Subject Review

Targeting Mdm2 and Mdmx in Cancer Therapy: Better Living through Medicinal Chemistry?

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Abstract

Genomic and proteomic profiling of human tumor samples and tumor-derived cell lines are essential for the realization of personalized therapy in oncology. Identification of the changes required for tumor initiation or maintenance will likely provide new targets for small-molecule and biological therapeutics. For example, inactivation of the p53 tumor suppressor pathway occurs in most human cancers. Although this can be due to frank *p53* gene mutation, almost half of all cancers retain the wild-type *p53* allele, indicating that the pathway is disabled by other means. Alternate mechanisms include deletion or epigenetic inactivation of the p53-positive regulator arf, methylation of the *p53* promoter, or elevated expression of the p53 regulators Mdm2 and Mdmx. This review discusses current models of p53 regulation by Mdm2 and Mdmx and presents the rationale for design of future Mdmx-specific therapeutics based on our knowledge of its structure and biological functions. (Mol Cancer Res 2009;7(1):1–11)

Models for Mdmx/Mdm2-Mediated Inhibition of p53

Genetic studies indicate that Mdm2 and Mdmx do nonredundant functions to keep p53 inactive during embryogenesis and throughout development (1, 2). There are currently two main models to explain why Mdm2 and Mdmx are unable to compensate for each other *in vivo*. In the first model, Mdm2 and Mdmx work independently to inhibit p53 activity. This could be due to a tissue- or temporal-specific requirement for either Mdm2 or Mdmx, as reported in hematopoietic and neuronal compartments, and might require biochemically distinct functions of the two proteins. For example, Mdm2 may be the primary determinant of p53 stability and abundance, whereas Mdmx may be needed to antagonize p53-dependent transcriptional control. Two recent reviews provide a more complete discussion of these alternatives (3, 4).

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In the second model, Mdm2 and Mdmx are proposed to form a complex that is more effective at inhibiting p53 transactivation or enhancing p53 turnover. Although the former possibility has not been excluded, several studies indicate that Mdm2 and Mdmx function as a heterodimeric pair to augment p53 degradation. Mdm2 is a member of the RING E3 ubiquitin ligase family and promotes proteasome-dependent degradation of p53. By binding to the target substrate and to an E2 ubiquitin-conjugating enzyme, RING E3s facilitate E2-to-substrate ubiquitin transfer (5). Similar to other RING E3s, it does not seem that Mdm2 forms a covalent link with ubiquitin during the reaction. Thus, Mdm2 does not have a "classic" catalytic site but acts as a molecular scaffold that presumably positions p53 for E2-dependent ubiquitination (Fig. 1). Specifically, the RING domain of Mdm2 is the primary binding site for E2s, whereas the Mdm2 NH_2 terminus and central domain are the contact sites for p53 (6-8). Interestingly, mouse p53 mutants lacking the major COOH-terminal ubiquitination sites exhibit normal half-lives *in vivo* (9, 10). This suggests that in their absence, alternative ubiquitination sites may suffice or that p53 ubiquitination is not required for p53 degradation.

The Mdm2 RING domain mediates Mdm2 homodimerization, and also heterodimerization with Mdmx. To date, Mdmx homodimers have not been found *in vitro* or *in vivo*, suggesting that free Mdmx may be monomeric. In quantitative analyses of normal human fibroblasts and mammary epithelial cells, total cellular Mdmx abundance was 1/5 to 1/10 that of Mdm2 (11). Because the binding affinity of the RING domains of Mdm2 and Mdmx seems to be higher than that of Mdm2 homodimers (12), we suggest that most Mdmx may be heterodimerized with Mdm2 in normal cells. The breast carcinoma cell line MCF7 produces copious amounts of Mdmx due to amplification and overexpression of its gene (13), and immunoprecipitation studies indicate that the Mdm2/Mdmx heterodimer is also the predominant form in these cells (14). Together, these data suggest that Mdm2:Mdmx stoichiometry is regulated such that formation of Mdm2/Mdmx heterodimers is favored. Intriguingly, although Mdmx also has a RING domain, it has no intrinsic ubiquitin ligase activity and binds poorly, if at all, to the E2s examined to date. Strikingly, although Mdmx cannot directly induce p53 ubiquitination, it can enhance Mdm2-dependent p53 ubiquitination and degradation (15, 16). The modulation of RING E3 ligase activity via heterodimerization seems to be a common mechanism of control. For example, activity of the RING E3 ligase Brca1 is stimulated following binding of BARD1, another RING domain protein with no intrinsic ubiquitin ligase activity (17). Similarly, the histone

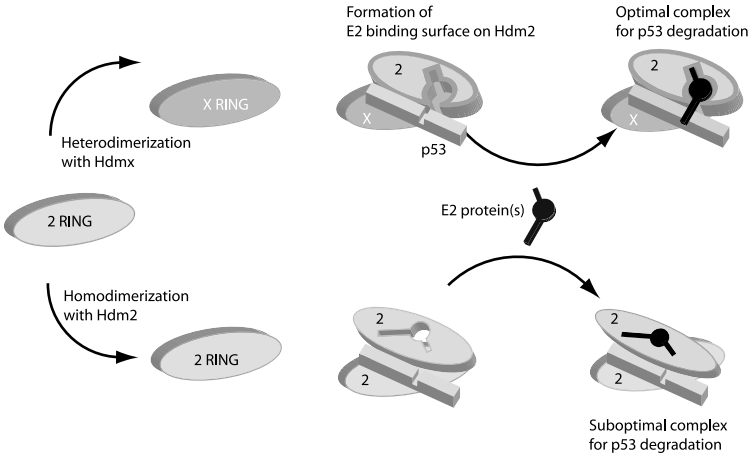

FIGURE 1. Mdm2/Mdmx heterodimers are more effective p53 ubiquitin ligases than Mdm2 homodimers. Mdm2 can homodimerize or heterodimerize with Mdmx via RING/RING interaction (Mdm2 and Mdmx RING depicted; the remainder of the protein is omitted for clarity). It is proposed that the Mdm2/Mdmx heterodimer (*upper scheme*) provides the optimal structure for E2-dependent p53 ubiquitination, whereas Mdm2 homodimerization (*lower scheme*) creates a suboptimal structure for p53 ubiquitination. In this schematic, the structural differences in homodimers versus heterodimers lead to different positioning of the E2 enzyme relative to p53. However, alternative explanations upstream of E2 recruitment, such as optimal p53 binding in the heterodimer, or downstream of E2 recruitment, such as more effective targeting of p53 to the proteasome by the heterodimer, cannot be excluded.

ubiquitin ligase Ring1b is stimulated by Bmi1 following RING-mediated heterodimerization (18).

