MDMX Overexpression Prevents p53 Activation by the MDM2 Inhibitor Nutlin*

The p53 tumor suppressor plays a key role in maintaining genomic stability and protection against malignant transformation. MDM2 and MDMX are both p53-binding proteins that regulate p53 stability and activity. Recent development of the MDM2 inhibitor Nutlin 3 has greatly facilitated functional analysis of MDM2-p53 binding. We found that although MDM2 is homologous to MDM2 and binds to the same region on p53 N terminus, Nutlin does not disrupt p53-DMMX interaction. The ability of Nutlin to activate p53 is compromised in tumor cells overexpressing MDMX.Combination of Nutlin with MDMX siRNA resulted in synergistic activation of p53 and growth arrest. These results suggest that MDMX is also a valid target for p53 activation in tumor cells. Development of novel compounds that are specific or optimized for dual-inhibition of MDM2 and MDMX are necessary to achieve full activation of p53 in tumor cells.

p53 is a transcription factor mutated in ~50% of human tumors. 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 acting as a ubiquitin E3 ligase. Overexpression of MDM2 abrogates the ability of p53 to induce cell cycle arrest and apoptosis. In about 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 anti-tumor agents (1).

MDMX is a p53-binding protein with significant sequence homology to MDM2 (2, 3). Unlike MDM2, MDMX does not have intrinsic E3 ligase activity and does not promote p53 degradation. However, MDMX binds to MDM2 through C-terminal RING domain interaction (4, 5) and stimulates the ability of MDM2 to ubiquitinate and degrade p53 (6, 7). MDMX-DMMX interaction can also lead to ubiquitination and degradation of MDMX (8–10); this may be an important mechanism for elimination of MDMX during DNA damage response. MDMX knock-out mice die in utero despite having endogenous MDM2 (11). This suggests that MDMX has a unique role in regulating p53 during embryonic development. MDMX overexpression has been found in a number of primary tumors or tumor cell lines with wild-type p53 (12, 13), suggesting that MDMX may contribute to p53 inactivation during tumorigenesis.

MDM2 and MDMX are both targeted by stress signaling pathways that activate p53, DNA damage by ionizing irradiation induces phosphorylation of MDM2 by ATM and c-Abl kinases and inhibits its ability to ubiquitinate p53. Recent studies showed that DNA damage also induces MDMX phosphorylation at the C-terminal region by ATM (14), Chk1 (15), and Chk2 (16). MDMX phosphorylation stimulates 14-3-3 binding (17, 18) and promotes MDMX nuclear translocation and degradation by MDM2 (19, 20). Mitogenic stress also induces MDMX degradation through induction of ARF expression. ARF binding to MDM2 selectively blocks p53-ubiquitination but promotes ubiquitination of MDMX (8).

The crystal structure of MDM2 in complex with an N-terminal peptide of p53 showed that the p53 peptide forms an amphipathic α-helix that interacts with a hydrophobic pocket on MDM2 (21). This model suggests that small molecules may be able to specifically compete for MDM2 binding and activate p53. Phage display has been used to identify peptides that can inhibit MDM2 p53 binding with IC50 at the 100 nm level in vitro (22). High throughput screening resulted in the recent development of Nutlins, which are cis-imidazoline analogs that can inhibit p53-DMMX binding with IC50 of 100–300 nm. Nutlin 3 showed the ability to activate p53 in cell culture at a concentration of 5–10 μM and inhibit tumor growth when given orally at 200 mg/kg (23), confirming the functional importance of MDM2-p53 interaction.

