Published OnlineFirst November 12, 2010; DOI: 10.1158/1535-7163.MCT-10-0581

Molecular Cancer Therapeutics

A Small-Molecule Inhibitor of MDMX Activates p53 and Induces Apoptosis

Hongbo Wang[1], Xujun Ma[1], Shumei Ren[1], John K. Buolamwini[2], and Chunhong Yan[1]

Abstract

The p53 inactivation caused by aberrant expression of its major regulators (e.g., MDM2 and MDMX) contributes to the genesis of a large number of human cancers. Recent studies have shown that restoration of p53 activity by counteracting p53 repressors is a promising anticancer strategy. Although agents (e.g., nutlin-3a) that disrupt MDM2–p53 interaction can inhibit tumor growth, they are less effective in cancer cells that express high levels of MDMX. MDMX binds to p53 and can repress the tumor suppressor function of p53 through inhibiting its *trans*-activation activity and/or destabilizing the protein. Here we report the identification of a benzofuroxan derivative [7-(4-methylpiperazin-1-yl)-4-nitro-1-oxido-2,1,3-benzoxadiazol-1-ium, NSC207895] that could inhibit *MDMX* expression in cancer cells through a reporter-based drug screening. Treatments of MCF-7 cells with this small-molecule MDMX inhibitor activated p53, resulting in elevated expression of proapoptotic genes (e.g., *PUMA*, *BAX*, and *PIG3*). Importantly, this novel small-molecule p53 activator caused MCF-7 cells to undergo apoptosis and acted additively with nutlin-3a to activate p53 and decrease the viability of cancer cells. These results thus show that small molecules targeting *MDMX* expression would be of therapeutic benefits. *Mol Cancer Ther; 10(1); 69–79. ©2010 AACR.*

Introduction

The tumor suppressor p53 is a gatekeeper of the genome, and maintains genetic stability on oncogenic challenges by inducing cell cycle arrest, apoptosis or senescence (1, 2). Accordingly, p53 inactivation is a major driving force for tumorigenesis. In spite of widespread gene mutations, p53 is inactivated by aberrant expression of its regulatory proteins in many human cancers (1). Indeed, MDM2, a major p53 repressor, which potently inhibits p53 transcriptional activity (3) while targeting the tumor suppressor for ubiquitin-dependent degradation (4, 5), is overexpressed in more than 10% of human cancers (1). On the other hand, overexpression of the MDM2 homologue MDMX (also referred to as MDM4) occurs in 18% to 19% of breast, lung, and colon cancers (6); 50% of head and neck squamous carcinomas (7); and 65% of retinoblastomas (8). Although MDMX contains the RING domain, it lacks the E3 ubiquitin ligase activity (9), and a prevailing view suggests that MDMX binds to p53 at its *trans*-activation domain thereby mainly repressing its transcriptional activity (1). However, emerging evidence suggests that MDMX can also regulate the stability of p53 through promoting MDM2-mediated degradation (10–14). Interestingly, overexpression of MDM2 and MDMX are mutually exclusive in cancer cells (6), suggesting that dysregulation of a major repressor is sufficient to inactivate p53 leading to tumor development. Because the *TP53* gene often remains wild-type in MDM2- or MDMX-overexpressing cancers, it has long been conceived that targeting these p53 repressors could restore p53 activity, thereby causing malignant cells to die or senesce (1, 15–17).

Indeed, recent studies have shown that small molecules disrupting the p53–MDM2 interaction can activate p53 leading to *in vivo* tumor regression (18, 19). A *cis*-imidazoline compound nutlin-3a, for example, can dissociate the p53–MDM2 complex by binding to the latter protein and consequently inducing nuclear accumulation of p53 (18). As a result of p53 activation, nutlin-3a sensitizes cancer cells to conventional cancer therapies (20–22) and inhibits growth of human tumor xenografts in nude mice (18). Notwithstanding these promising anticancer activities, nutlin-3a cannot induce apoptosis in cancer cells that express high levels of MDMX protein (e.g., MCF-7), presumably due to its inability to disrupt the p53–MDMX interaction (23–25). These studies thus indicate a need to develop agents targeting *MDMX* overexpression in cancer cells (26, 27). Although MDMX siRNA (28) or a peptide simultaneously disrupting the

Authors' Affiliations: [1]Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York; and [2]Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee

Note: Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org).

Corresponding Author: Chunhong Yan, Albany Medical College, MC 165, 47 New Scotland Avenue, Albany, NY 12208. Phone: 518-262-6157, Fax: 518-262-6659. E-mail: YanC@mail.amc.edu.

doi: 10.1158/1535-7163.MCT-10-0581

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interaction of p53 with MDM2 or MDMX (29) can inhibit tumor growth and sensitize MCF-7 cells to nutlin-3a–induced apoptosis (24), small molecules exhibiting similar activities are more desirable for cancer therapy.

Given that *MDMX* overexpression in cancer is mainly caused by aberrant transcription (30), we applied a recent high-throughput drug screening (HTS) strategy (31) to search for small molecules that can inhibit *MDMX* transcription. We identified a small molecule that could downregulate *MDMX* expression in various cancer cells. Most importantly, we found that this MDMX-targeting agent dramatically increased the p53 activity leading to expression of proapoptotic p53-target genes. This small molecule thus represents a new class of p53 activators that can restore the activity of the tumor suppressor leading to eradication of cancer cells.

Materials and Methods

Cell culture and transfections

MCF-7 and A549 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, whereas LNCaP cells were routinely cultured in RPMI1640 medium. Breast cancer lines MDA-MB-175VII, ZR-75-1, ZR-75-30, MDA-MB-231, BT474, and BT549 were obtained from Dr. Ceshi Chen (Albany Medical College, Albany, NY), and cultured as recommended by the American Type Culture Collection. These cell lines have not been tested and authenticated by us. For transfections, cells were plated at 90% to 95% confluence, and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation.

