# Efficient p53 Activation and Apoptosis by Simultaneous Disruption of Binding to MDM2 and MDMX

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## Abstract

**The p53 tumor suppressor plays a key role in protection against malignant transformation. MDM2 and MDMX are important regulators of the transcriptional activity and stability of p53 by binding to its $NH_2$ terminus. Recent studies suggest that inhibition of both MDM2 and MDMX is necessary for robust activation of p53 in certain tumor cells. However, small-molecule MDM2 inhibitors such as Nutlin fail to inhibit MDMX despite significant homology between the two proteins. The therapeutic efficacy of such compounds may be compromised by MDMX overexpression. To evaluate the feasibility and biological effects of simultaneously disrupting p53 binding to MDM2 and MDMX, we used phage display to identify a novel peptide that can inhibit p53 interactions with MDM2 ($IC_{50}$ = 10 nmol/L) and MDMX ($IC_{50}$ = 100 nmol/L). Expression of a scaffold protein (thioredoxin) displaying this peptide sequence by adenovirus disrupts both MDM2 and MDMX interaction with p53, resulting in efficient p53 activation, cell cycle arrest, and apoptosis of tumor cells overexpressing MDM2 and MDMX. Intratumoral injection of the adenovirus also induces growth suppression of tumor xenografts in mice in a p53-dependent fashion. These results show the therapeutic potential of targeting both MDM2 and MDMX in cancer, and provide a novel structural motif for the design of potent p53 activators.** [Cancer Res 2007;67(18):8810–7]

## Introduction

MDM2 is a ubiquitin E3 ligase for p53 and an important regulator of p53 stability and activity by forming a negative feedback loop (1, 2). Overexpression of MDM2 abrogates the ability of p53 to induce cell cycle arrest and apoptosis (3). In ~30% of human osteogenic sarcomas and soft tissue sarcomas, MDM2 is overexpressed due to gene amplification. In tumors without *MDM2* amplification, hyperactivation of MDM2 due to silencing of ARF expression also leads to p53 inactivation. Therefore, MDM2 is a key factor in tolerance of wild-type p53 in nearly 50% of tumors, making it an attractive target for the development of novel antitumor agents (4).

The MDM2 homologue MDMX also binds to p53 and inhibits p53-dependent transcription (5). Loss of *MDM2* or *MDMX* leads to embryonic lethality, which can be rescued by deletion of *p53* (6–8). Therefore, expression of both MDM2 and MDMX is necessary for regulation of p53 during development. Unlike MDM2, MDMX does not have significant intrinsic E3 ligase activity (9). However, MDMX forms heterodimers with MDM2 through COOH-terminal RING domain interactions, which stimulates the ability of MDM2 to ubiquitinate and degrade p53 (10–13). Another consequence of MDMX-MDM2 heterodimer formation is that MDMX can be ubiquitinated and degraded by MDM2 (14–16); this is an important mechanism for controlling MDMX level during p53 stress response.

Recent studies suggest that the major mechanism of p53 regulation by MDMX is the formation of inactive MDMX-p53 complexes. Under nonstress conditions, MDM2 and p53 have short half-lives whereas MDMX is relatively stable. Therefore, elimination of MDMX is important for efficient p53 activation during stress response. DNA damage induces MDMX phosphorylation by ATM and Chk2 at several COOH-terminal serine residues ($Ser^{342}$, $Ser^{367}$, and $Ser^{403}$) generating a docking site for 14-3-3. These modifications stimulate MDMX degradation by MDM2, which facilitates p53 activation (17–20). Ribosomal stress resulting from disruption of rRNA biogenesis also activates p53, in part, by promoting MDMX degradation through L11-MDM2 binding, which enhances MDMX ubiquitination (21–24). MDMX overexpression leads to sequestration of p53 into inactive complexes and abrogates p53-mediated cell cycle arrest in response to ribosomal stress (24).

*MDMX* overexpression has been found in 40% of tumor cell lines (25), and in breast, colon, and lung tumor samples with 18.5% frequency (26). It is amplified in 4% of glioblastomas (27) and 5% of breast tumors (26). More recently, ~60% of retinoblastomas have been found to have *MDMX* overexpression or gene amplification (28). *MDMX* overexpression prevents oncogenic *ras*-induced premature senescence in mouse fibroblasts and cooperates with activated *ras* to confer tumorigenic potential in nude mice (26). RNAi-mediated knockdown of *MDMX* in HCT116 tumor cells suppresses tumor xenograft formation in nude mice (24). Because *MDM2* and *MDMX* overexpression or deregulation mainly occurs in tumors that retain wild-type *p53,* they are appealing targets for cancer drug discovery.

The extensive validation of MDM2 as a drug target resulted in the development of Nutlin, which can activate p53 by disrupting MDM2-p53 complex in tumor cells and tumor xenograft models (29). MDM2 and MDMX showed ~50% amino acid sequence identity in their p53-binding domains. However, recent studies reveal that Nutlin is inefficient for disruption of MDMX-p53 interaction and failed to activate p53 in cells overexpressing MDMX (30–32). Knockdown of MDMX cooperates with Nutlin to activate p53 in tumor cells and induces growth arrest. These results suggest that development of novel inhibitors optimized for dual-inhibition of MDM2 and MDMX is necessary to achieve full activation of p53.

In this study, we used phage display to identify a novel peptide that can inhibit p53 interactions with MDM2 ($IC_{50}$ = 10 nmol/L) and MDMX ($IC_{50}$ = 100 nmol/L) *in vitro*. Expression of a scaffold protein (thioredoxin) displaying this peptide sequence by recombinant adenovirus for the first time achieved disruption of both MDM2 and MDMX interaction with p53, resulting in efficient p53 activation and apoptosis of MDMX-overexpressing tumor cells in

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culture and in mice in a p53-dependent fashion. These results show the advantage of targeting both MDM2 and MDMX for p53 activation and induction of apoptosis in cancer cells, and provide a novel structural motif for the design of potent p53 activators.