Mdm2 requires only ubiquitin and E1/E2 enzymes to catalyze RING-dependent autoubiquitylation and p53 ubiquitylation *in vitro* (19). However, p53 inactivation by Mdm2 is inefficient in the absence of Mdmx because embryonic lethality or tissue-specific induction of cell cycle arrest or apoptosis occurs postnatally. We presume that these phenotypes result from inefficient p53 degradation when Mdmx is not expressed. Consistent with this view, p53 degradation in the absence of Mdmx seems to require high Mdm2 concentrations to maximize formation of homodimers that can interact with E2s (e.g., see refs. 8, 20). In addition, Mdm2 overexpression is required to rescue the embryonic lethality engendered by loss of Mdmx (21). Monomeric forms of Mdm2 are relatively ineffective E3 ligases (22, 23), and Mdmx seems to lower the concentration at which Mdm2 autoubiquitylation and p53 ubiquitylation and degradation occur (15). These data indicate that whereas Mdm2 alone can ubiquitylate p53 and induce p53 degradation, the process is more efficient after heterodimerization with Mdmx. This effect is lost when residues close to the Mdmx RING domain (but which are dispensable for dimerization) are mutated. Structural analysis of the heterodimer suggests that these residues are part of an extended surface that is formed on dimerization. As these sites are distal to the E2 binding site, it has been proposed that structural requirements beyond dimerization and E2 recruitment are required for optimal E3 ligase function (see Fig. 1; ref. 8).

As attractive as this model is, there are observations suggesting that it is still incomplete. For example, if Mdmx is stoichiometrically limiting for p53 degradation, increasing its abundance should lead to lower p53 levels, yet this is not always observed (24, 25). Thus, it is possible that other factors needed for p53 degradation are limiting, such as the biologically relevant E2, or factors needed to deliver p53 to the proteasome. Although various E2 enzymes are used in *in vitro* assays, the true constellation of E2s used by Mdm2 *in vivo* is unknown. It is possible that different E2 enzymes may be recruited to Mdm2 homodimers compared with Mdm2/Mdmx heterodimers. Thus, depending on the E2 that is bound, either Mdm2 or p53 may be preferentially ubiquitylated and degraded. It is also possible that "presentation" of p53 to the recruited E2 is more effective in an Mdm2/Mdmx heterodimer compared with an Mdm2 homodimer. An intriguing possibility is that the ability of Mdmx to enhance Mdm2 autoubiquitylation is actually required for effective p53 ubiquitylation. This would explain the paradoxical observation that Mdmx enhances Mdm2 autoubiquitylation and p53 ubiquitylation. Although this may be difficult to prove, there are indications that increased Brca1 autoubiquitylation enhances its activity toward substrates (26). On the other hand, it is challenging to explain how Mdm2 and p53 exhibit similar half-lives in unstressed cells, whereas Mdmx is far more stable (27, 28). It is tempting to speculate that the relative stability of Mdmx derives from its preferential deubiquitylation by the ubiquitin-specific protease HAUSP in unstressed cells. Phosphorylation of Mdm2 and Mdmx after DNA damage induces HAUSP dissociation, which increases Mdm2 autoubiquitination and transubiquitination of Mdmx and accelerates the turnover of both (28-31).

Determining the functional importance of the Mdm2/Mdmx heterodimer requires the development of *in vivo* mouse models, where stoichiometry is preserved and in which tissue-specific effects can be evaluated. Recently, a knock-in mouse expressing Mdm2 with a mutant RING domain was reported. Although this mutant can bind p53, it cannot target p53 for degradation. Consistent with previous reports, the authors concluded that the major function of Mdm2 *in vivo* is to control p53 level rather than suppress p53-dependent transactivation (32). However, the RING mutation that was generated causes profound structural changes to the Mdm2 RING domain, and this might prevent effective heterodimerization with Mdmx. This lends support to the idea that Mdm2/Mdmx is critical for suppression of p53 during development and in adult somatic tissues. It is also possible that the substantial structural perturbations in this RING domain mutant lead to its degradation mediated by the unfolded protein response, which could explain why it does not effectively antagonize p53. Greater insight into the contribution of heterodimerization and E3 ligase activity should come from studies of more subtle mutations that preserve the RING structure but disable the ability of Mdm2 to homodimerize or heterodimerize or mutations that preserve dimerization but prevent ligase function.

Modulating Mdm2 and Mdmx Levels Profoundly Affects p53 Activity *In vivo*

The expression levels of Mdm2 and Mdmx are critical for maintaining appropriate p53 activity to enable normal development. Indeed, too little Mdm2 or Mdmx elicits lethality, whereas an excess of either protein can be oncogenic. For example, mice heterozygous for either *Mdm2* or *Mdmx* are viable, but all double heterozygous mice die within 3 weeks postpartum (33). Deletion of one *p53* allele rescues the lethality of Mdm2/Mdmx compound heterozygotes, illustrating the importance of gene dosage in the p53 pathway during development.

Although ~50% of human cancers retain the wild-type *p53* allele, many express increased levels of either Mdm2 or Mdmx (see ref. 3 for review). These data suggest that, in addition to genetic mutation, p53 can be functionally inactivated by either Mdm2 or Mdmx overexpression. Consistent with the observation that Mdm2 and Mdmx are overexpressed in cancer, several studies indicate that both proteins are bona fide oncogenes. For example, a 2-fold increase in Mdm2 expression in transgenic mice is tumorigenic and leads to a similar spectrum of tumors as those found in human tumors with amplified *Mdm2* (34, 35). Although we await data from murine models of Mdmx overexpression, xenograft studies indicate that elevated Mdmx cooperates with oncogenic ras to transform cells (13). These observations underscore the effect of subtle changes in Mdm2 and Mdmx levels on tumorigenesis. Indeed, a single nucleotide polymorphism (SNP) in the *Mdm2* promoter increases *Mdm2* mRNA and protein levels 2- to 4-fold and is associated with poor prognosis in some human tumors (36). This SNP is associated with early onset of diverse tumor types in premenopausal women, suggesting a role for both gender and estrogen signaling in Mdm2-dependent cancer susceptibility (37, 38). This extends previous observations of elevated Mdm2 expression in estrogen receptor α–positive breast carcinoma lines (39). Corresponding SNPs in the *Mdmx* promoter have not been found to date, but there is some evidence that mitogenic signaling can increase *Mdmx* mRNA levels (40). However, additional studies are required to determine whether response elements and/or SNPs in the *Mdmx* gene contribute to aberrant Mdmx expression in cancer. Regardless of the mechanism, increased *Mdmx* mRNA and protein have been described across a wide spectrum of tumors (see Table 1). In cases of *Mdmx* gene amplification, a comparison with neighboring loci indicates that Mdmx is most likely the selected oncogene (41). In general, elevated *Mdmx* mRNA occurs in tumors with wild-type p53, and Mdmx knockdown leads to p53-dependent growth arrest or apoptosis in both breast carcinoma and retinoblastoma cell lines (13, 42, 43). These data suggest that, in the absence of changes in other p53 regulatory proteins such as Mdm2 or arf, Mdmx-dependent inhibition of p53 is critical for tumorigenesis.