Development of the luciferase reporter assay and drug screening

This was done essentially as described previously (31, 32). Briefly, a fragment of the *MDMX* promoter (−1991 to +120, relative to the transcription start site) was amplified by PCR using genomic DNA derived from normal human fibroblasts WI-38 and cloned into the pCM-luc vector constructed previously (32). The plasmid containing the *MDMX* promoter and a FRT sequence was then cotransfected with the Flp-expressing pOG44 plasmid into HT1080/F55 cells (33), followed by selection for Hygromycin B-resistant clones. A random resistant clone was lysed for luciferase activity assay to confirm that the *MDMX* promoter was active and then expanded for drug screening. To determine the suitability of the recombinant cells for drug screening, cells plated in 96-well plates were treated with 1 μmol/L actinomycin D for 16 hours and lysed for luciferase activity assays. The relative promoter activity was then used to calculate the value of Z′-factor, which was defined in ref. 34 and used to measure reproducibility of an assay to determine the suitability of the assay for HTS. For drug screening, recombinant cells plated in 96-well plates were treated with 5 μmol/L of individual compounds from the NCI Diversity-Set library for 6 hours and lysed for luciferase activity assays (31). Compounds decreasing the luciferase activity to 50% or more were further tested for their cytotoxicity using MTT assays as described (31) and subjected to a counter-screen using a cytomegalovirus (CMV) reporter to exclude general transcription inhibitors. The CMV promoter inserted into the same F55 genomic locus was prepared similarly as described above. A similar strategy was also employed to engineer the P1 or P2 promoter of the *MDM2* gene (35) into the F55 locus.

Immunoblotting and cycloheximide chase assays

Immunoblotting was carried out as previously described (36). The following antibodies were used: DO-1 (for p53; Santa Cruz Biotechnology), p-S15-p53 (Cell Signaling), MDMX (Bethyl Laboratories,), PARP (H-250; Santa Cruz), p21 (BD Biosciences), MDM2 (SMP-14; Santa Cruz), and β-actin (Sigma-Aldrich). For cycloheximide chase assays, cells were treated with or without 100 μg/mL of cycloheximde (Sigma-Aldrich) for 0.5, 1, 2, and 3 hours, and then lysed for immunoblotting as described (37).

Quantitative RT-PCR

Total RNA was prepared, reverse transcribed and subjected to real-time PCR assays essentially as described previously (33). The sequences of primers used for amplifying MDMX, p21, PUMA, BAX, and PIG3 cDNA are available on request.

shRNA knockdown

Knockdown was done using a Lentivector-based shRNA system (pSIH-H1 shRNA Cloning and Lentivector Expression system; System Biosciences) as described previously (38). The targeted sequences for MDMX, p53, and MDM2 were 5′-GTG ATG ATA CCG ATG TAG A-3′, 5′-GAC TCC AGT GGT AAT CTA C-3′, and 5′-GGA ATT TAG ACA ACC TGA A-3′ based on publications (23, 39, 40), respectively. For negative controls, a luciferase-targeted sequence (5′-CTT ACG CTG AGT ACT TCG A-3′) was cloned into the Lentivector.

Flow cytometry and TUNEL staining

MCF-7 cells treated with dimethyl sulfoxide (DMSO), nutlin-3a, or the MDMX inhibitor were permeabilized with cold 70% ethanol overnight, and stained with a solution containing 50 μg/mL propidium iodide (PI; Sigma-Aldrich) and 20 μg/mL RNase A at 37°C for 20 minutes. The cells were then subjected to flow cytometry analysis as described previously (41). The FlowJo software was used to calculate percentages of cells in each cell cycle phase. For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining, MCF-7 cells treated with the MDMX inhibitor for 2 days were fixed with 4% paraformadelhyde for 1 hour, and then subjected to dUTP labeling using *In Situ* Cell Death Detection Kit TMR Red (Roche) according to the manufacturer's protocol. For quantitation, at least 300 cells

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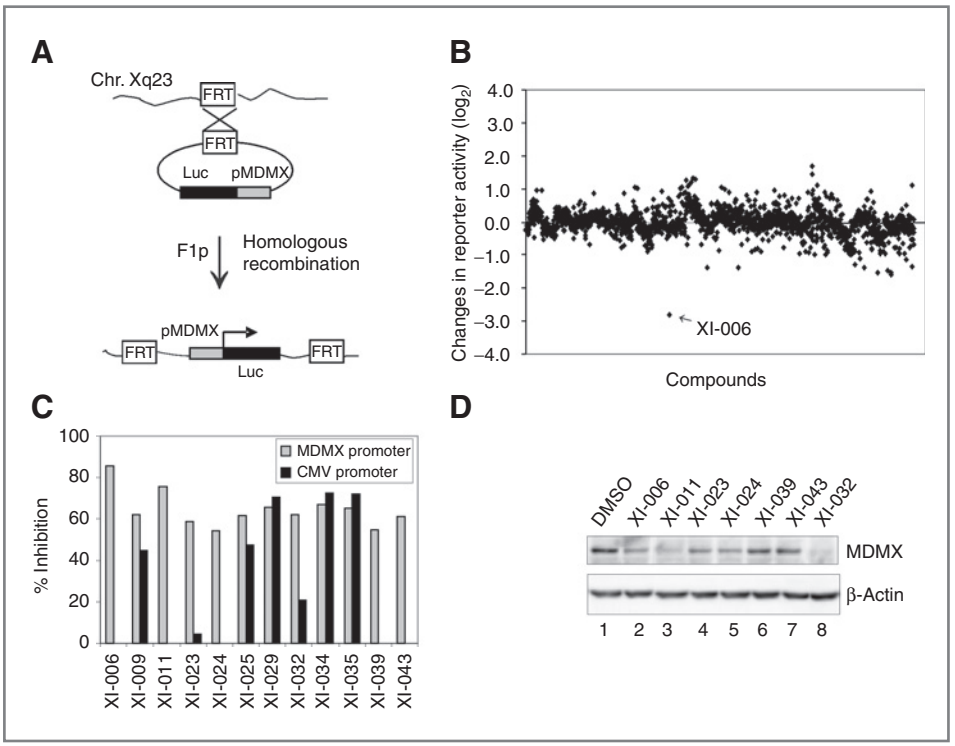

Figure 1. Discovery of small molecules inhibitory for *MDMX* expression through a promoter-based drug screening. A, schematic showing recombination between FRT fragments allowing for integration of a luciferase reporter gene driven by a *MDMX* promoter into a chromosomal location at Xq23 (33). B, recombinant cells carrying the integrated *MDMX* promoter were plated into 96-well plates and treated with 5 μmol/L of compounds derived from the NCI Diversity-Set chemical library for 6 hours. Cells were then lysed for luciferase activity assays. The fold changes in the luciferase activity were converted into logarithm values (binary logarithm, i.e., log2) and plotted for each compound. C, recombinant cells carrying the *MDMX* promoter or a CMV promoter were treated with the screening hits for 6 hours and lysed for luciferase activity assays. D, HT1080 cells were treated with the indicated compounds for 24 hours, and lysed for immunoblotting to measure *MDMX* expression levels.

were randomly chosen and the numbers of TUNEL-positive cells were counted.