Nutlin was identified based on its ability to inhibit MDM2-p53 binding. MDM2 and MDMX showed ~80% similarity in their p53-binding domains. Previous study using peptide inhibitors suggested that the p53-binding site on MDMX is similar to MDM2 (24), suggesting that MDM2 inhibitors should also cross-inhibit MDMX. Alternatively, elevated MDM2 levels following Nutlin treatment may be sufficient to degrade MDMX. Surprisingly, we found that Nutlin 3 is inactive for inhibition of MDMX-p53 binding and failed to induce MDMX degradation in several tumor cell lines.
lines. p53 activation by Nutlin 3 is compromised in cells overexpressing MDMX. Simultaneous targeting of MDM2 and MDMX cooperates to activate p53 in tumor cells and induces growth arrest. These results suggest that MDMX is also an important inhibitor of p53 in tumor cells and indicate a need to develop MDMX-specific inhibitors.

MATERIALS AND METHODS

ELISA Assay—GST-MDM2 and GST-MDMX containing full-length human MDM2 and MDMX and His₆-tagged human p53 were expressed in Escherichia coli and affinity purified by binding to glutathione-agarose and Ni²⁺-nitrilotriacetic acid beads under non-denaturing conditions. ELISA plates were incubated with 2.5 μg/ml His₆-p53 in phosphate-buffered saline (PBS) for 16 h. 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 h. Compounds were dissolved in Me₂SO. GST-HDM2 and MDMX (5 μg/ml) were mixed with compounds in PBSMT + 10% glycerol + 10 mM dithiothreitol and added to the wells. The plates were washed with PBST after incubating for 1 h at room temperature, incubated with MDM2 antibody 5B10 and MDMX antibody 8C6 in PBSMT for 1 h, followed by washing and incubation with horseradish peroxidase-rabbit-anti-mouse Ig antibody for 1 h. The plates were developed by incubation with TMB peroxidase substrate (KPL) and measured by absorbance at 450 nm.

GST Pulldown Assay—[³⁵S]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 ~10 μg of E. coli produced GST-p53 mutants in lysis buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM 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 autoradiography.

Western Blot—Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and centrifuged for 5 min at 10,000 × g, and the insoluble debris were discarded. Cell lysate (10–50 μg of protein) was fractionated by SDS-PAGE and transferred to Immobilon P filters (Millipore). The following antibodies were used: 3C9 for MDM2; 8C6 for MDMX; DO-1 for p53; p21 antibody (Oncogene Research Products); p53 phospho-Ser-15 antibody (Cell Signaling Technology). The filter was developed using horseradish peroxidase-conjugated secondary antibodies and ECL-plus reagent (Amersham Biosciences).

Cell Lines and Reagents—Tumor cell lines A549 (lung), U2OS (breast), JEG3 (placenta), and HCT116-p53+/+ and HCT116-p53−/− (colon) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. All MDMX constructs used in this study were based on human cDNA clones. To inhibit MDMX expression by RNA interference, double-stranded oligonucleotide (5’GATCCCGTGA-TGATACCAGTTAGATTCA-AGAGATCTACATCGGTATC-ATCACTTTTTTGGAAA, MDMX sequence underlined) was cloned into the pSilencer vector (OligoEngine). Cells were transfected with the MDMX shRNA plasmid and selected with 0.5–1 μg/ml puromycin to obtain the clonal cell line used in Fig. 4a. To transiently inhibit MDMX expression, cells were transfected with 200 nM control siRNA (AATTC-TCCGAACTGCTGAGCTG) and MDMX siRNA (AGATTCAGCT-GGTTATTAA) using Oligofectamine (Invitrogen). After 48 h of transfection, cells were treated with Nutlin for 16 h and analyzed. Lentivirus vector expressing MDMX was generated using the ViraPower™ T-REx™ system (Invitrogen). Tetracycline-inducible expression of MDMX was
achieved by first infecting cells with the T-REX regulator lentivirus and selection with Blasticidin, followed by infection with the MDMX lentivirus and selection with Zeocin. MDMX expression was induced with 0.1–1 μg/ml tetracycline. Nutlin 3 was purchased from Cayman Chemical Company (Ann Arbor, MI).