Genetic inactivation of *Mdm2* and *Mdmx* can also profoundly affect tumorigenesis. For example, decreasing Mdm2 expression by as little as 20% reduces adenoma formation in $APC^{min/+}$ mice, whereas a 70% decrease dramatically suppresses spontaneous tumor formation (44). Deleting one allele of either *Mdm2* or *Mdmx* also suppresses c-myc–induced lymphomagenesis in mice (33, 45). These data provide encouragement for efforts to develop antagonists of both Mdm2 and Mdmx for therapeutic purposes.

Understanding the mechanism by which Mdm2 and Mdmx inhibit p53 function is critical for the development of novel chemotherapeutic agents. Many aspects of basic research into p53 regulation, including *in vivo* mouse models, structural biology, biochemistry, and cell biology, provide a wealth of data, which will guide the design of small-molecule activators of p53. The following sections present findings from such studies that provide a rationale for targeting both Mdm2 and Mdmx to activate p53.

Inhibition of Mdmx in Cancer

There are at least three strategies to inhibit the oncogenic activity of Mdmx in cancer. First, reducing Mdmx protein levels should increase p53 activity to mitigate Mdmx oncogenicity. Second, development of small molecules that relieve Mdmx-dependent inhibition of p53 would restore p53-dependent cell cycle arrest and/or apoptosis. In the absence of optimal Mdmx antagonists, we envisage an alternative approach using existing Mdm2 antagonists in combination with agents that sensitize cells to p53-dependent apoptosis. Below, we describe all three strategies in more detail and highlight areas that require further investigation.

Reduction of Mdmx Protein Levels

Down-regulation of *Mdmx* mRNA using RNA interference *in vitro* can activate p53, induce cell cycle arrest, and suppress colony outgrowth (13). Because lentiviral-mediated *Mdmx* knockdown can reduce tumor xenograft growth *in vivo*, targeting *Mdmx* mRNA in human cancers may be a valid therapeutic approach (25). However, improving small interfering RNA–mediated therapy, particularly in the delivery of such agents to deep-seated tumors, will be required to make this approach feasible (46). An alternative approach is to target the transcription or translation of the *Mdmx* mRNA. To develop specific inhibitors of *Mdmx* transcription, a detailed analysis of the promoter region and associated transcriptional activators is required. Recent studies indicate the Ets and Elk transcription factors are putative regulators of *Mdmx* expression (40).

Table 1. Tumors with Either Increased Mdmx Copy Number or Increased Mdmx mRNA

Tumor Type	% of Cases	Reference
Retinoblastoma	60%	(42)
Glioblastoma	4%	(41)
Colon	19%	(13)
Lung	18%	(13)
Breast	19%	(13)
Melanoma	14%	(13)
Hepatocellular carcinoma	50%	(104)
Head and neck	50%	(105)
Sarcoma	22%	(43)
Bladder cancer	25%	(106)
All cell lines	40%	(107)

NOTE: Percentages indicate the overall frequency of changes, irrespective of mechanism.

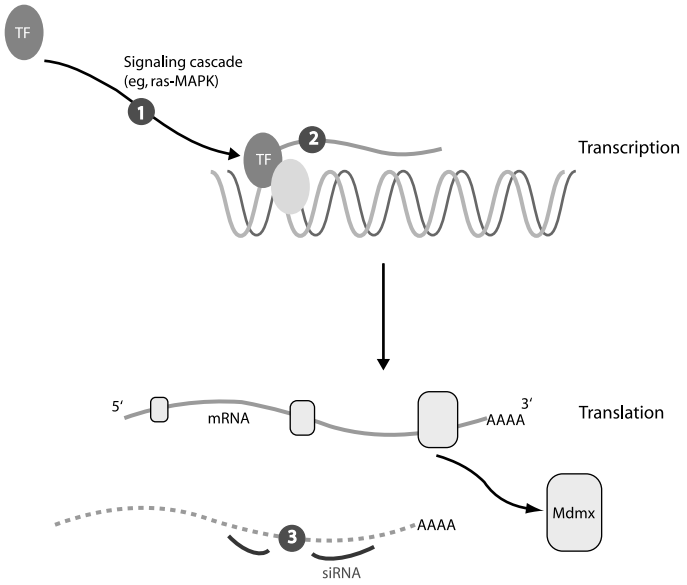

FIGURE 2. Blockade of Mdmx at the level of transcription or translation. Inhibition of mitogen-activated signaling pathways (*1*) may prevent transcription factor recruitment to the Mdmx promoter, thereby lowering *Mdmx* mRNA. Disruption of transcription factor complexes (*2*) on the DNA could also reduce *Mdmx* transcription. Degradation or reduced translation of Mdmx mRNA following small interfering RNA strategies (*3*) will also lead to decreased Mdmx protein.

However, because these are general transcriptional coactivators, directly targeting them will likely generate many off-target effects (see Fig. 2 for potential strategies to target Mdmx synthesis in tumor cells).

Given the difficulty and limitations of targeting a specific mRNA to reduce Mdmx protein level, an alternative method is to destabilize the Mdmx protein itself. Activating pathways that promote Mdmx proteolysis, or conversely inhibiting those that prevent Mdmx degradation, may achieve this. Most studies have focused on the degradation of Mdmx following DNA damage. The emerging view is that damage-induced phosphorylation of the Mdmx COOH terminus, and a p53-dependent increase in Mdm2 levels, triggers Mdmx degradation (30, 31, 47). The COOH-terminal modifications lead to dissociation of HAUSP, a deubiquitylating enzyme that normally stabilizes Mdmx (38). Thus, targeting the HAUSP/Mdmx interaction might be expected to destabilize Mdmx. Whether this would translate into a favorable response in tumors with high Mdmx levels remains unclear because HAUSP has other cellular targets, including Mdm2 and p53 (48, 49).