## Materials and Methods

**Phage display.** An M13 phage library (Ph.D.-12, New England Biolabs) encoding random 12-mer peptides at the $NH_2$ terminus of pIII coat protein ($2.7 \times 10^9$ sequences) was used. Glutathione *S*-transferase (GST)-MDM2-1-150 and GST-MDMX-1-200 fusion proteins containing the p53 binding domain of human MDM2 and MDMX were expressed in *E. coli* and loaded onto glutathione-agarose beads. The loaded beads were incubated with blocking buffer [0.1 mol/L $NaHCO_3$ (pH 8.6), 5 mg/mL bovine serum albumin (BSA), 0.02% $NaN_3$] for 1 h at 4°C, washed with TBST [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20], and incubated in TBST at 4°C with $4 \times 10^{10}$ phages. Bound phages were eluted with 0.2 mol/L glycine (pH 2.2), 1 mg/mL BSA and neutralized with 1 mol/L Tris (pH 9.1). The eluted phages were amplified as instructed by the manufacturer. The binding/amplification process was repeated for four cycles for both targets. Phage DNA was prepared and the region of interest was sequenced.

**Fusion protein construction.** *E. coli* thioredoxin was used as a scaffold to display structurally constrained peptides. Double-stranded oligonucleotide (5′-GTCCGCCTCTGAGTTTGACGTTTGAGCATTATTGGGCGCAGTTGACGTCGGAAAACG) encoding pDI was cloned into the RsrII site of pBAD/Thio vector (Invitrogen). The complete thioredoxin-coding region with the peptide insert was amplified by PCR (using 5′-GGTCGACCCATGGGATCTGATAAAATTATTCATC-3′, 5′-GCTCGAGGGCCAGGTTAGCGTCG-3′ primers), cleaved with *Sal*I and *Xho*I, and cloned into pShuttle-IRES-hrGFP-1 vector (Stratagene). The plasmid was linearized with *Pme*I and cotransformed into *E. coli* BJ5183 with adenoviral backbone plasmid pAdEasy-1. Recombinant plasmids were linearized with *Pac*I and transfected into AD-293 cells (Stratagene) to generate viruses. Recombinant adenoviruses were purified by ultracentrifugation on $CsCl_2$ gradients and titered using the Adeno-X Rapid Titer Kit (Clontech).

**ELISA assay.** GST-MDM2-1-150 and GST-MDMX-1-200 containing human MDM2 and MDMX, respectively, and His6-tagged human p53 expressed in *E. coli* were used in ELISA as previously described (30).

**GST pull-down assay.** $^{35}$S[Methionine]-labeled MDMX and MDM2 were generated using the TNT *in vitro* transcription/translation kit (Promega). Five microliters of the translation products were mixed and incubated with glutathione-agarose beads loaded with 5 μg of GST-p53-1-52 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] for 2 h at 4°C. The beads were washed with lysis buffer, fractionated by SDS-PAGE, and bound MDMX and MDM2 were detected by autofluorography.

**Cell lines and antibodies.** Tumor cell lines H1299 (lung, p53-null), U2OS (bone), MCF-7 (breast), JEG-3 (placenta), and DLD-1 (breast) were maintained in DMEM with 10% fetal bovine serum. HCT116-$p53^{+/+}$ and HCT116-$p53^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein (Department of Oncology, Johns Hopkins University, Baltimore, MD); retinoblastoma Y79 cell line was provided by Dr. George Blanck (Department of Biochemistry, University of South Florida, Tampa, FL); and normal human foreskin fibroblasts were provided by Dr. Jack Pledger (Moffitt Cancer Center, Tampa, FL). A U2OS stable cell line with MDMX overexpression and U2OS stable cell line, expressing tetracycline-regulated human MDM2 and MDMX were previously described (14, 30). The following antibodies were used in the experiment: 3G9 (mouse) and a rabbit polyclonal serum for MDM2 Western blot and immunoprecipitation; DO-1 (PharMingen) and FL393 (Santa Cruz Biotechnology) for p53 Western blot; 8C6 monoclonal or a rabbit polyclonal serum for MDMX Western blot and immunoprecipitation; and anti-p21WAF (Santa Cruz Biotechnology) for p21. p53 ubiquitination assay was done as previously described (14).

**Animal studies.** Athymic-NCr-nu female mice, between 7 and 8 weeks of age, were inoculated s.c. on both flanks with $5 \times 10^6$ HCT116 cells. Tumor formation was followed for 4 to 6 days. When tumor size reached ~0.2 $cm^3$, the mice were injected with $5 \times 10^{10}$ plaque-forming units (pfu) of Ad-DI or control adenoviruses everyday for a total of five times. Tumor volume was calculated with the formula $[(\text{length} + \text{width}) / 2]^3 \times 0.5236$. Tumors recovered after termination of the experiments were fixed in formalin and paraffin sections were analyzed by immunohistochemical staining with rabbit antibodies against p53 and FLAG.

## Results

**Identification of a high-affinity MDM2 and MDMX binding peptide.** To identify novel peptide inhibitors of MDM2 and MDMX, phage display was used to screen a 12-mer library to obtain peptides that bind to the p53-binding domains of MDM2 and MDMX. GST-MDM2 and GST-MDMX were used as baits and the binding/amplification process was repeated for four cycles for both targets. Phage DNA was prepared and the variable region was sequenced. The results showed that 7 of 10 MDMX-selected and 4 of 10 MDM2-selected phages contain the same insert (LTFEHYWAQLTS; Table 1). This peptide was named pDI for peptide dual inhibitor. The remaining phages contain unrelated and inactive sequences when tested by ELISA and were not further characterized.