RESULTS

Nutlin Inhibits the Binding of p53 to MDM2 but Not to MDMX—Nutlin 3 was developed to target MDM2 and appears to activate p53 specifically by disrupting MDM2-p53 binding. It does not cause DNA damage-related stress and phosphorylation of p53 (25). Our experiments also confirmed that Nutlin inhibits MDM2-p53 binding in vitro and induces p53 target gene expression and cell cycle arrest in a p53-dependent fashion at concentrations of 2–10 μM (Figs. 1a and 2a and data not shown). To determine whether Nutlin also disrupts MDMX-p53 interaction, an ELISA assay was used to detect binding between immobilized His₆-p53 and free GST-DM2 or GST-MDMX. As expected, Nutlin inhibited p53-DM2 binding with IC₅₀ of ~800 nm (Fig. 1a). Surprisingly, MDMX-p53 interaction was completely resistant to Nutlin at concentrations up to 30 μM, which is close to its solubility limit in the ELISA assay (Fig. 1b). As a positive control, a previously developed peptide optimized for binding to MDM2 (12/1) inhibited both MDM2 and MDMX (~4-fold less efficient for MDMX) (24). Therefore, MDMX is at least 40-fold less sensitive to inhibition by Nutlin compared with MDM2. To test whether Nutlin can inhibit MDMX-p53 binding in vivo, JEG3 and MCF7 cells expressing high levels of endogenous MDMX were treated with Nutlin or MG132. p53 was immunoprecipitated from cell lysates and probed with MDM2 or MDMX antibodies. Coimmunoprecipitation of MDM2 with p53 was blocked by Nutlin, whereas MDMX-p53 co-IP was completely unaffected (Fig. 1c). Therefore, Nutlin failed to inhibit MDMX-p53 binding in vitro and in vivo.

To determine whether MDM2 and MDMX interactions with p53 have subtle differences that account for the different sensitivities to Nutlin, a panel of GST-p53 fusion proteins with point mutations and deletions in the transactivation domain were tested for binding to in vitro translated MDM2 and MDMX. MDM2 and MDMX were presented as a mixture to glutathione agarose beads loaded with GST-p53 mutants to compare the capture efficiency under identical binding and washing conditions. The results showed that the different p53 mutations reduced MDM2 and MDMX binding to the same extent (Fig. 1d). Therefore, MDM2 and MDMX interact with the same amino acid residues on p53. However, the ability of Nutlin to block p53 binding is significantly different, presumably due to lower binding affinity of Nutlin to MDMX.

MDM2 Induced by Nutlin Does Not Cause MDMX Degradation in Tumor Cells—MDM2 can be rapidly ubiquitinated and degraded by MDM2 in cotransfection experiments or in cells treated with ionizing irradiation (8). Nutlin treatment induces MDM2 expression due to activation of p53, which is expected to lead to degradation of MDMX. However, we found that in several cell lines (JEG3, MCF7, HCT116, U2OS) the MDMX level did not decrease despite an increase in MDM2 level (Fig. 2). On the contrary, a moderate increase in MDMX level was often observed after treatment with Nutlin (Fig. 2, c and d). Analysis of MDMX mRNA level by semi-quantitative RT-PCR showed no increase in MDMX transcription level. Furthermore, MDMX half-life analysis by cycloheximide block did not reveal a significant change in protein stability (data not shown). These results ruled out the possibility that increased MDMX transcription compensated for rapid degradation by MDM2.

Recent studies showed that efficient degradation of MDMX by MDM2 requires DNA damage induced phosphorylation at multiple serine residues (14, 16). Phosphorylation-specific antibody against a Chk2 site on MDMX (S342) and ATM site on p53 (S15) did not detect significant increase of phosphorylation on these residues following Nutlin treatment (Fig. 2b). This was consistent with a recent report that Nutlin does not induce DNA damage (25). To test whether MDMX phosphorylation following irradiation was sufficient to sensitize degradation by MDM2 after Nutlin treatment, cells were treated