Results

Identification of small molecules inhibitory for *MDMX* expression through drug screening

To search for small molecules that inhibit *MDMX* expression, we employed a recent FRT-based recombinant technology (31) to insert a firefly luciferase gene fused with a cloned *MDMX* promoter into a well-characterized genomic location in HT1080 cells (33), and generated recombinant cells carrying a luciferase reporter directed by the *MDMX* promoter (Fig. 1A). As expected, actinomycin D, a general transcription inhibitor, decreased the reporter activity, and such effects were highly reproducible (Supplementary Fig. S1), indicating that the recombinant cells were suitable for HTS (34). We therefore employed these cells to screen the NCI Diversity-Set chemical library (42) for small molecules that can diminish the *MDMX* promoter activity. A total of 44 compounds (2.2%) were found to reduce the luciferase activity to at least 50% (log2 scale < −1.0; Fig. 1B). Of the 44 compounds, 32 reduced the luciferase activity merely by decreasing cell viability (measured by MTT assays, data not shown) and thus were excluded from further investigation. We also excluded 5 compounds that decreased (>30%) the activity of a constitutively-active CMV promoter integrated into the same genomic location (Fig. 1C) as they might inhibit gene transcription in general. The remaining 7 compounds were used to treat HT1080 cells for immunoblotting to validate their effects on *MDMX* expression. The results show that 3 compounds, XI-006 (NSC207895), XI-011 (NSC146109), and XI-032 (NSC25485) significantly inhibited *MDMX* expression (Fig. 1D, lanes 2, 3, and 8). Of them, XI-006 [7-(4-methylpiperazin-1-yl)-4-nitro-1-oxido-2,1,3-benzoxadiazol-1-ium], a benzofuroxan derivative (Fig. 2A), is less toxic according to the NCI toxicity database (http://dtp.nci.nih.gov), and thus was chosen for further investigation. We confirmed that XI-006 decreased the *MDMX* promoter activity in a dose-dependent manner (Fig. 2B), suggesting that this small molecule likely inhibited *MDMX* expression through repressing *MDMX* transcription.

Small-molecule MDMX inhibitor activates p53

Intrigued by the possibility that this MDMX-targeted agent could be used for cancer therapy, we treated *MDMX*-overexpressing MCF-7 cells (24) with the small

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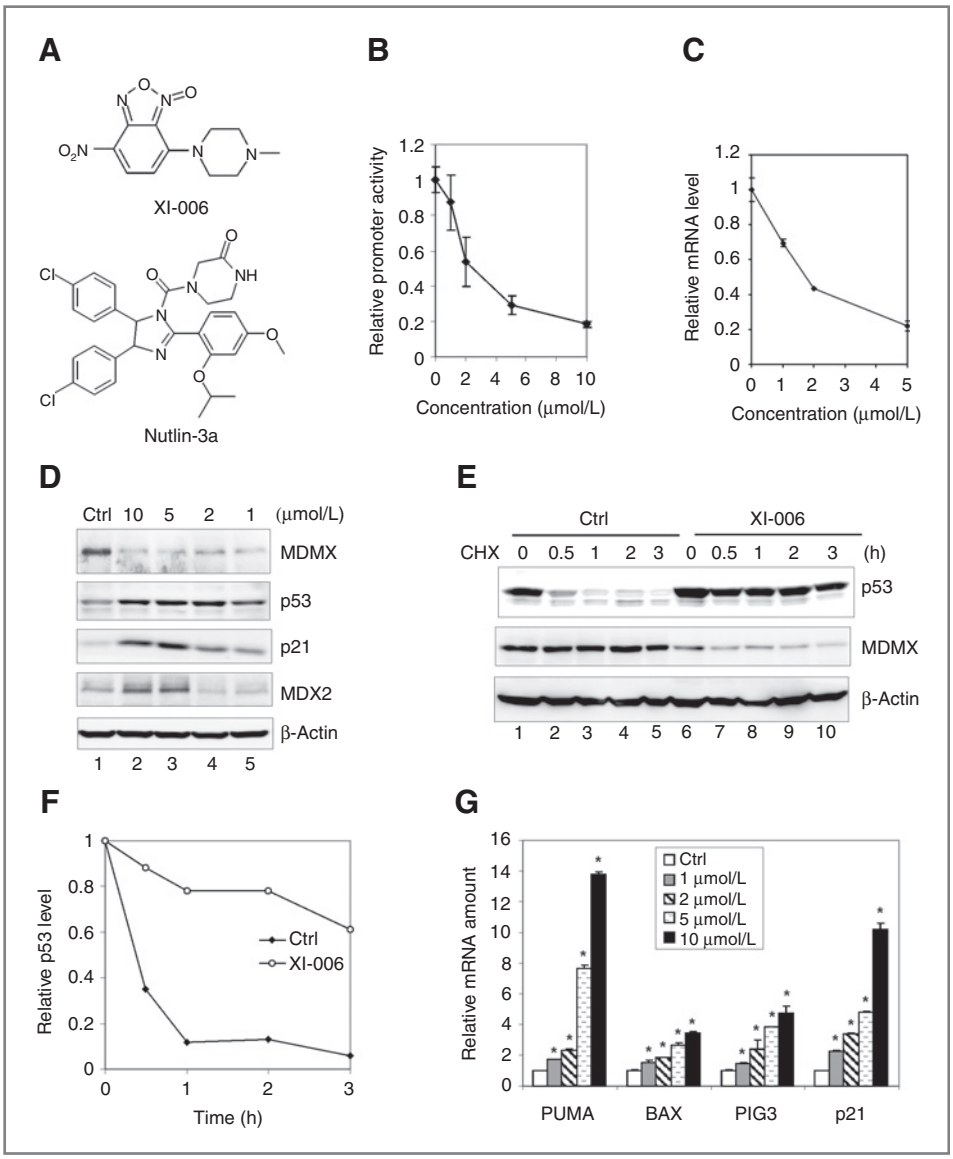