The results above showed that screens using GST-MDM2 or GST-MDMX resulted in the selection of the same peptide. This suggests that pDI has such high affinity for both MDM2 and MDMX that it outcompeted any potential isoform-specific peptides by a significant margin. The pDI peptide (LTFEHYWAQLTS) is distinct from the p53 peptide (16-QETFSDLWKLLP-28) and the previously identified 12/1 peptide (MPRFMDYWEGLN) that interacts with MDM2 and MDMX (33), but retains three key p53 hydrophobic residues ($Phe^{19}$, $Trp^{23}$, and $Leu^{26}$, underlined) that contact the MDM2 pocket (34, 35). In an ELISA assay, pDI inhibited MDM2-p53 and MDMX-p53 interactions with $IC_{50}$ of 10

**Table 1.** Peptide sequences selected by phage display

| MDMX selected phages | MDM2 selected phages |
|---|---|
| T-MX-1: F A P L N R T V E T S P | T-M2-1: Q Q M H L M S Y A P G P |
| T-MX-2: L T F E H Y W A Q L T S | T-M2-2: T I R P S T T M D S P T |
| T-MX-3: L T F E H Y W A Q L T S | T-M2-3: Y A N P Q M E K A F E S |
| T-MX-4: Y A V S S S P R V A A L | T-M2-4: L T F E H Y W A Q L T S |
| T-MX-5: L T F E H Y W A Q L T S | T-M2-5: L P N L T W A L M P G A |
| T-MX-6: L T F E H Y W A Q L T S | T-M2-6: Y A N P Q M E K A F A S |
| T-MX-7: L T F E H Y W A Q L T S | T-M2-7: L T F E H Y W A Q L T S |
| T-MX-8: L T F E H Y W A Q L T S | T-M2-8: L T F E H Y W A Q L T S |
| T-MX-9: V V H V P N S A T P P R | T-M2-9: L L A D T T H H R P W T |
| T-MX-10: L T F E H Y W A Q L T S | T-M2-10: L T F E H Y W A Q L T S |



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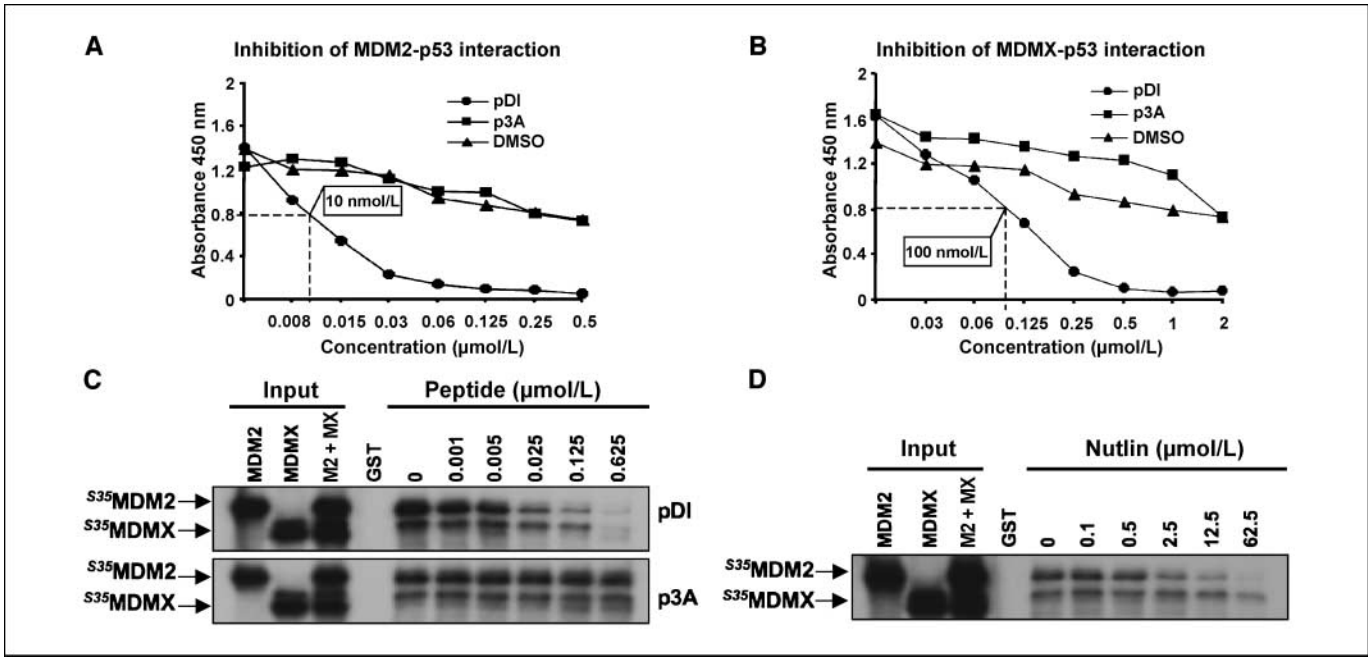


**Figure 1.** Disruption of MDM2 and MDMX-p53 interactions by a novel peptide. *A* and *B,* phage display–selected pDI and control p3A peptides were tested in an ELISA assay of GST-MDM2 and GST-MDMX binding to immobilized His6-p53. *C* and *D,* glutathione beads loaded with GST-p53-1-52 fusion protein were incubated with a mixture of *in vitro* translated MDM2 and MDMX and different concentrations of peptides or Nutlin. Binding of MDM2 and MDMX to GST-p53-1-52 in the presence of inhibitors was determined by autofluorography after washing and SDS-PAGE.

and 100 nmol/L respectively, which is 15-, 60-, and 300-fold better than 12/1, Nutlin, and p53 peptide on MDM2 (Fig. 1*A* and *B*; Table 2). Mutation of the three key hydrophobic residues to alanine (p3A) abrogated MDM2 and MDMX inhibition, suggesting that pDI mimics p53 binding to MDM2 and MDMX. In a different assay, pDI also inhibited GST-p53 capture of *in vitro* translated MDM2 and MDMX with different efficiency (Fig. 1*C*). In contrast, Nutlin only blocked MDM2 but had no effect on MDMX-p53 interaction (Fig. 1*D*). The 10-fold difference in $IC_{50}$ for MDM2 and MDMX suggests that MDMX may bind p53 with higher affinity than MDM2, which is consistent with its mechanism of p53 inhibition by sequestration.