Mdmx can also be down-regulated via nongenotoxic mechanisms. For example, Nutlin-3a (a small-molecule antagonist of Mdm2 that activates p53; see below and ref. 50) leads to down-regulation of Mdmx in the absence of damage-associated modifications (51). The down-regulation occurs concomitantly with increases in Mdm2 levels in normal human fibroblasts and is inhibited by p53 knockdown, suggesting that increased levels of Mdm2 can lead to Mdmx degradation (24, 51). Interestingly, however, Nutlin-induced Mdmx degradation is attenuated in a subset of cancer cells despite robust induction of Mdm2 (51, 52), and Nutlin does not significantly down-regulate Mdmx in mouse embryonic fibroblasts.[1] These data indicate that factors in addition to Mdm2 levels may determine the stability of Mdmx. Regardless of the initial stimulus, the mechanism by which Mdmx is actually targeted to the proteasome for degradation is unknown. Identification of factors that facilitate or inhibit Nutlin- or genotoxin-induced Mdmx degradation might therefore provide additional targets for small-molecule destabilization of Mdmx.

Small-Molecule Inhibition of the Mdmx/p53 or Mdm2/Mdmx Interaction

Mdm2 was identified as a p53-negative regulator 4 years before the discovery of Mdmx (6, 34, 53). Thus, more is known about structure-function studies for Mdm2/p53 than for Mdmx/p53. Because Mdm2 and Mdmx are highly homologous proteins, it is not surprising that many findings about Mdm2 also apply to Mdmx. Nonetheless, there is an emerging appreciation that subtle structural differences between the structures of Mdm2 and Mdmx necessitate changes in the design of small molecules to create potent inhibitors of the Mdmx/p53 interaction.

Crystallographic studies show that the NH_2 terminus of p53 inserts into a hydrophobic cleft in the Mdm2 NH_2 terminus and that three p53 residues (F19, L22, and L26, the "FWL" motif) are critical for this interaction (54). These data constitute the basis of numerous structure-based studies designed to identify inhibitors of the Mdm2/p53 interaction. Naturally, peptides based on the amino acid sequence around the FWL motif were among the first tested and have been used with varying success (55, 56). Many peptide iterations have been explored, with modification and substitution of noncritical amino acids in the p53 peptide sequence for those that improve solubility, cell permeability, and stability. The p53 peptides adopt an α-helical conformation when bound to Mdm2 but are unstructured in solution, which effectively decreases binding affinity (57). Recently, "peptide stapling" to increase α-helicity and enhance affinity for Mdm2 has been successfully shown for a new

[1] M. Wade, unpublished observations.

generation of p53 peptidomimetics (57). Whether the *in vitro* potency of these p53-activating peptides can be translated to *in vivo* efficacy remains to be determined.

Perhaps the most promising developments for p53 reactivation are from the nonpeptide small-molecule inhibitor class of compounds. These molecules have been identified both by high-throughput screening of compound libraries and by computer-assisted, structure-based drug design. A previous review by Patrick Chene on this topic ominously implied that the patience of pharmaceutical companies might run thin should results not be forthcoming (58). Ironically, data from the first potent Mdm2 antagonists, the *cis*-imdazoline Nutlins, were published just 1 month later (50). Nutlins occupy the hydrophobic p53-binding pocket of Mdm2 and inhibit the Mdm2/p53 interaction, which stabilizes and activates p53. A combination of high-throughput screening and medicinal chemistry was used to optimize the efficacy of Nutlin-related molecules, and these drugs are now finding broad application in many academic and preclinical studies. More recently, spiro-oxindole Mdm2 antagonists ("MI" compounds) were developed following computer-assisted screening of small-molecule libraries (59, 60). Other screening efforts have identified additional small-molecule antagonists of the Mdm2/p53 interaction, although these do not exhibit the potency of Nutlins or MI compounds. We refer the reader to other recent articles that discuss these molecules in detail (61-67).

Because the p53-binding regions of Mdm2 and Mdmx are similar, our laboratory and others investigated whether antagonists designed to target Mdm2 would also disrupt the Mdmx/p53 interaction. This is likely to be clinically relevant because overexpression of Mdm2 and Mdmx is a mutually exclusive event in a significant fraction of human tumors (3). Surprisingly, neither Nutlin nor MI-219 appreciably antagonizes the Mdmx/p53 interaction in cancer cell lines, and high Mdmx reduces apoptosis and/or cell cycle arrest induced by these drugs (24, 51, 68, 69). The binding data have subsequently been confirmed by *in vitro* assays that show that each compound binds more effectively to Mdm2 than to Mdmx (42, 59). Differences in amino acid sequences and recent nuclear magnetic resonance (NMR) studies may provide an explanation for these observations. For example, Mdmx has a Pro^{95}-Ser^{96}-Pro^{97} motif at the end of an α-helical section in the p53-binding domain, which is absent in Mdm2. This was speculated to cause structural differences between the Mdmx and Mdm2 NH_2 termini (70). Recently, Popowicz and colleagues proposed that these proline residues shift the α-helical domain in Mdmx relative to Mdm2 and cause two residues to protrude into the p53-binding cleft of Mdmx, making it shallower and less accessible to molecules such as Nutlin (71, 72). Additional NMR data suggested that Nutlin competes with p53 for binding to Mdm2 but not to Mdmx. This may be because Nutlin is unable to stably interact with the relatively shallow p53-binding pocket of Mdmx. Amino acids 16 to 24 of Mdm2, which are immediately adjacent to the p53-binding site, are also implicated in control of ligand binding. This region constitutes a "flexible lid" that makes contact with the p53-binding site (73, 74). Although p53 peptides seem only to bind efficiently to the "open" lid conformation, the small size and chemical structure of Nutlin allow it to bind to the "closed" lid, which is the predominant form identified by NMR (75). This may contribute to the efficacy of Nutlin compared with p53 peptides. Notably, these lid residues are not conserved in the Mdmx NH_2 terminus, but it is presently unclear whether this contributes to the relative insensitivity of Mdmx to the Nutlins. However, structural studies of the Mdmx lid should also be considered to design optimal Mdmx-specific antagonists.