FIGURE 2. Nutlin does not induce MDMX degradation. a. HCT116 cells with and without p53 were treated with Nutlin for 8 h and analyzed for expression levels of indicated markers. b. U2OS overexpressing stably transfected MDMX were treated with 8 μM Nutlin for 8 h or irradiated with 10 Gy for 4 h. MDMX was analyzed by immunoprecipitation with 8C6 followed by Western blot with P3342 antibody, which detects Chk2-phosphorylated Ser-342. c and d, cells were pretreated with 5 μM Nutlin for 4 h followed by γ irradiation for 4 h in the presence of Nutlin and analyzed for MDMX level and p53 activity.
with Nutlin in combination with 1–8 Gy γ irradiation. The results showed that γ irradiation cooperated with Nutlin in the induction of p21 and MDM2 expression. However, MDMX degradation following irradiation and Nutlin treatment was not more efficient than irradiation alone (Fig. 2, c and d). Therefore, MDMX phosphorylation is not the rate-limiting factor that prevents degradation by Nutlin-induced MDM2. These observations suggest the presence of additional unidentified mechanisms that control MDMX degradation by MDM2.

The Ability of Nutlin to Activate p53 and Inhibit Proliferation Is Compromised by MDMX—Because Nutlin does not inhibit MDMX-p53 binding or cause significant degradation of MDMX, its ability to activate p53 may be affected by the level of MDMX and MDMX/p53 ratio after stabilization of p53. Previous studies of Nutlin often used the SJSA cell line with amplified MDM2 but a very low levels of MDMX. To determine whether the ability of Nutlin to activate p53 is attenuated by MDMX overexpression, cell lines with different MDMX levels were compared. The results suggested a correlation between high MDMX level and reduced p53 activation and p21WAF1 induction (Fig. 3a). When the ability of Nutlin to induce cell cycle arrest was analyzed by MTT and FACS assays, MDMX-overexpressing cell lines also showed reduced growth arrest (Fig. 3, b and c). In a 7-day colony formation assay, continuous presence of Nutlin failed to inhibit the growth of JEG3 or U2OS cells transfected with MDMX (data not shown). Therefore, Nutlin is less effective against tumor cells overexpressing MDMX.

To further test the effect of MDMX overexpression on p53 activation by Nutlin, U2OS cells expressing tetracycline-inducible MDMX were treated with Nutlin. A physiologically relevant increase in MDMX level (lower than JEG3 cells) resulted in significant reduction of p21 expression after Nutlin treatment (Fig. 3d). Furthermore, MDMX overexpression in U2OS cells by stable transfection (∼30-fold above endogenous level, ∼5-fold above JEG3 level) completely blocked p21 induction by Nutlin (Fig. 4a). These results showed that p53 activation by Nutlin is attenuated by MDMX overexpression, most likely due to inability of Nutlin to inhibit MDMX-p53 binding.

MDMX has been shown to cooperate with MDM2 to promote p53 degradation (6), although its effect on p53 stability is moderate compared with MDM2 (26). In our experiments, MDMX overexpression or knockdown also showed a moderate effect on p53 stabilization by Nutlin (Figs. 3d and 4a), suggesting that it also prevents p53 activation by mechanisms unrelated to degradation. To determine the amount of stabilized p53 that was bound to MDMX, JEG3 cells were treated with Nutlin and analyzed by MDMX immunodepletion and p53 Western blot to quantify the free and MDMX-bound p53. The results showed that ∼30–50% of p53 in JEG3 cells was bound to MDMX, and this ratio was not altered by Nutlin (Fig. 3e). This is likely a conservative estimate of the fraction of MDMX-bound p53, since some of the complexes are expected to dissociate during washing. In contrast, only a negligible fraction of p53 was depleted using MDM2 antibody (Fig. 3f), suggesting that MDM2 mainly acts by degrading p53 in this cell line. Therefore, in MDMX-overexpressing cells, a large fraction of p53 stabilized by Nutlin was stoichiometrically sequestered by MDMX into non-functional complexes. These results suggest that MDMX inactivates p53 by a combination of effects on its stability and activity.
MDMX Knockdown Cooperates with Nutlin to Activate p53—Since Nutlin does not inhibit MDMX-p53 binding, we tested whether combining Nutlin with MDMX siRNA results in more efficient activation of p53. A U2OS clonal cell line with stable knockdown of MDMX (∼80% reduction) was generated by stable transfection with an MDMX shRNA plasmid. U2OS cells apparently can tolerate a significant reduction of MDMX and the consequent small increase in p53 and p21 levels in normal cell culture. Additionally, an overexpression cell line (30-fold above endogenous level) was created by stable transfection with MDMX cDNA plasmid. These modifications did not lead to significant changes in the rates of cell proliferation.