**Construction of an MDM2 and MDMX inhibitory protein.** After unsuccessful attempts to activate p53 in cells by fusion or conjugation of pDI to the Antennapedia cell-permeable peptide, the pDI sequence was inserted into the active center of FLAG-tagged *E. coli* thioredoxin protein that serves as a display scaffold (ref. 36; Fig. 2*A*). Adenoviruses expressing the fusion proteins with wild-type pDI (Ad-DI) and control p3A (Ad-3A) sequences were constructed (Fig. 2*B*). The fusion proteins expressed in infected cells showed diffused cytoplasmic and nuclear staining, consistent with their small sizes ($\sim$15 kDa), and has a half life of $\sim$1 h, which was unrelated to MDM2 binding (data not shown).

The pDI peptide was selected based on its ability to bind MDM2 and MDMX. To test whether insertion of pDI sequence into thioredoxin conferred the ability to binding MDM2 and MDMX, cells infected with Ad-DI virus were immunoprecipitated with FLAG antibody and analyzed for the coprecipitation of endogenous MDM2 and MDMX. The results showed that the FLAG-DI protein, but not FLAG-3A, coprecipitated with both MDM2 and MDMX when expressed in MCF-7 cells (Fig. 2*C*). Furthermore, FLAG-DI expression disrupted MDM2-p53 and MDMX-p53 coprecipitations (Fig. 2*D*). As expected, Nutlin failed to disrupt MDMX-p53 complex in the same assay. Infection with Ad-DI did not cause p53 $Ser^{15}$ phosphorylation (data not shown), suggesting that the fusion protein disrupted p53 binding to MDM2 and MDMX by a competitive mechanism without triggering DNA damage signaling. These results showed that the FLAG-DI fusion protein was expressed at levels sufficient to compete with p53 for binding to endogenous MDM2 and MDMX.

**Activation of p53 by the MDM2 and MDMX inhibitory protein.** Disruption of MDM2-p53 and MDMX-p53 complexes should result in p53 stabilization and activation. As expected, infection of tumor cells or normal human foreskin fibroblasts expressing wild-type p53 with Ad-DI resulted in significant increase in p53 level and induction of p53 targets (p21, MDM2, and PUMA; Fig. 3*A* and *B*). The effects were not observed using Ad-3A or Ad-vector control viruses, indicating that they were dependent on the pDI sequence in the fusion protein. Infection of p53-null (HCT116-$p53^{-/-}$) or mutant (DLD1) cell lines failed to induce p53 target genes, indicating that the effect of Ad-DI was p53 dependent (Fig. 3*A* and *B*). Reverse transcription-PCR analysis showed that

**Table 2.** Inhibition of MDM2 and MDMX-p53 binding by peptides in ELISA

| Peptides (compound) | Sequences | $IC_{50}$ for MDM2 (μmol/L) | $IC_{50}$ for MDMX (μmol/L) |
|---|---|---|---|
| p53pep | QETFSDLWKLLP | 3.00 | 27.50 |
| 12/1 | MPRFMDYWEGLN | 0.15 | 1.25 |
| Nutlin | | 0.60 | No inhibition |
| pDI | LTFEHYWAQLTS | 0.01 | 0.10 |
| p3A | LTAEHYAAQATS | No inhibition | No inhibition |



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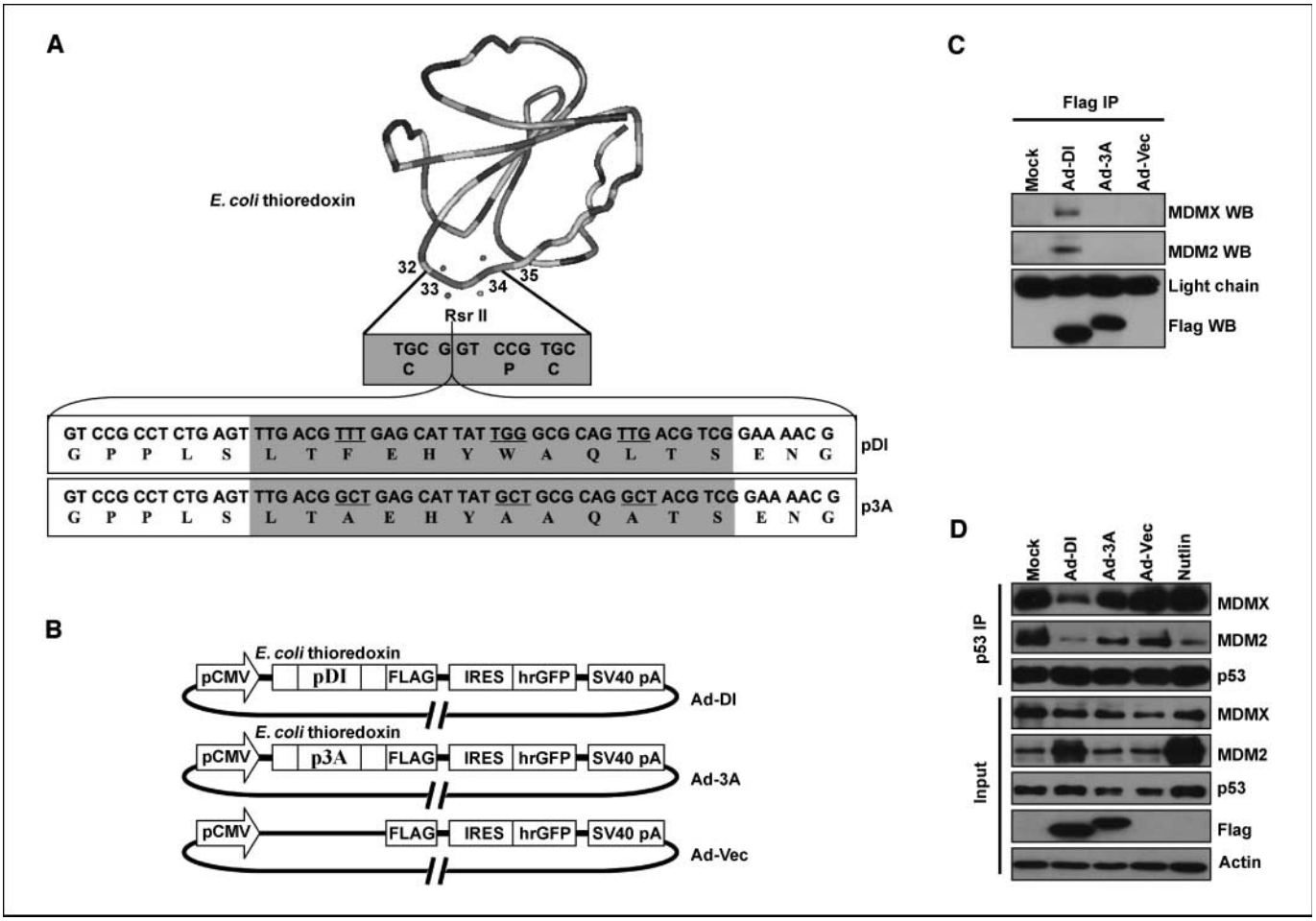