Despite these observations, Nutlin has been used successfully in a murine model of retinoblastoma, where overexpressed Mdmx inhibits p53 function (42). This may be due to the achievement of high drug concentration at the site of the tumor. However, development of either Mdmx-specific or dual specificity Mdm2/Mdmx inhibitors will clearly be beneficial. Experimental support that such an approach is feasible has emerged from studies of phage-displayed peptides. Extending results of previous studies (56), Hu and colleagues

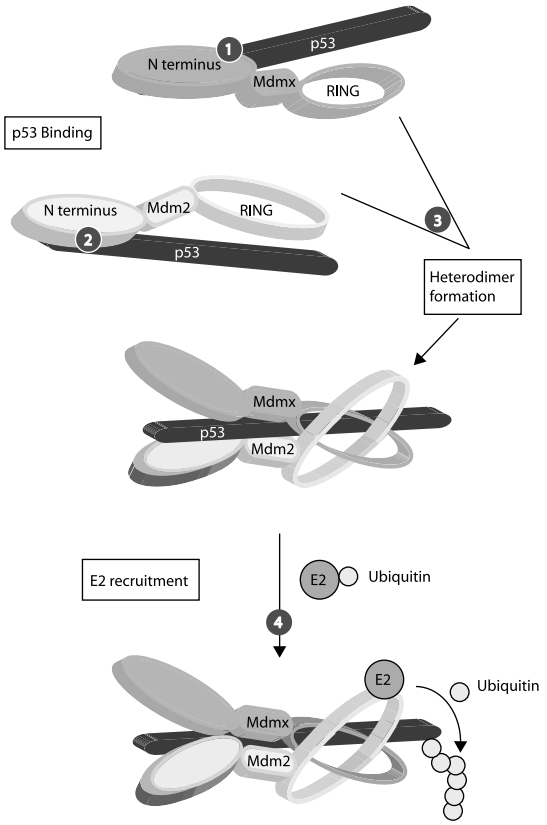

FIGURE 3. Mdmx and Mdm2 may be functionally inhibited at multiple steps to reactivate p53. Numbered circles indicate potential sites at which small-molecule antagonists may be used. First, the interaction of the Mdmx or Mdm2 NH_2 termini with p53 can be antagonized using small molecules such as Nutlin (*1* and *2*). Second, antagonism of the interaction between the Mdm2 and Mdmx RING domains (*3*) may prevent the formation of an optimal E3 ligase complex. Whether such antagonists would interact with Mdm2/Mdmx monomers or with heterodimers remains to be determined. Third, the recruitment of E2-conjugating enzyme carrying activated ubiquitin to Mdm2/Mdmx is a potential target for antagonists (*4*). Note that each heterodimer has the potential to bind at least two p53 molecules via the NH_2 termini of both Mdm2 and Mdmx. However, no structural data are available for the NH_2-terminal regions of Mdm2/Mdmx heterodimers in complex with p53, and therefore, only one p53 molecule is shown for simplicity.

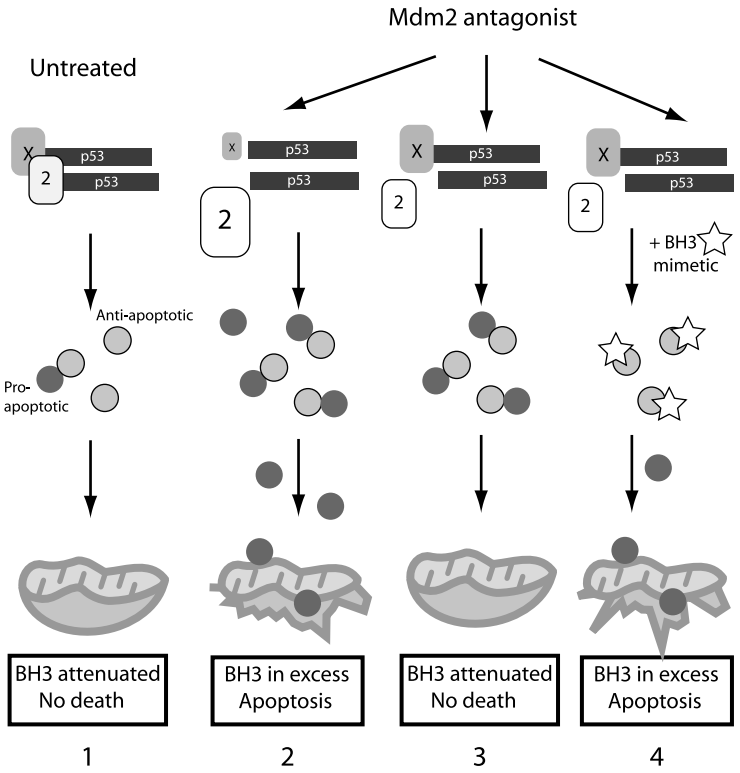

FIGURE 4. BH3-mediated sensitization to Mdm2 antagonists. In unstressed cells (*1*), proapoptotic BH3 proteins are attenuated by the activity of antiapoptotic homologues (*dark* and *light circles*, respectively). Following antagonism of Mdm2, three scenarios can be envisioned. In *2*, activation of p53 induces sufficient Mdm2 to decrease Mdmx levels and leads to sufficient proapoptotic BH3 activation to induce apoptosis. If Mdmx is not degraded or antagonized (*3*), the amount of p53-induced BH3s may not reach the threshold required for apoptosis. One potential mechanism to abrogate Mdmx-dependent protection against apoptosis is to lower the threshold at which BH3 activation will induce apoptosis by titrating antiapoptotic proteins from the system. This can be achieved using BH3 mimetic compounds (*stars, 4*).

isolated a peptide that exhibits dual Mdm2/Mdmx specificity (76). Using adenoviral delivery, this peptide was shown to activate p53 in cell lines and tumor xenografts. Adenoviral therapy may be limited to certain tumors *in vivo*, and improvements to this new class of peptides that permit an alternative delivery method will be required to increase the range of treatable tumors. Notably, a stapled peptide that targets both Mdm2 and Mdmx (57)[2] may serve as a lead compound in this regard.

Additional strategies to prevent Mdm2-dependent ubiquitination of p53 have also been explored. *In vitro* chemical screens have identified inhibitors of Mdm2 ubiquitin ligase activity (77-79). Some of the identified compounds preferentially target Mdm2 ubiquitination of p53 but not Mdm2 autoubiquitination, whereas others have no measurable selectivity. Although both classes of compounds are promising leads for further development, several questions remain related to efficacy, specificity, and mechanism of action. For example, the HL198 series of Mdm2 ubiquitin ligase inhibitors stabilize p53 as predicted yet also exhibit some p53-independent effects. Furthermore, compounds selective for Mdm2-dependent ubiquitination of p53 have not yet been shown to stabilize p53 or induce p53-dependent cell death *in vivo* (79).