Figure 2. A small-molecule MDMX inhibitor activates p53 leading to activation of proapoptotic gene expression. A, the chemical structures of XI-006 and nutlin-3a. B, recombinant cells carrying the *MDMX* promoter were treated with varying amounts of XI-006 for 6 hours and lysed for luciferase activity assays. Data are depicted as average ± SD values of 3 determinations. C, MCF-7 cells were treated with XI-006 overnight and lysed for qRT-PCR assays to determine the *MDMX* mRNA levels. Data are depicted as average ± SD values of 3 determinations. D, MCF-7 cells were treated with DMSO (Ctrl) or XI-006 for 16 hours and lysed for immunoblotting to measure expression levels of MDMX, p53, p21, and MDM2. E, after treatment with 5 μmol/L of XI-006 for 16 hours, MCF-7 cells were incubated with 100 μg/mL of cycloheximide. Cells were lysed at the indicated time and subjected to immunoblotting assays. F, relative p53 levels in E was estimated by densitometry analysis. G, MCF-7 cells were treated as in D. Total RNA was prepared, reverse transcribed, and cDNA subjected to qRT-PCR assays for measuring levels of PUMA, BAX, PIG3, and p21 mRNA. Data are depicted as average ± SD values of 3 determinations. *, $P < 0.05$ versus the DMSO control (Student's *t* test).

molecule and determined whether p53 could be activated through inhibition of *MDMX* expression. Consistent with its capability to repress the *MDMX* promoter (Fig. 2B), XI-006 decreased both the MDMX mRNA (Fig. 2C) and protein levels (Fig. 2D) in MCF-7 cells. Importantly, this novel MDMX inhibitor induced expression of p21, a well-characterized p53-target gene, in a dose-dependent manner (Fig. 2D), indicating that inhibition of *MDMX* expression by the small molecule indeed activated p53. Expression of MDM2, another p53 downstream gene, was also induced, despite to a less extent, by XI-006 (Fig. 2D, lanes 2 and 3 versus lane 1). Interestingly, XI-006 appeared to increase the p53 levels in MCF-7 cells (Fig. 2D), suggesting that the small molecule could stabilize p53 as well. Indeed, the MDMX inhibitor extended the half-life of p53 from 20 to 30 minutes to more than 3 hours as revealed by cycloheximide chase assays (Fig. 2E and F). These results are reminiscence of previous reports showing that MDMX downregulation by siRNA increases p53 levels (10, 29). Interestingly, in addition to MCF-7 cells, XI-006 also activated p53 and induced p21 and MDM2 expression in LNCaP prostate and A549 lung cancer cells (Supplementary Fig. S2A and B).

One consequence of p53 activation is *trans*-activation of a group of genes (e.g., *PUMA*, *BAX*, and *PIG3*) that link p53 to cell death signaling pathways leading to eradication of cancer cells. Indeed, results from qRT-PCR assays show that XI-006 increased the mRNA levels of *PUMA*, *BAX*, and *PIG3* in a dose-dependent manner (Fig. 2G). Although MDMX can regulate the functions of other genes (43, 44), the induction of proapoptotic gene expression by XI-006 in MCF-7 cells was most likely through activating p53 because these effects were largely impaired by shRNA-mediated p53 downregulation

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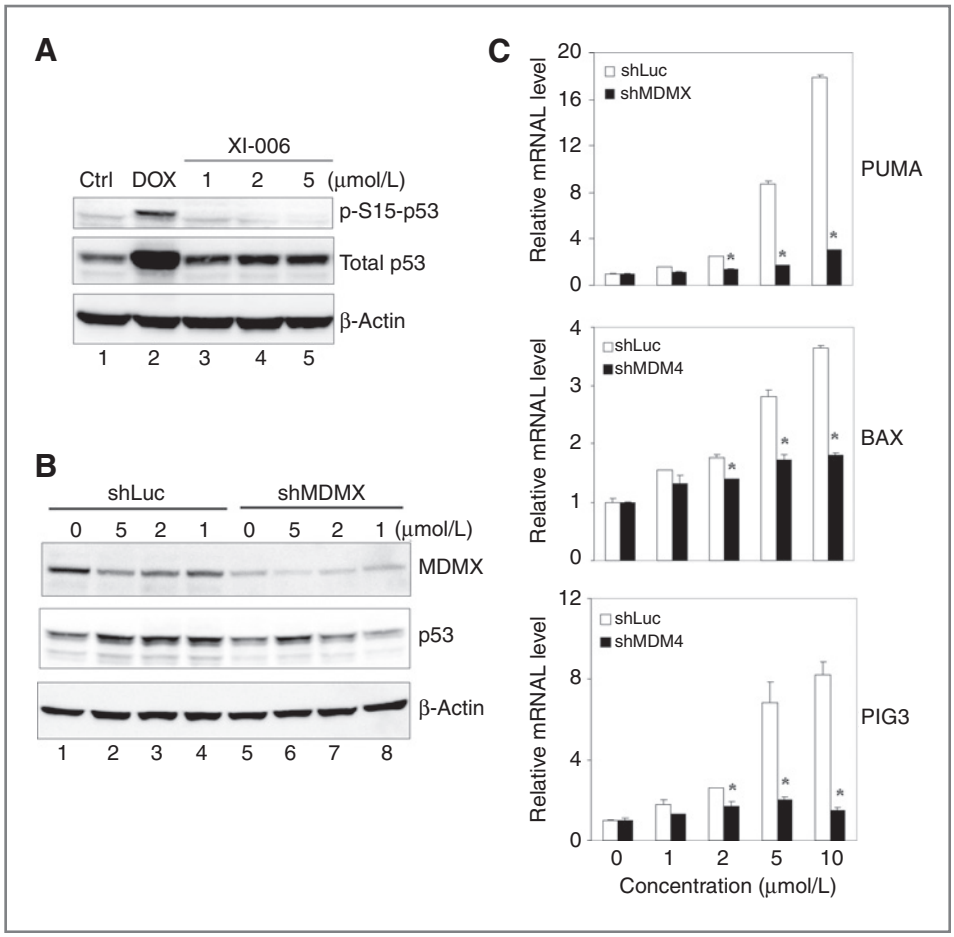

Figure 3. Activation of p53 by XI-006 is a consequence of inhibition of *MDMX* expression. A, MCF-7 cells were treated with 0.5 μg/mL of DOX or XI-006 as indicated for 16 hours and lysed to measure p53 phosphorylation levels. B, MCF-7 cells infected with Lentiviruses expressing shRNA specific to luciferase (shLuc) or MDMX (shMDMX) were treated with varying amounts of XI-006 for 24 hours, and subjected to immunoblotting for p53 and MDMX expression. C, MCF-7 cells expressing shMDMX or shLuc were treated as in B and subjected to qRT-PCR analysis to measure the levels of the indicated mRNA. Data are depicted as the average ± SD values of 3 determinations. *, $P < 0.05$ versus the corresponding shLuc cells (Student's *t* test).