**Figure 2.** Design of recombinant adenovirus for expression of peptide epitopes. *A,* schematic representation of the scaffold protein *E. coli* thioredoxin with pDI and p3A inserted between $Gly^{33}$ and $Pro^{34}$ at the active center. *B,* the coding regions of thioredoxin-DI and thioredoxin-3A were fused to FLAG epitope in the adenovirus genome. The cytomegalovirus (CMV) promoter drives expression of FLAG-DI or FLAG-3A, and an internal ribosome entry site (IRES) element allows coexpression of hrGFP from the same transcript. *C,* MCF-7 cells were infected with Ad-DI, Ad-3A, and Ad-Vec (MOI, 300) for 48 h and cell lysate was immunoprecipitated with FLAG antibody and blotted for coprecipitation of MDM2 and MDMX. *D,* MCF-7 cells were treated with Ad-DI, Ad-3A, and Ad-Vec (MOI, 300) or Nutlin (10 μmol/L) for 48 h and MG132 (30 μmol/L) was added 4 h before harvest. Cell lysate was immunoprecipitated with p53 antibody and blotted for coprecipitation of MDM2 and MDMX.

the increase in p21 and MDM2 levels was associated with increase in mRNA (data not shown). Ad-DI infection also activated a stably integrated p53-responsive reporter, BP100-luc, in HCT116 and MCF-7 cells (Fig. 3*C*), indicating activation of p53 transcriptional function. Although FLAG-DI was diffusely localized to both nucleus and cytoplasm, MDM2 and p53 induced by its expression were predominantly nuclear. As expected, Ad-DI infection inhibited p53 ubiquitination by MDM2 (data not shown), consistent with its ability to disrupt MDM2-p53 binding. Cell cycle analysis by fluorescence-activated cell sorting (FACS) revealed an ~10-fold reduction of S-phase population in the Ad-DI–infected HCT116-$p53^{+/+}$ cells but not in HCT116-$p53^{-/-}$ cells (Fig. 3*D*). Ad-DI infection also induced significant apoptosis in HCT116-$p53^{+/+}$ cells, but was much less effective in HCT116-$p53^{-/-}$ cells (Fig. 3*D*). Therefore, the cell cycle arrest and apoptosis activities of Ad-DI are mediated by activation of p53.

**Activation of p53 in cells overexpressing MDMX.** Nutlin does not disrupt MDMX-p53 binding when applied at practical concentrations dictated by solubility and nonspecific toxicity (5–10 μmol/L). Therefore, p53 activation by Nutlin is attenuated in cells overexpressing MDMX (30–32). To test whether Ad-DI is more efficient in activating p53 in cells overexpressing MDMX, JEG3 (high MDM2 and MDMX) and Y79 (high MDMX) cells were treated with the virus (24, 28). The results showed that Ad-DI infection resulted in significant apoptosis in both cell lines, whereas Nutlin was less effective (Fig. 4*A*). These results suggested that FLAG-DI is able to overcome physiologic levels of MDM2 and MDMX overexpression.

To further test the ability of FLAG-DI in overcoming higher levels of MDM2 and MDMX overexpression, U2OS cells expressing tetracycline-regulated MDM2 (~8× endogenous level) and MDMX (~8× endogenous level) were treated with Ad-DI and Nutlin. The results showed that, as expected, Nutlin remained highly effective even when MDM2 is overexpressed, whereas MDMX overexpression completely abrogated the ability of Nutlin to activate p53 (Fig. 4*B*). In contrast, the activity of Ad-DI was only moderately inhibited by MDM2 and MDMX overexpression, consistent with a competitive mechanism of FLAG-DI action. Similar assay using U2OS overexpressing even higher levels of MDMX (30× endogenous level, noninducible) also showed the ability of Ad-DI to activate p53 under conditions when Nutlin was completely ineffective (data not shown). In addition, FACS



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and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays showed that Ad-DI induced significant apoptosis in U2OS-MDMX cells, which were completely resistant to Nutlin (Fig. 4*C* and *D*). Treatment of normal human foreskin fibroblasts with Ad-DI did not cause apoptosis (data not shown). This is expected because most nontransformed cell types do not undergo apoptosis on p53 activation (37). These results suggested that FLAG-DI is an efficient activator of p53 in tumor cells over-expressing MDM2 and MDMX due to its ability to neutralize both proteins.

**Antitumor effects of MDM2 and MDMX inhibition.** To test the antitumor potential of Ad-DI, HCT116 tumor xenografts ( $\sim 0.2$ cm$^3$) were treated with daily single intratumoral (i.t.) injection of $5 \times 10^{10}$ pfu Ad-DI or control virus for 5 consecutive days. This resulted in 90% suppression of tumor growth for the following 16 days by Ad-DI but not by control viruses or buffer (Fig. 5*A*). The differences in