The requirement for both an E1 ubiquitin-activating enzyme and an E2 ubiquitin-conjugating enzyme to mediate Mdm2-dependent p53 ubiquitination and degradation suggests additional strategies for p53 activation (see Fig. 3). Indeed, E1 and E2s are potentially druggable targets because they have "conventional" catalytic sites. For example, Yang and colleagues recently described a small-molecule inhibitor of a human E1 enzyme (80). However, numerous E2/E3 enzymes can use ubiquitin activated by a particular E1. Consequently, such drugs may affect multiple cellular processes, leading to undesirable p53-independent effects.

Because the Mdm2/Mdmx heterodimer may be a more effective ubiquitin ligase for p53 than homodimeric Mdm2 (refs. 14, 16, 22, 23 and see above), perturbing the functional interaction between Mdm2 and Mdmx should activate p53. Consistent with this hypothesis, Stad and colleagues showed that overexpression of the Mdmx RING domain alone was sufficient to stabilize both wild-type and mutant p53 (81). More recently, Kawai and colleagues showed that wild-type p53 is stabilized and activated following overexpression of the Mdmx RING domain but not by an Mdm2 binding-deficient Mdmx RING mutant (14). Together, these data suggest that antagonism of endogenous Mdm2/Mdmx heterodimers should activate endogenous p53. The RING/RING interaction may be difficult to "drug," however, because it lacks a defined catalytic site, or a hydrophobic pocket or groove to which small molecules can bind. Whether the Mdm2 or Mdmx RING domains have ligand binding pockets or grooves is not clear from published crystal structures (8, 20). Furthermore, the RING domain does not contain a typical catalytic site; rather, Mdm2 acts as a molecular scaffold to promote ubiquitination. Perhaps NMR-based screening efforts (82) using the Mdm2 or Mdmx RING domain may identify "hotspots" for ligand binding. Ligand binding sites can also be created during

[2] F. Bernal et al., unpublished data.

dimerization of E3 ligases with other proteins (83). It is possible that Mdm2/Mdmx heterodimerization may create such sites, although whether this would be accessible to small molecules remains to be determined.

Combination Therapies in Cells with High Mdmx

Combination chemotherapy is widely used to treat cancer patients because the probability that resistance will emerge is decreased when multiple drugs targeting different pathways are used simultaneously. Historically, the drug combinations were determined empirically, but with the advent of genomics and proteomics, this strategy may be tailored according to individual patient profiles.

Although the current Mdm2 antagonists are not optimal for targeting Mdmx, their efficacy may be improved using a combination regimen. This has been shown in the mouse retinoblastoma model, where the DNA-damaging agent topotecan is used in combination with Nutlin-3a (42). Although the mechanism for this synergy is unclear, topotecan might potentiate the effects of Nutlin by triggering down-regulation of Mdmx. Indeed, other studies using DNA-damaging agents in combination with Nutlin have yielded similar synergistic results (24, 84, 85). Additionally, inducing Mdmx degradation using doxorubicin can also sensitize Nutlin-resistant cells to undergo apoptosis (52). Genotoxins likely also synergize with Mdm2 inhibitors through additional mechanisms. For example, Nutlin and fludarabine synergize in cases of chronic lymphocytic leukemia even in cases where ataxia-telangiectasia mutated (a kinase that triggers Mdmx down-regulation) is mutated (86). We have generated a knock-in mouse in which three serine residues that are phosphorylated by damage kinases are mutated to alanine.[3] This *in vivo* model will provide a rigorous test of the contribution of Mdmx down-regulation to genotoxin-based combination chemotherapy.

Although combining Mdm2 antagonists with genotoxic agents reduces tumor burden, collateral DNA damage may lead to undesirable effects in nonmalignant tissues and could increase the risk of secondary malignancies. Therefore, in cancers where Mdmx is a determinant of sensitivity to Mdm2 antagonists, induction of DNA damage to down-regulate Mdmx is not an optimal chemotherapeutic strategy. A more suitable approach may be to activate damage-independent mechanism(s) that abrogates Mdmx-dependent protection against apoptosis. We have observed that Mdmx level is a determinant of sensitivity to Mdm2 antagonists in several tumor cell lines (51, 68). In many cases, this is associated with reduced p53 transcriptional output or an inability to down-regulate Mdmx protein. We hypothesized that the level of p53-induced proapoptotic proteins in these cell lines fails to exceed the buffering capacity of antiapoptotic family members. A prediction from this hypothesis is that antagonism of anti-apoptotic bcl-2 proteins would sensitize these resistant cell lines to Nutlin-induced apoptosis. Indeed, combined treatment with Nutlin and ABT-737 (a small-molecule BH3 mimetic; ref. 87) significantly increased apoptosis compared with either agent alone in cells with high levels of Mdmx (68). This particular combination has also proven effective in primary isolates from acute myelogenous leukemia patients (88), although whether Mdmx is a determinant of sensitivity to Nutlin in this particular tumor type is not known. As illustrated in Fig. 4, combined treatment with Mdm2 antagonists and BH3 mimetics may therefore be a novel strategy to induce regression of tumors with wild-type p53.

Preferential Selection of Mdm2 and Mdmx Overexpression during Tumorigenesis

Basic research and profiling of clinical samples indicate the importance of both Mdm2 and Mdmx as oncogenes in human cancer, yet many questions remain. For example, what are the selective pressures that lead to increased Mdm2 in some tumor types but increased Mdmx in others? In tumors that retain both arf and p53, Mdmx rather than Mdm2 may be selected for because only Mdmx is refractory to arf inhibition (89). Additionally, up-regulation of either Mdm2 or Mdmx may be determined by additional oncogenic events, such as ras activation (40, 90). Finally, selection for Mdm2 and Mdmx overexpression may be more likely in tissues that have an obligate requirement for these proteins in the nonneoplastic state.

The Role of Heterodimerization in Restricting p53 Activity

If the Mdm2/Mdmx heterodimer is the most effective p53 ubiquitin ligase, then an increase in either Mdm2 or Mdmx might be selected for to keep p53 at a level compatible with tumor progression. Whether Mdm2 or Mdmx is selected for may depend on which protein is stoichiometrically limiting for heterodimer formation in a particular tissue. The situation is likely complex, however, because our preliminary studies indicate that Mdmx is the stoichiometrically limiting component in most cells studied, yet the overall frequency of *Mdm2* and *Mdmx* amplification in tumors is similar (3, 11). Analysis of the stoichiometric relationship between Mdm2, Mdmx, and p53 across a large number of clinical samples and matched normal tissues may provide further insight in this regard. Mouse models represent an additional approach to determine whether Mdm2/Mdmx heterodimerization is required for p53 regulation *in vivo*. Generation of Mdm2 or Mdmx mutants that preclude heterodimerization would be a direct test of this hypothesis. Such a mutation should preserve as much of the structure of the native protein as possible to avoid artifacts introduced by conformational changes.