(Supplementary Fig. S3). Therefore, the small-molecule MDMX inhibitor activated p53 leading to the expression of proapoptotic genes.

P53 activation by XI-006 is caused by inhibition of *MDMX* expression

Because DNA damage induced by small molecules can result in p53 activation, we sought to address an important question as to whether p53 activation by XI-006 was indeed a consequence of inhibition of *MDMX* expression. We thus treated MCF-7 cells with XI-006 and measured levels of serine 15-phophorylated p53 (p-S15-p53), a major cellular marker for DNA damage (45, 46), to determine whether the MDMX inhibitor could induce DNA damage at concentrations sufficient to activate p53 (Fig. 2D and 2G). As expected, a known DNA-damaging agent doxorubicin (DOX) induced p53 phosphorylation (Fig. 3A, lane 2). However, treatments with XI-006 failed to result in accumulation of phospho-p53 protein (Fig. 3A, lanes 3–5) although the cellular p53 levels were still elevated after treatments (Fig. 3A). These results thus argue against the notion that the MDMX inhibitor activates p53 through inducing DNA damage. To provide further evidence supporting that XI-006 activated p53 through inhibition of MDMX, we knocked down *MDMX* expression using Lentivector-based shRNA (shMDMX) and determined whether this treatment would compromise p53 activation by XI-006. Indeed, downregulation of *MDMX* expression largely impaired the increase of p53 levels (Fig. 3B) and subsequent PUMA, BAX, and PIG3 activation (Fig. 3C), showing that p53 activation by the MDMX-targeted agent was caused, at least in part, by inhibition of *MDMX* expression. Of note, knockdown of *MDMX* expression by the shRNA alone appeared insufficient to increase the cellular p53 levels and activate p53 (Fig. 3B, lane 5 versus lane 1)—an observation consistent with several (6, 23, 24) but in contrast to other reports (10, 29). The reason for this apparent discrepancy is unclear, but might be related to the complexity of the p53 regulatory network (refs. 17, 27; also see Discussion).

XI-006 induces apoptosis and inhibits cancer cell growth

Because the MDMX inhibitor induced proapoptotic gene expression, we did flow cytometry assays to determine whether activation of p53 by XI-006 could induce apoptosis. Consistent with a previous report (24), the MDM2 inhibitor nutlin-3a caused a significant

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reduction in the percentage of S-phase cells but failed to promote apoptosis in MCF-7 cells (Fig. 4A and 4B). Conversely, treatment with XI-006 resulted in a significant increase in the numbers of sub-G_0/G_1 cells although G_2 arrest was also apparent (Fig. 4A), indicating that the MDMX inhibitor, unlike nutlin-3a, could induce apoptosis. Indeed, TUNEL staining assays confirmed that XI-006 treatments resulted in increased number of apoptotic (i.e., TUNEL-positive) cells (Supplementary Fig. S4; Fig. 4C). Moreover, the cleavage of poly(ADP-ribose) polymerase (PARP)—a biochemical marker for apoptosis—was largely induced by XI-006 (Fig. 4D). These results thus support the notion that inhibition of *MDMX* expression by small molecules can promote apoptosis resulting in eradication of cancer cells. Indeed, although treatments of MCF-7 cells with 5 μmol/L of XI-006 for 5 days resulted in more than 40% of cells dying via apoptosis (Fig. 4E), this MDMX-targeted agent dose-dependently reduced cell viability (Fig. 4F). Moreover, XI-006 appeared more effective in decreasing cell viability than nutlin-3a (Fig. 4F, see the structure of nutlin-3a in Figure 2A). Similar results were observed in A549 and LNCaP cells (Supplementary Fig. S2C and D). Interestingly, compound 3268, an analogue of XI-006 (Supplementary Fig. S4A), which was inactive in inhibiting *MDMX* expression and activating p53 (Supplementary Fig. S4B), failed to induce apoptosis (Fig. 4E), alter cell cycle progression (Fig. 4B), or decrease cell viability (Fig. 4F). It is important to note that XI-006 and compound 3268 have close predicted logP (logarithm of the oil–water partition coefficient) values around 1.5, arguing against the possibility that the lack of activity of the latter compound was likely due to poor cell entry.

XI-006 induces apoptosis through inhibition of *MDMX* expression and activation of p53

To confirm that the apoptosis induced by the small molecule was indeed caused by inhibition of *MDMX* expression and activation of p53, we knocked down *MDMX* expression or p53 expression with shRNA in MCF-7 cells, and determined whether these treatments could impair the induction of PARP cleavage. Indeed, downregulation of either *MDMX* expression (Fig. 5A) or p53 expression (Fig. 5B) abolished the induction of PARP cleavage by the small-molecule MDMX inhibitor. Interestingly, XI-006 appeared to be less effective in decreasing the viability of breast cancer cells that carry wild-type p53 but express low levels of MDMX (i.e., MDA-MB-175VII, ZR-75-1, ZR-75-30; Fig. 5C and 5D), consistent with the notion that XI-006 affects cancer cell growth mainly through regulating MDMX functions. On the contrary, although p53 activation is required for the induction of apoptosis in MCF-7 cells by XI-006 (Fig. 5B), the MDMX inhibitor decreased the viability of several breast cancer cells that express mutant p53 (Supplementary Fig. S6). These results are not unexpected, given that MDMX can also bind to and regulate p63 and p73 (44, 47, 48), two other p53 family members that can alternatively *trans*-activate proapoptotic genes in the absence of functional p53 (49). It might also be possible that XI-006 targets pathway(s) that is less dependent on MDMX in these p53-mutated cells.