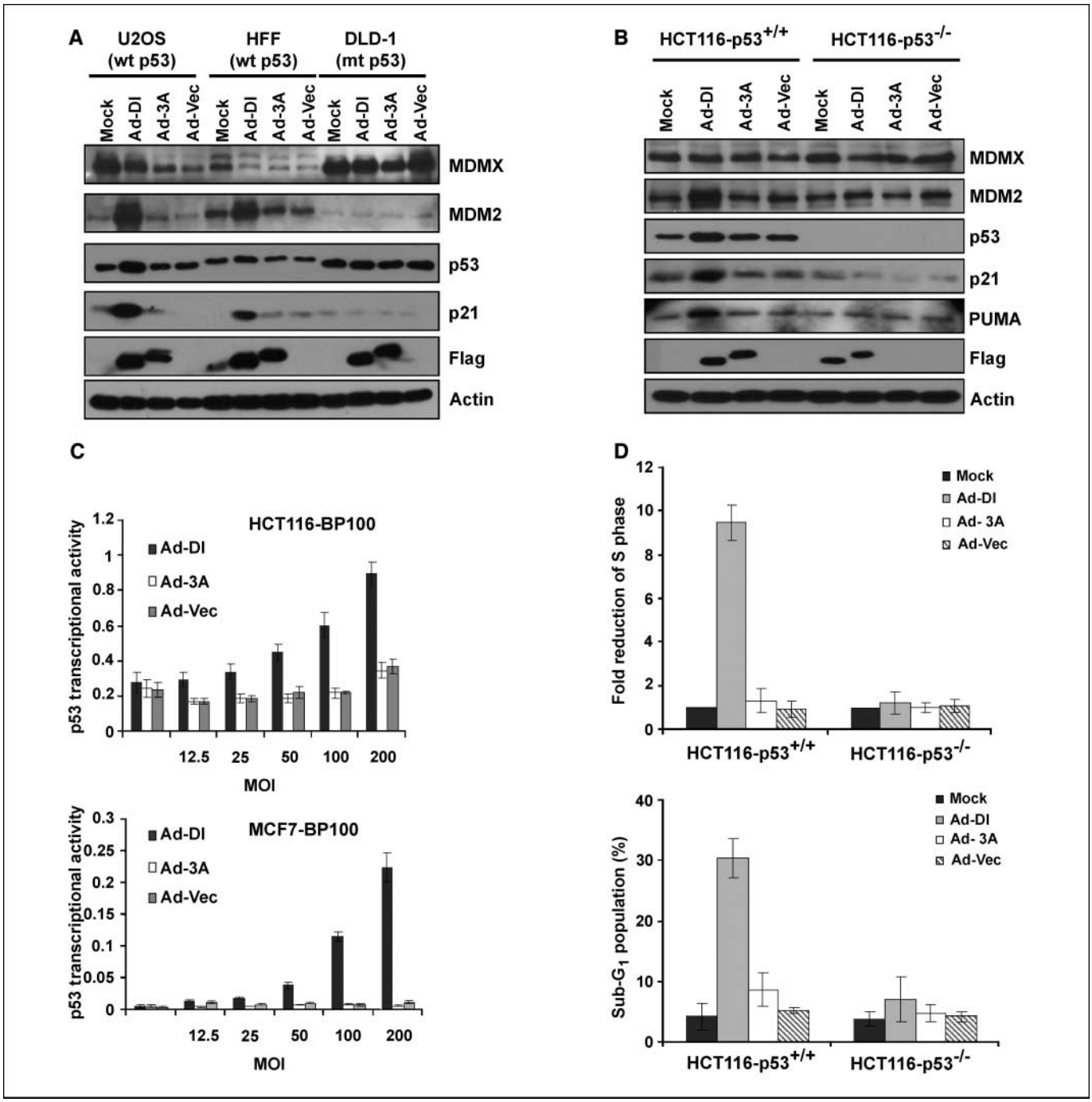


**Figure 3.** Inhibition of MDM2 and MDMX-p53 binding by FLAG-DI activates p53. *A* and *B,* cells were infected with the recombinant adenoviruses (MOI, 100) for 48 h and analyzed by Western blot. *C,* HCT116 and MCF-7 cells stably transfected with the p53-responsive reporter plasmid BP100-luc were infected with Ad-DI at the indicated MOI for 48 h and luciferase activity was determined ($n = 3$). *D,* HCT116-*p53*$^{+/+}$ and HCT116-*p53*$^{-/-}$ cells were infected with Ad-DI (MOI, 300) for 48 h. Reduction of S phase was analyzed by propidium iodide staining and FACS. Apoptosis was measured by the level of sub-$G_1$ fraction in FACS analysis.



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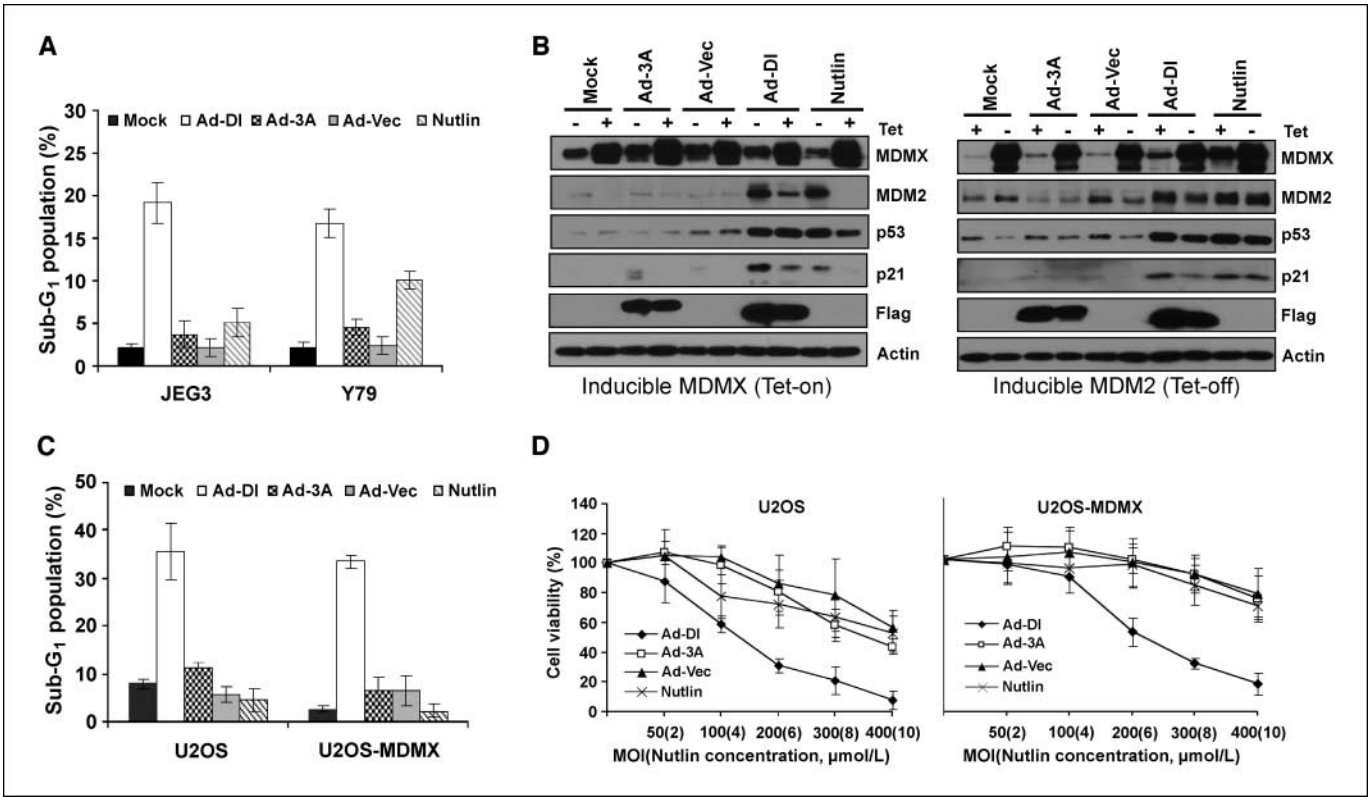


