MDM2 to promote p53 degradation and in turn induces p53 stabilization and activation. Similar results were also observed for compounds #**2**, #**4**, and #**6** (data not shown). The concentrations required for induction of p53 target genes in Western blot experiments were slightly higher than that needed for antiproliferation effects or for p53 activation in luciferase assay. This is likely due to differences in assay format and treatment timing.

The interaction between MDM2 and p53 is weakened by phosphorylation of the p53 NH_2 terminus. Many DNA-damaging agents activate p53 by inducing phosphorylation of p53 at the NH_2- and COOH-terminal serine residues (3, 22), whereas compounds that directly block p53/MDM2 binding should not induce p53 phosphorylation. When p53 from HCT116 cells was analyzed with antibodies that specifically recognize phosphorylated Ser^{15} and Ser^{46}, camptothecin-treated positive control had significantly increased phosphorylation of Ser^{15} and weak increase on Ser^{46} compared with p53 stabilized by the MG132 proteasome inhibitor. In contrast, no increase in phosphorylation was detected after treatment with #**1**, despite stronger p53 activation by this compound (Fig. 4C). These results suggest that #**1** does not induce DNA damage. Additional experiments showed that active compounds #**2**, #**4**, and #**6** also did not induce p53 Ser^{15} phosphorylation compared with camptothecin (Fig. 4D). Therefore, p53 stabilization and activation by terphenyl derivatives do not involve phosphorylation of Ser^{15}, suggesting that the compounds do not induce DNA damage and activation of the ATM kinase.

In summary, we have found that synthetic low molecular weight inhibitors designed to mimic the NH_2-terminal α-helix of p53 block p53/MDM2 interaction *in vitro* and stabilize and activate p53 *in vivo*. Many agents, such as topoisomerase 2 inhibitors and CDK inhibitors, are also known to activate p53 by partially inhibiting RNA polymerase II and in turn reducing the expression level of MDM2 (23, 24). Terphenyl compounds are unlikely to belong to such a category because they did not repress MDM2 expression level in cells without p53 and induced MDM2 in the presence of p53 (Fig. 4A). More importantly, terphenyl compounds that activated p53 in cell culture were also active in ELISA assay, whereas compounds that were ELISA-negative were also inactive in cell culture (Table 1). Therefore, the ability to activate p53 correlates with the ability to inhibit MDM2-p53 binding.

It is often expected that significantly higher drug concentrations are needed to induce a biological effect

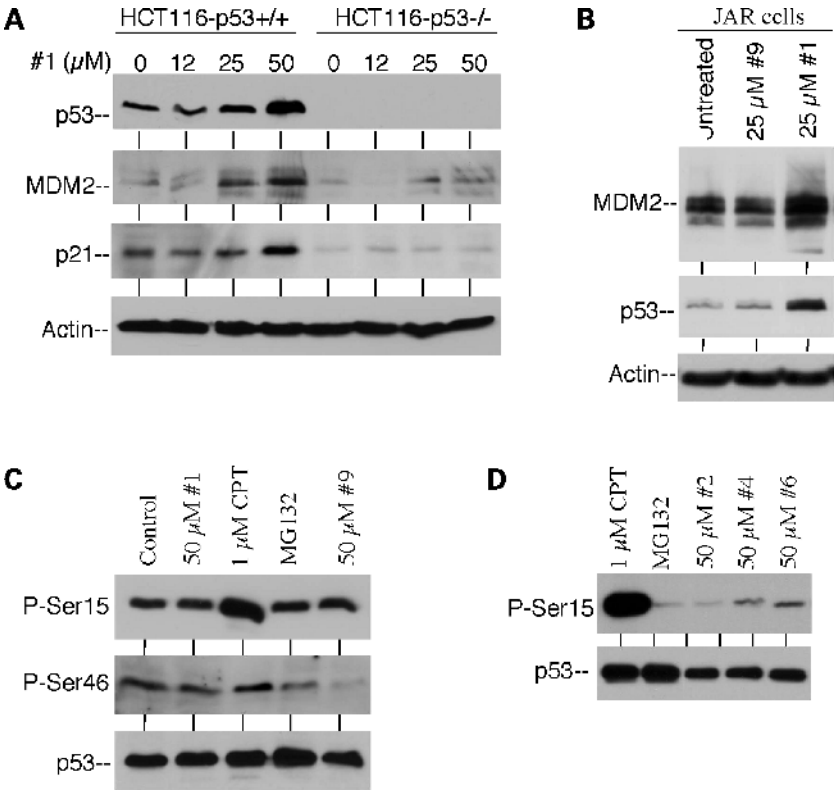

Figure 4. Induction of p53 accumulation and activation by terphenyl derivatives. **A,** HCT116 cells with or without endogenous wild-type p53 were treated with #**1** and analyzed by Western blot for p53, MDM2, and p21WAF1 protein levels. MDM2 and p21WAF1 induction by #**1** occurred in a p53-dependent manner. **B,** terphenyl #**1** induces p53 accumulation in tumor cells with MDM2 amplification. JAR cells were treated with #**1** or control #**9** for 20 h and MDM2 and p53 levels were determined by Western blot. **C** and **D,** terphenyl compounds do not induce p53 Ser^{15} phosphorylation. HCT116 cells were treated with 50 μmol/L #**1**, #**2**, #**4**, #**6**, control #**9**, or 1 μmol/L CPT for 8 h and loading was adjusted to obtain similar levels of total p53. p53 was analyzed by phospho-Ser^{15} and Ser^{46}-specific antibodies or by DO-1, which recognizes total p53. Ser^{15} and Ser^{46} phosphorylation was only induced by CPT but not by terphenyl compounds.

in vivo compared with *in vitro* assays due to membrane permeability and stability limits. It is somewhat surprising that the potency of terphenyl compounds *in vivo* closely parallel their ELISA activity. It is possible that the hydrophobic nature of these compounds limit their potency in ELISA but facilitates cellular entry and accumulation. Alternatively, the compounds may be more capable of disrupting intracellular p53-MDM2 interactions compared with the *E. coli*-produced recombinant proteins in ELISA. However, given the relatively weak potency of the compounds, we cannot exclude the possibility that other indirect mechanisms also contributed to p53 activation.

In a separate study, the interaction of active terphenyl compounds with the p53-binding pocket on MDM2 has also been detected by displacement of fluorescent p53 peptide and by induction of chemical shift changes at residues that form the p53-binding pocket in ^{15}N-HSQC nuclear magnetic resonance (25). These results are consistent with a mechanism of p53 activation by direct competition for MDM2 binding. Importantly, several terphenyl compounds identified here are membrane-permeable and able to induce p53 accumulation and activation in tumor cells. These results identify a novel and interesting scaffold for further synthesis of more specific and potent inhibitors of MDM2. Development of novel inhibitors based on different scaffolds should also increase the probability of obtaining compounds with properties suitable for therapeutic applications.

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