XI-006 enhances the effects of nutlin-3a on cell viability

Nutlin-3a can disrupt the p53–MDM2 interaction but is less effective in dissociating MDMX from p53 (23–25). Because the binding of MDMX to p53 can repress p53 *trans*-activation activity, it is assumed that MDMX-targeted agents could cooperate with nutlin-3a to activate p53 leading to cell death (23, 24). To explore this possibility, we applied the same strategy described in Figure 1A to develop a p53 reporter cell line in which a p53-responsive promoter (the P2 promoter of the *MDM2* gene; ref. 35) fused with a luciferase gene was integrated into the same genomic location as the *MDMX* promoter. For controlling reporter specificity, a cell line carrying a corresponding p53-nonresponsive promoter (the *MDM2* P1 promoter; ref. 35) in the same genomic locus was also developed. These cells were treated with XI-006 (2 μmol/L) in combination with or without 5 μmol/L of nutlin-3a for 24 hours and then lysed for luciferase activity assays. The results show that both the MDM2 and MDMX inhibitors increased the activity of the p53-responsive P2 promoter but not the P1 promoter, consistent with their p53-activating activities. Interestingly, the MDMX inhibitor XI-006 enhanced nutlin-3a-induced p53 activation (Fig. 6A). However, the effects were additive rather than synergistic (Fig. 6A). To corroborate these results, we treated MCF-7 cells with these inhibitors and determined p53 activation by immunoblotting. Again, the MDMX inhibitor modestly increased the levels of p53 and p21 induced by nutlin-3a (Fig. 6B, lane 4 versus lane 2). Of note, consistent with a previous report (23), nutlin-3a slightly increased the MDMX level, arguing against the notion that the MDM2 inhibitor can be used to downregulate *MDMX* expression for cancer treatments. To further investigate the cooperation between the MDM4 inhibitors and nutlin-3a, we treated cells with varying amounts of nutlin-3a in combination with 2 μmol/L of XI-006 for 4 days and measured cell viability using MTT assays. We found that XI-006 decreased cell survival in combination with low concentrations of Nutltin-3a in an additive manner (Fig. 6C). Moreover, their effects on nutlin-3a–induced decrease of cell viability were almost eliminated when the MDM2 inhibitor was at a high concentration (10 μmol/L). Therefore, the small-molecule MDMX inhibitor could cooperate with the MDM2 inhibitor to activate p53 and decrease cell survival.

Discussion

Given that MDMX is a major p53 repressor and its overexpression has been found in nearly 20% of human cancers (1, 27), it has been conceived that targeting *MDMX* expression could be an appealing strategy for the treatment of cancer. Through a promoter-based

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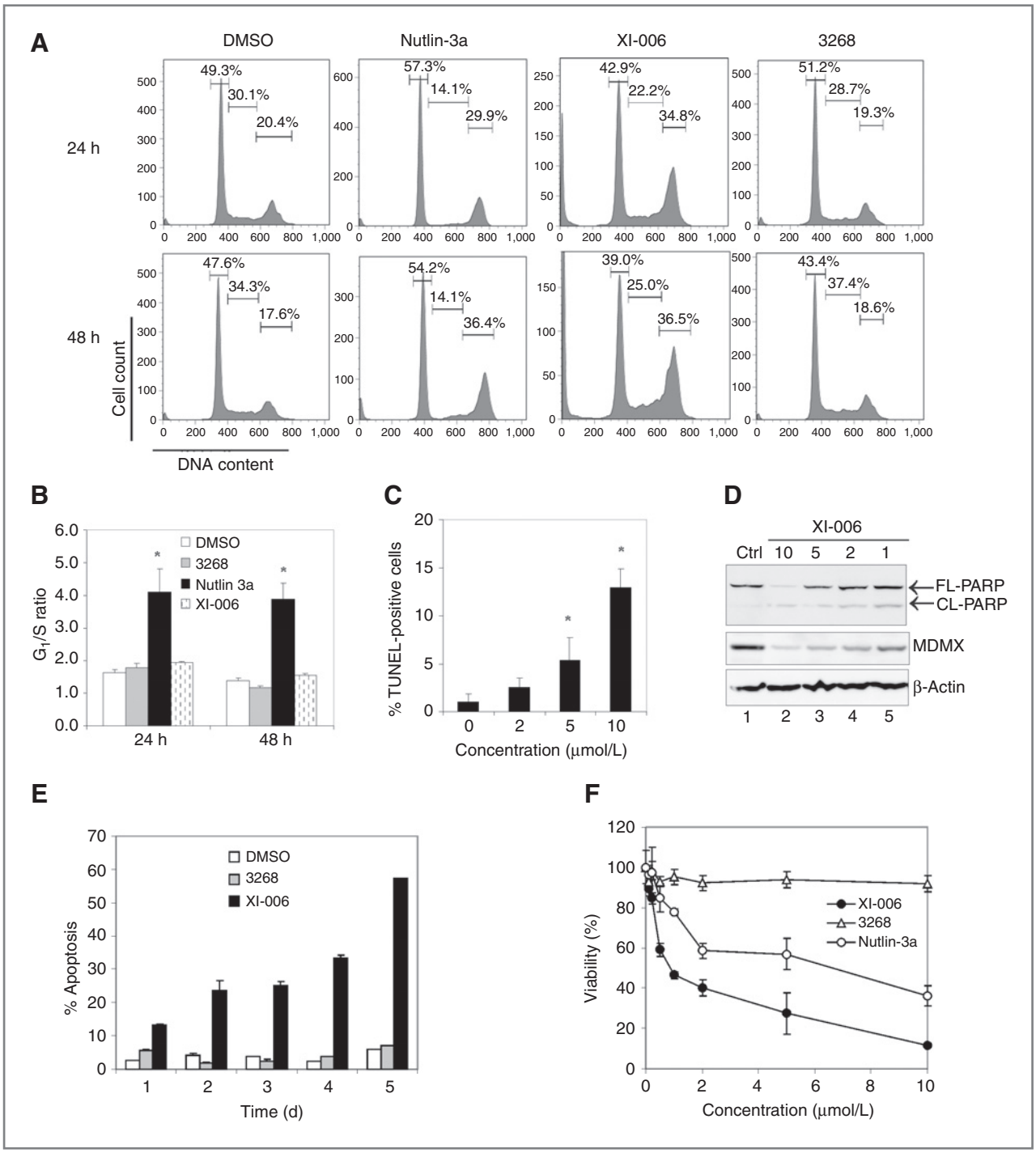

Figure 4. XI-006 induces apoptosis in MCF-7 cells. A and B, MCF-7 cells treated with 5 μmol/L of nutlin-3a, XI-006, or its analog compound 3268, or DMSO for 24 or 48 hours were stained with PI and subjected to flow cytometry analysis. A, numbers inserted in graphs indicate percentages of cells at different stages of the cell cycle. B, the ratios of the numbers of G_1 and S phase cells are shown. C, MCF-7 cells treated with XI-006 for 48 hours were subjected to TUNEL staining assays. At least 300 cells were randomly chosen and the numbers of TUNEL-positive cells were counted. D, MCF-7 cells were treated with XI-006 for 2 days, and subjected to immunoblotting to detect cleaved (CL-PARP) and full-length PARP (FL-PARP). E, MCF-7 cells were treated with 5 μmol/L of XI-006, compound 3268, or DMSO for different days and subjected to flow cytometry to determine percentages of apoptotic cells (sub-G_0/G_1 cells). F, MCF-7 cells were treated with XI-006, compound 3268, or nutlin-3a for 4 days. Cell viability was measured by MTT assays.