A Case for Mdmx-Specific Antagonists?

Sensitivity to current Mdm2 antagonists can be attenuated in cells with high levels of Mdmx, particularly when Mdmx is not down-regulated following treatment (51). This suggests that designing Mdmx-specific antagonists may be of therapeutic benefit. However, whether such drugs will be as effective as current Mdm2 antagonists is presently unclear. For example, Mdm2 retains its ubiquitin ligase activity when bound by current antagonists, and this leads to down-regulation of other Mdm2 targets on treatment (51, 91, 92). It is unknown whether

[3] Y. Wang and G.M. Wahl, in preparation.

Table 2. Variant Transcripts Expressed from the Mdmx Locus

Transcript	Notes	Reference
Hdmx-A	Deletion of the acidic domain	(108)
Hdmx-G	Deletion of the p53-BD	(108)
Hdmx-211	Deletion of aa27-353	(109)
Mdmx-S/Hdmx-E	Retains only the p53-BD Binds p53 more effectively than FL-Mdmx	(108, 110)
XALT1	Induced by DNA damage, retains only p53-BD	(111)
XALT2	Induced by DNA damage, lacks p53-BD	(111)

Abbreviations: H, human; M, mouse; p53-BD, p53-binding domain; FL, full-length.

this is required for the cytotoxicity of Mdm2 antagonists, but analogies may be drawn with the recent finding that E3 ligase activity is required for inhibitor of apoptosis protein antagonist-induced apoptosis (93). The outcome of treatment with Mdmx-specific antagonists remains to be determined. Such compounds may be effective in tumor cells where the majority of p53 is bound only to Mdmx, or in cells in which Mdmx acts as molecular scaffold to bring Mdm2, E2 enzyme, and p53 into contact. However, the ensuing activation of p53 will lead to an increase in Mdm2 levels, raising the possibility that increased Mdm2/p53 interaction will negate the effect of Mdmx-specific antagonists. Such a finding would support the use of dual specificity Mdm2 and Mdmx inhibitors. Hu and colleagues recently provided proof of principle for efficacy of this type of inhibitor (76). However, this peptidic compound worked efficiently only when delivered by adenovirus and so may find limited application in therapy in its present form. Nevertheless, this is a promising indication that dual inhibition of Mdm2/Mdmx by small molecules will be effective.

It is currently unclear whether inhibitors of the Mdm2/Mdmx interaction will be as potent as Mdm2/p53 or Mdmx/p53 antagonists. If the cooperative model of p53 regulation by Mdm2 and Mdmx is correct, such inhibitors may be extremely potent. However, disruption of Mdm2/Mdmx interaction does not prevent the individual proteins from interacting with the p53 NH_2 terminus and suppressing p53-dependent transactivation. Therefore, cell fate may be determined by the degree to which p53 accumulates versus the ability of free Mdm2 and Mdmx to inhibit it.

There are likely to be additional factors that can modulate sensitivity to compounds such as the Nutlins. For example, differential p53 target gene regulation and deregulated E2F-1 activity are also reported to determine cell fate after Nutlin treatment (94-96). These data emphasize the need for multiparametric analyses of tumor biopsies to generate accurate predictions of drug sensitivity for a particular tumor type. There are also several alternate transcripts of both *Mdm2* and *Mdmx* that are found predominantly in tumor cells or are induced in response to genotoxic stress (see Table 2). Given that the proteins encoded by these mRNAs retain the ability to interact with either full-length Mdm2, Mdmx, or p53, they may also influence the response to Mdm2/Mdmx antagonists. Further analyses of the various Mdm2 and Mdmx isoforms are required to address this possibility.

Achieving a Favorable Therapeutic Index with Mdm2/Mdmx Inhibitors

The therapeutic index of a drug is the ratio given by the dose that causes toxicity in 50% of the patient population divided by the minimal effective dose for 50% of the population. Thus, a higher therapeutic index is more favorable because toxicity (in this case) to tumor cells will be achieved at drug concentrations much lower than those that would damage normal tissues. Because Mdm2 and Mdmx are present in both normal and tumor tissues, one concern is that targeting them would give a prohibitively low therapeutic index. Indeed, murine models indicate that complete loss of Mdm2 protein function in the adult mouse is a lethal event, raising concerns about using such drugs in human patients (32, 97). However, long-term (up to 3 weeks) systemic administration of both Nutlin and MI-219 in mice resulted in surprisingly little toxicity in tissues that are most sensitive to p53-induced apoptosis (50, 59, 94). A plausible reason for this difference is that the genetic ablation of Mdm2 function results in permanent and irreversible production of high enough levels of p53 to exceed the buffering capacity of Mdmx. By contrast, drugs such as Nutlins or MI do not bind and inhibit Mdm2 irreversibly, and the *Mdm2* gene is continuously activated by p53 in the drug-treated tissues. Therefore, Mdm2 antagonists may initially stabilize p53 and produce a biological response, but p53 activation will persist only as long as the drug is administered. It is possible that in normal tissues the response may be acute and transient due to clearance of the drug or activation of the p53-Mdm2 negative feedback loop.

Other hypotheses have been put forward to explain the differential sensitivity of tumor versus normal tissues to Mdm2 antagonists. For example, a short hairpin RNA screen identified 53BP1 as a critical mediator of Nutlin-induced senescence (98). 53BP1 transduces DNA damage signals to p53, and 53BP1 foci (which are indicative of ongoing DNA damage) are present in many tumor cells but not in their normal counterparts (103-105). Thus, the combination of Nutlin-induced p53 and damage-induced signaling may preferentially inhibit the growth of tumor cells that experience ongoing genomic instability. Additionally, deregulated E2F-1 activity may preferentially sensitize tumor cells to Mdm2 antagonists (96). Specifically, E2F-1–dependent gene transcription is postulated to cooperate with Nutlin-induced p53 activity to induce apoptosis. Finally, overexpression of Mdm2 itself may be a determinant of sensitivity to Nutlin in some tumor types (94). In this case, the tumor cells may be "addicted" to the high levels of Mdm2 needed to restrict p53 activity. Once Mdm2-p53 interaction is prevented, and p53 activation occurs, the tumor cell may be acutely sensitive to the effects of p53-induced gene products.