**Figure 4.** Activation of p53 by FLAG-DI in cells overexpressing MDM2 and MDMX. *A,* JEG3 and Y79 cells were treated with Ad-DI (MOI, 300) or Nutlin (10 μmol/L) for 48 h and analyzed by FACS for apoptotic sub-$G_1$ population. *B,* U2OS expressing inducible MDMX (Tet-on) and MDM2 (Tet-off) were treated with 0.1 μg/mL tetracycline for 18 h, followed by treatment with Ad-DI (MOI, 100) or Nutlin (10 μmol/L) for additional 24 h, and p53 activation markers were analyzed by Western blot. *C,* U2OS and U2OS-MDMX cells were treated with Ad-DI (MOI, 300) and Nutlin (10 μmol/L) for 48 h and analyzed by FACS for sub-$G_1$ population. *D,* U2OS and U2OS-MDMX cells were treated with Ad-DI and Nutlin at indicated concentrations for 5 d and analyzed by MTT assay for cell viability.

tumor suppression effects of Ad-DI ($n$ = 10), Ad-3A ($n$ = 6), Ad-vector ($n$ = 8), and buffer ($n$ = 17) were statistically significant ($P$ < 0.01). Similar treatment of larger tumors (>0.5 $cm^3$) resulted in moderate growth inhibition in only a subset of animals (data not shown), most likely due to limited access of the virus to distant parts of the tumor. Strong induction of p53, MDM2, and p21 was observed in the extract of tumors 48 h after injection with Ad-DI (Fig. 5*B*). Immunohistochemical staining of tumor serial sections showed localized expression of FLAG-DI in the nucleus and cytoplasm of tumor cells near the sites of injection, which correlates with significant p53 staining in the same area (Fig. 5*C*).

Consistent with cell culture results, the antitumor effect of Ad-DI was strictly dependent on p53. The growth of HCT116-*p53*$^{-/-}$ tumor xenograft was not inhibited by Ad-DI ($n$ = 6) and Ad-3A ($n$ = 6, $P$ > 0.05; Fig. 5*D*). Furthermore, Ad-DI also inhibited the growth of tumor xenograft formed by a modified HCT116-*MDMX* cell line overexpressing MDMX (~5-fold; Fig. 5*D*; ref. 24; $P$ < 0.01, $n$ = 5) and induced p53 activation, as determined by Western blot and immunohistochemical staining (data not shown). Ad-DI and control viruses were well tolerated in mice after i.t. administration, with no observable weight loss or pathologic changes of different organs (data not shown).

## Discussion

Recent studies suggest that MDM2 regulates p53 mainly by promoting its degradation, whereas MDMX acts by sequestration of p53 (38, 39). Although current understanding of the role of MDMX in cancer is still limited, cell culture experiments suggest that MDMX is a significant player in suppressing p53 activity in at least a subset of tumors. Several studies of MDMX expression in clinical samples also strongly implicate its involvement in cancer development. These observations suggest a need to further evaluate the potential of MDMX as a therapeutic target. Currently, the most attractive approach for targeting MDMX is to use small molecules to disrupt MDMX-p53 association. MDMX knockout and RNAi provided valuable evidence for the functional importance of MDMX in regulating p53. However, because MDMX also interacts with other molecules such as MDM2 and casein kinase 1, MDMX depletion does not provide the best simulation of disrupting MDMX-p53 binding.

The MDM2 inhibitor Nutlin was developed specifically for MDM2 (40, 41). Interestingly, we and others showed that Nutlin is at least 30-fold less efficient in disrupting MDMX-p53 binding (30–32). When applied at practical concentrations, Nutlin is likely to function only by inhibiting MDM2. MDMX is also insensitive to a class of small-molecule MDM2 inhibitors that are α-helical mimics based on the terphenyl scaffold (42).[1] These observations suggest that the p53-binding pockets on MDM2 and MDMX have differences that affect the binding of small molecules. Such differences may also compromise the effect of other small molecules optimized for MDM2.

[1] B. Hu and J. Chen, unpublished results.