When the modified U2OS cell lines were treated for 24 h with Nutlin, p53 in cells with reduced MDMX level was more efficiently activated by low concentrations of Nutlin, resulting in higher levels of p21 induction and more efficient growth arrest in a 4 day MTT assay (Fig. 4, a and b). Overexpression of MDMX abrogated p21 induction without blocking p53 stabilization by Nutlin. To confirm the results using stable MDMX shRNA, MCF7 and JEG3 cells were also transiently transfected with a synthetic MDMX siRNA followed by treatment with Nutlin. Significantly stronger induction of p21 and Bax expression were observed after transient knockdown of MDMX, whereas Apaf-1 induction was less predictable (Fig. 4, c and d). MDMX knockdown cooperated with Nutlin to induce more efficient growth arrest; no significant increase in apoptosis was observed (Fig. 4e). These results suggested that simultaneous targeting of both MDM2 and MDMX is needed to fully activate p53 in a subset of tumor cells expressing high levels of MDMX. However, the decision between cell cycle arrest or apoptosis after p53 activation is unlikely to be determined by MDMX alone.

DISCUSSION

Previous study using peptides isolated from phage display concluded that MDM2 and MDMX have similar p53-binding sites, since the peptides isolated using MDM2 as a target also inhibits MDMX. Here we show that different point mutations in the p53 N-terminal region have identical effects in disrupting MDM2 and MDMX binding to p53. These observations suggest that the overall p53-binding surface on MDM2 and MDMX are indeed very similar when interacting with long peptide sequences. However, MDM2 and MDMX have surprising differences in the sensitivity to small molecules such as Nutlin.
MDMX is also insensitive to a different class of small molecule MDM2 inhibitors that are α-helical mimics based on the terphenyl scaffold (27). These results suggest that small molecule inhibitors require more precise fit with their targets or demand certain conformational changes to achieve the tight binding through a small contact area. In such cases, minor differences in structure or flexibility of MDM2 and MDMX result in large differences in affinity for Nutlin.

The lack of MDMX down-regulation upon MDM2 induction by Nutlin suggests that MDM2 level is not rate-limiting for MDMX degradation in the cell lines tested. Recent studies showed that although MDM2 is necessary for ubiquitination and degradation of MDMX, the efficiency of degradation is under complex control. MDMX phosphorylation, 14-3-3 binding, and degradation of MDMX, the efficiency of degradation is highly organ-specific (28). The significance of MDMX in human cancer development and treatment response is still an area of active investigation. The results described in this report, however, suggest that MDMX is highly organ-specific (28). The significance of MDMX in human cancer development and treatment response is still an area of active investigation. The results described in this report, however, suggest that MDMX is resistant to Nutlin.

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REFERENCES

1. Bond, G. L., Hu, W., and Levine, A. J. (2005) Curr. Cancer Drug Targets 5, 3–8
2. Shvarts, A., Steegenga, W. T., Riteco, N., van Laar, T., Dekker, P., Bazuine,
3. B. Hu, D. M., Gilkes, B., Farooqi, S. M., Sebti, and J. Chen, unpublished observations.
4. G. Wahl, personal communication.