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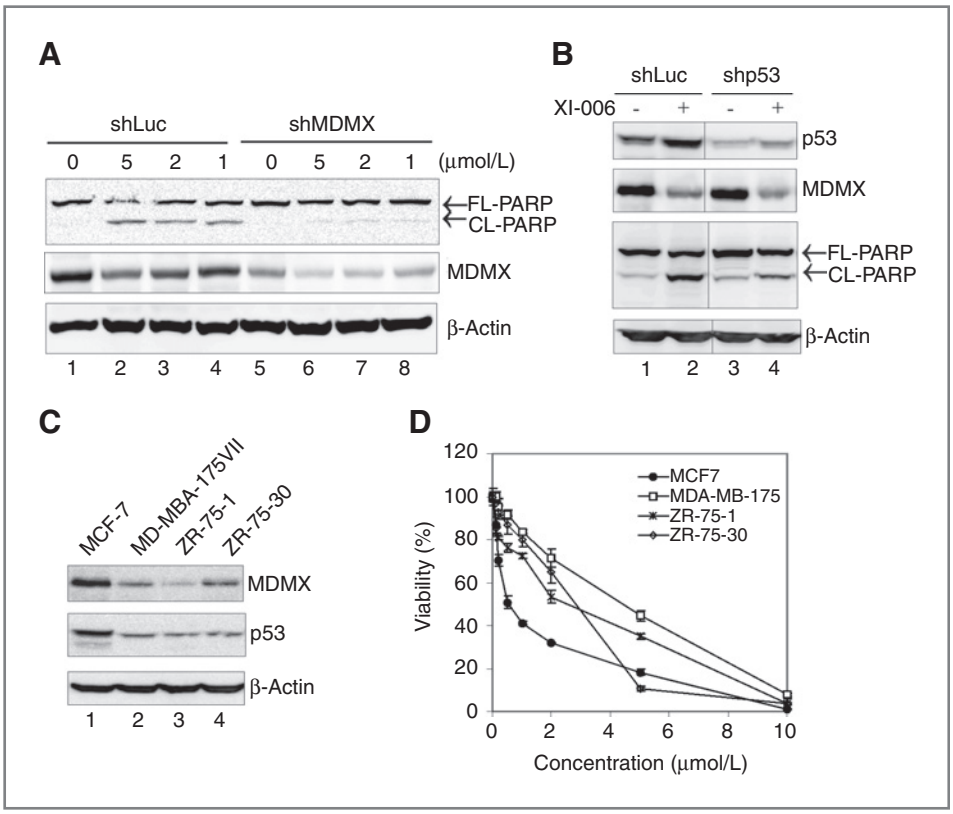

Figure 5. XI-006 induces apoptosis through inhibition of *MDMX* expression and activation of p53. A, MCF-7 cells infected with Lentiviruses expressing shLuc or shMDMX were treated with XI-006 for 2 days and lysed for immunoblotting assays as in Figure 4C. B, MCF-7 cells were infected with shLuc or shRNA specific to p53 (shp53), treated with 5 μmol/L of XI-006 for 2 days, and lysed for immunoblotting assays. C, breast cancer cell lines carrying wild-type p53 were lysed and subjected to immunoblotting assays. D, indicated cells plated in 96-well plates were treated with XI-006 for 3 days and subjected to MTT assays for measuring cell viability.

drug screening, we have identified a small molecule that can activate p53 by inhibiting *MDMX* expression and consequently induce the expression of proapoptotic genes leading to cancer cell death. This study thus provides the first evidence supporting the notion that small molecules targeting *MDMX* expression could be of therapeutic benefit to cancer patients. Interestingly, this small molecule was also identified in an earlier drug screening designed to search for small-molecule p53 activators (50), and thus its p53-activating activities has been independently validated. Therefore, XI-006 can serve as a lead compound for further development of a new class of therapeutic agents targeting *MDMX* expression in cancer.

One of the major thrusts of this study is to identify small molecules that can antagonize the repressive effects of MDMX on p53 *trans*-activation (27) to enhance the anticancer efficacies of MDM2 inhibitors (e.g., nutlin-3a) in *MDMX*-overexpressing cancer cells (23–25). However, we found that the identified MDMX inhibitor additively, rather than synergistically, enhanced p53 activation induced by nutlin-3a. Similar additive anticancer effects were also recently reported for a small-molecule inhibitor that directly binds to MDMX and disrupts the p53–MDMX interaction (51). Intriguingly, although both MDM2 and MDMX inhibitors can activate p53, the MDMX inhibitor differ from nutlin-3a in that it could *trans*-activate proapoptotic genes in MCF-7 cells leading to cancer cell death, in line with the notion that MDMX-targeted agents could achieve better outcomes in treatments of *MDMX*-overexpressing cancers (23–25).

XI-006 is a benzofuroxan derivative and was shown in early reports that it can inhibit biosynthesis of nucleic acids and proteins (52) and exhibit cytotoxicity against murine leukemia cells (53). However, XI-006 unlikely downregulated MDMX expression through inhibition of general protein/DNA synthesis as the levels of several other tested proteins including p21 and MDM2 were not decreased by the small molecule. Rather, current evidence is in line with the notion that the small molecule inhibited *MDMX* expression through targeting transcription of the *MDMX* gene. In this regard, XI-006 decreased not only the activity of the *MDMX* promoter (Fig. 2B) but the *MDMX* mRNA level (Fig. 2C). Moreover, although MDM2 can promote MDMX degradation (54), induction of MDM2 expression by XI-006 (Fig. 2C) was rather a consequence than a cause of *MDMX* inhibition because the MDMX inhibitor retained its ability to decrease *MDMX* expression levels in cells where *MDM2* or p53 expression was knocked down by shRNA (Supplementary Fig. S7). Indeed, MDMX stability was not significantly altered by the 2 small molecules (Fig. 2E). Interestingly, knockdown of *MDM2* expression could slightly compromise the inhibition of *MDMX* expression caused by XI-006 treatments (Supplementary Fig. S7B), suggesting that MDM2 induced by activated p53 could lead to a further decrease in the MDMX protein levels through promoting proteolysis of the latter protein.