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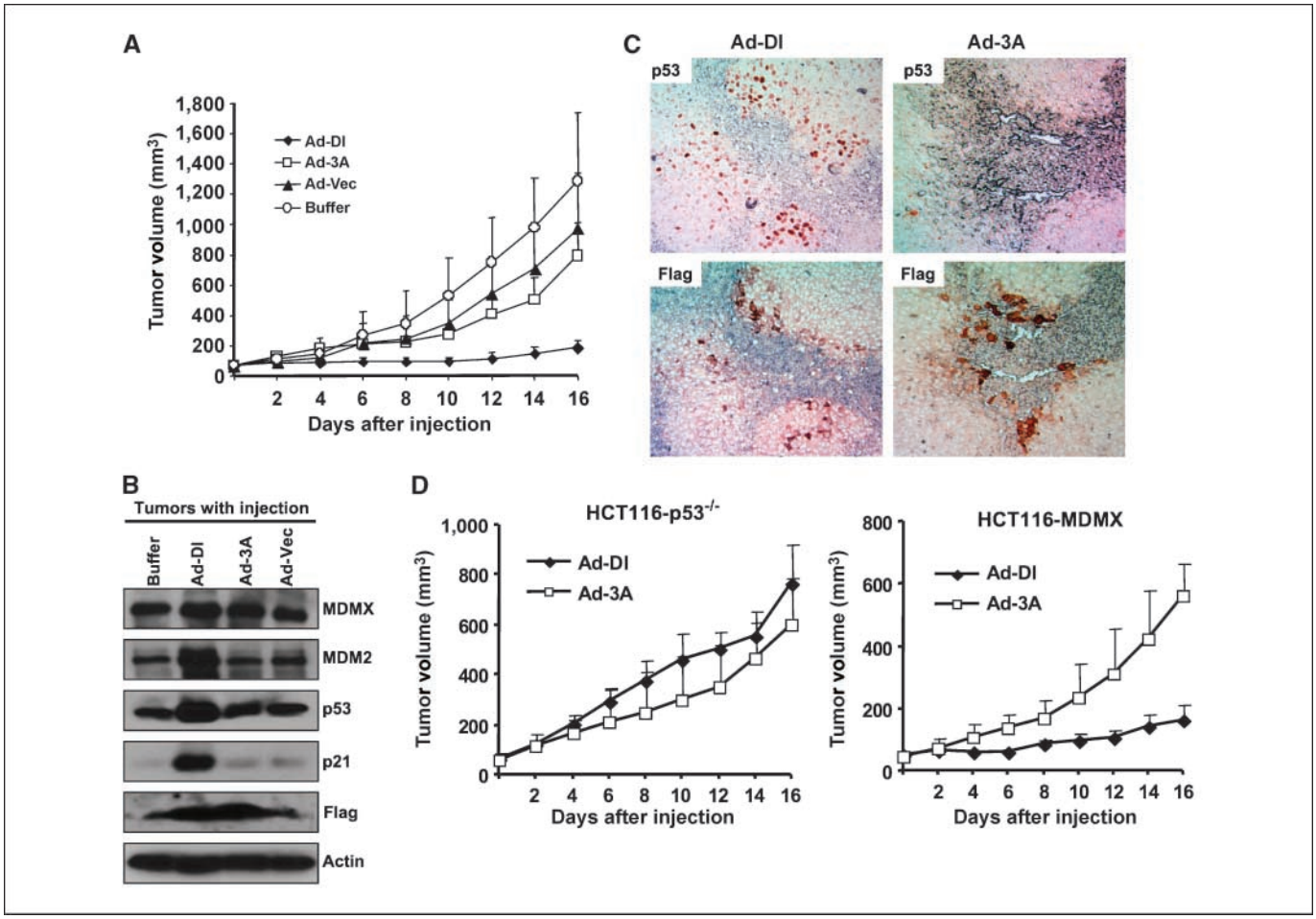


**Figure 5.** Antitumor activity of Ad-DI. *A,* nude mice bearing HCT116-*p53*$^{+/+}$ xenografts were treated with Ad-DI, Ad-3A, Ad-vector, or buffer by daily i.t. injections ($5 \times 10^{10}$ pfu/injection) for 5 consecutive days. The tumor volumes were measured every 2 d after completion of treatment cycle. *B,* representative tumor samples recovered 48 h after injection were analyzed by Western blot. *C,* representative tumor specimens 48 h after injection with Ad-DI and Ad-3A were stained for p53 and FLAG in serial sections. *D,* HCT116-*p53*$^{-/-}$ and HCT116-MDMX tumor xenografts were treated with i.t. injection of $5 \times 10^{10}$ Ad-DI or Ad-3A for 5 consecutive days. Tumor growth was measured for the indicated time frame after completion of injections.

For therapeutic applications, it is beneficial to have an inhibitor that is dual-specific for MDM2 and MDMX. Our attempts to screen for MDM2 and MDMX binding peptides in this study resulted in the identification of the same sequence motif. This finding suggests that MDM2 and MDMX have similar binding specificity to peptide sequences, which is distinct from their interactions with small molecules. It is possible that peptides rely on extensive contacts with the p53 binding pockets and is not sensitive to minor differences that affect small-molecule ligands. Interestingly, in both ELISA and GST pull-down assays, disruption of MDMX-p53 interaction always requires ~10-fold higher concentrations of the pDI peptide. This suggests that MDMX may bind p53 with higher affinity than MDM2, which is consistent with its mechanism of p53 inhibition by forming stable complexes.

Our results showed that simultaneous inhibition of MDM2 and MDMX binding to p53 has strong proapoptotic potential in cell culture and can efficiently suppress tumor growth *in vivo*. The ability of Ad-DI to induce apoptosis is a significant contrast to Nutlin, which induces cell cycle arrest in most tumor cell lines (41). It is possible that the ability of FLAG-DI to target both MDM2 and MDMX caused p53 activation over the apoptotic threshold. These results provide a proof-of-principle for the antitumor potential of MDM2/MDMX dual inhibitors. The potent pDI peptide also provides a novel motif for structural study of MDM2 and MDMX interactions with a high-affinity ligand and should aid the design of small-molecule inhibitors. Cancer therapy using the Ad-DI virus may be limited by the efficiency of delivery to large tumors. However, it is possible that gene therapy is a useful approach against certain tumors such as retinoblastoma, 60% of which overexpress MDMX (28). In such cases, the small tumor sizes, enclosed environment, and a need to avoid genotoxicity and preserve vision may make it a potential therapeutic option (43).

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# Efficient p53 Activation and Apoptosis by Simultaneous Disruption of Binding to MDM2 and MDMX

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