Published OnlineFirst November 12, 2010; DOI: 10.1158/1535-7163.MCT-10-0581

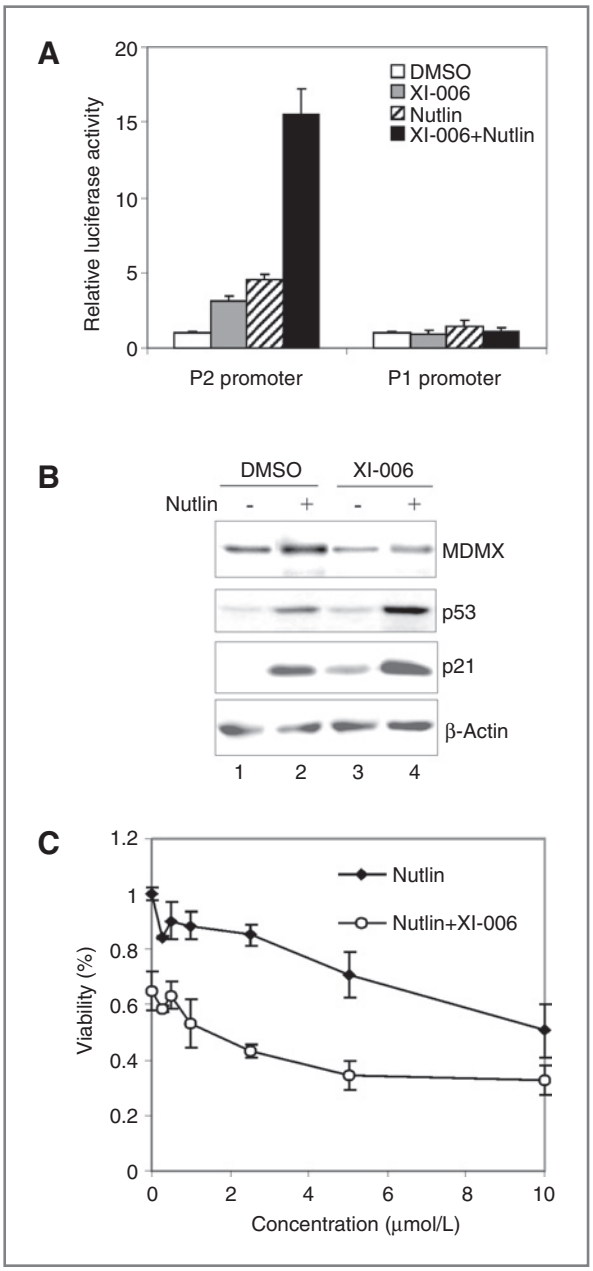

Figure 6. XI-006 additively enhances p53 activation and anticancer effects of nutlin-3a. A, the p53-responsive P2 or p53-nonresponsive P1 promoter of the *MDM2* gene was integrated into the same chromosomal location as in Figure 1A. Recombinant cells were treated with 5 μmol/L of nutlin-3a with or without 2 μmol/L of XI-006 for 6 hours and lysed for luciferase activity assays. B, MCF-7 cells were treated with 5 μmol/L of nutlin-3a with or without 2 μmol/L of XI-006 for 24 hours and lysed for immunoblotting to measure MDMX, p53 and p21 levels. C, MCF-7 cells were treated with varying amounts of nutlin-3a with or without 2 μmol/L of XI-006 for 4 days and subjected to MTT assays to measure cell viability. Data are depicted as average ± SD values of 3 determinations.

Further exploration of the mechanism(s) by which XI-006 inhibits *MDMX* transcription is difficult because the *cis-/trans*-regulatory elements contributing to *MDMX* transcriptional regulation are currently unknown.

An interesting finding in this study is that inhibition of *MDMX* expression by XI-006 not only enhanced the p53 transcriptional activity, but blocked p53 degradation. This finding argues against a prevailing model in which MDMX only plays a role in repression of p53 *trans*-activation (1, 27). However, increase of p53 stability by downregulation of *MDMX* expression is not without precedent (6, 23, 24). Indeed, recent evidence strongly supports that MDMX is required for efficient ubiquitination and degradation of p53 mediated by MDM2 (12–14). MDMX can form a heterodimer with MDM2 via the RING domain (17), and such an interaction is essential for MDM2 to exert its E3 ubiquitin ligase activity toward p53 (12–14). Because we have shown that knockdown of *MDMX* expression by shRNA could abolish p53 activation induced by XI-006 (Fig. 3B) and that the compound unlikely stabilizes p53 through inducing DNA damage (Fig. 3A), it was unlikely that the observed p53-stabilizing effects (Fig. 2B) were caused by an activity aside from inhibition of *MDMX* expression. Our results thus provide an additional support for the emerging role that MDMX plays in regulating p53 stability (12–14). If MDMX promotes p53 degradation, one question arises as to why MDMX shRNA failed to stabilize p53 (Fig. 3B, lane 5 versus lane 1). It is important to note that a siRNA sharing the same target sequence as the shRNA used in this study had a negligible effect on the p53 stability in MCF-7 but largely increased p53 levels in U2OS cells (23), suggesting that siRNA/shRNA, unlike the MDMX-targeted small molecule, might affect p53 stability in a cell-context–dependent manner.

In summary, we have identified a small molecule that can inhibit *MDMX* expression. This agent represents a new class of small-molecule p53 activators that can induce apoptosis leading to elimination of malignant cells. This small-molecule MDMX inhibitor can also serve as a molecular probe for a better understanding of the p53-MDMX interplay and consequences of *MDMX* overexpression in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Hua Lu, Jiandong Chen, Guillermina Lozano, J. P. Blaydes, and Ceshi Chen for providing reagents and comments.

Grant Support

This study was supported by a Department of Defense Grant PC061106 (W81XWH-07-1-0095) and an NIH Grant R01CA139107 to C. Yan.

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Received June 21, 2010; revised October 12, 2010; accepted October 25, 2010; published OnlineFirst November 12, 2010.

Published OnlineFirst November 12, 2010; DOI: 10.1158/1535-7163.MCT-10-0581

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Published OnlineFirst November 12, 2010; DOI: 10.1158/1535-7163.MCT-10-0581

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Mol Cancer Ther 2011;10:69-79. Published OnlineFirst November 12, 2010.