Although the future for Mdm2 inhibitors is promising, there are additional concerns that require further investigation. Because these antagonists are such potent activators of p53-dependent cell cycle arrest and apoptosis, there may be a strong selective pressure for loss of p53 function either by *p53* gene mutation or by epigenetic changes. Additionally, there are reports that inhibition of Mdm2 can also lead to stabilization of mutant forms of p53 (102, 103). Thus, it is conceivable that treatment with Mdm2 inhibitors as single agents may lead to tumor relapse or secondary tumor formation. This scenario may

be avoided if Mdm2 antagonists are given in combination with agents that induce tumor cell apoptosis independently of p53 or with agents that are able to reactivate mutant p53. As has been the case with other rationally designed agents, the more we understand about mechanism of action, the better we will be prepared to develop strategies to deal with the inevitable resistance that is likely to emerge. Given the rapid pace of progress in this area, we are encouraged that p53 agonists will provide powerful additions to the targeted therapeutic armamentarium.

Disclosure of Potential Conflicts of Interest

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References

1. Parant J, Chavez-Reyes A, Little NA, et al. Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. Nat Genet 2001;29:92–5.

2. Montes de Oca Luna R, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 1995; 378:203–6.

3. Toledo F, Wahl GM. Regulating the p53 pathway: *in vitro* hypotheses, *in vivo* veritas. Nat Rev Cancer 2006;6:909–23.

4. Marine JC, Dyer MA, Jochemsen AG. MDMX: from bench to bedside. J Cell Sci 2007;120:371–8.

5. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002;82: 373–428.

6. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992;69:1237–45.

7. Ma J, Martin JD, Zhang H, et al. A second p53 binding site in the central domain of Mdm2 is essential for p53 ubiquitination. Biochemistry 2006;45: 9238–45.

8. Linke K, Mace PD, Smith CA, Vaux DL, Silke J, Day CL. Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. Cell Death Differ 2008;15:841–8.

9. Krummel KA, Toledo F, Lee CJ, Wahl GM. The C-terminal lysines fine-tune p53 stress responses in a mouse model, but are not required for stability control or transactivation. Proc Natl Acad Sci U S A 2005;102:10188–93.

10. Feng L, Lin T, Uranishi H, Gu W, Xu Y. Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity. Mol Cell Biol 2005;25:5389–95.

11. Wang YV, Wade M, Wong E, Li YC, Rodewald LW, Wahl GM. Quantitative analyses reveal the importance of regulated Hdmx degradation for p53 activation. Proc Natl Acad Sci U S A 2007;104:12365–70.

12. Tanimura S, Ohtsuka S, Mitsui K, Shirouzu K, Yoshimura A, Ohtsubo M. MDM2 interacts with MDMX through their RING finger domains. FEBS Lett 1999;447:5–9.

13. Danovi D, Meulmeester E, Pasini D, et al. Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. Mol Cell Biol 2004;24:5835–43.

14. Kawai H, Lopez-Pajares V, Kim MM, Wiederschain D, Yuan ZM. RING domain-mediated interaction is a requirement for MDM2's E3 ligase activity. Cancer Res 2007;67:6026–30.

15. Linares LK, Hengstermann A, Ciechanover A, Muller S, Scheffner M. HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. Proc Natl Acad Sci U S A 2003;100:12009–14.

16. Gu J, Kawai H, Nie L, et al. Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. J Biol Chem 2002;277:19251–4.

17. Hashizume R, Fukuda M, Maeda I, et al. The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. J Biol Chem 2001;276:14537–40.

18. Buchwald G, van der Stoop P, Weichenrieder O, Perrakis A, van Lohuizen M, Sixma TK. Structure and E3-ligase activity of the Ring-Ring complex of polycomb proteins Bmi1 and Ring1b. EMBO J 2006;25:2465–74.

19. Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J Biol Chem 2000;275:8945–51.

20. Kostic M, Matt T, Martinez-Yamout MA, Dyson HJ, Wright PE. Solution structure of the Hdm2 C2H2C4 RING, a domain critical for ubiquitination of p53. J Mol Biol 2006;363:433–50.

21. Steinman HA, Hoover KM, Keeler ML, Sands AT, Jones SN. Rescue of Mdm4-deficient mice by Mdm2 reveals functional overlap of Mdm2 and Mdm4 in development. Oncogene 2005;24:7935–40.

22. Uldrijan S, Pannekoek WJ, Vousden KH. An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. EMBO J 2007;26: 102–12.

23. Poyurovsky MV, Priest C, Kentsis A, et al. The Mdm2 RING domain C-terminus is required for supramolecular assembly and ubiquitin ligase activity. EMBO J 2007;26:90–101.

24. Patton JT, Mayo LD, Singhi AD, Gudkov AV, Stark GR, Jackson MW. Levels of HdmX expression dictate the sensitivity of normal and transformed cells to Nutlin-3. Cancer Res 2006;66:3169–76.

25. Gilkes DM, Chen L, Chen J. MDMX regulation of p53 response to ribosomal stress. EMBO J 2006;25:5614–25.

26. Mallery DL, Vandenberg CJ, Hiom K. Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. EMBO J 2002;21: 6755–62.

27. Maki CG, Huibregtse JM, Howley PM. *In vivo* ubiquitination and proteasome-mediated degradation of p53. Cancer Res 1996;56:2649–54.

28. Meulmeester E, Maurice MM, Boutell C, et al. Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. Mol Cell 2005;18:565–76.

29. Stommel JM, Wahl GM. Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation. EMBO J 2004;23:1547–56.

30. Pereg Y, Shkedy D, de Graaf P, et al. Phosphorylation of Hdmx mediates its Hdm2- and ATM-dependent degradation in response to DNA damage. Proc Natl Acad Sci U S A 2005;102:5056–61.

31. Kawai H, Wiederschain D, Kitao H, Stuart J, Tsai KK, Yuan ZM. DNA damage-induced MDMX degradation is mediated by MDM2. J Biol Chem 2003; 278:45946–53.

32. Itahana K, Mao H, Jin A, et al. Targeted inactivation of Mdm2 RING finger E3 ubiquitin ligase activity in the mouse reveals mechanistic insights into p53 regulation. Cancer Cell 2007;12:355–66.

33. Terzian T, Wang Y, Van Pelt CS, Box NF, Travis EL, Lozano G. Haploinsufficiency of Mdm2 and Mdm4 in tumorigenesis and development. Mol Cell Biol 2007;27:5479–85.

34. Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B. Amplification of a gene encoding a p53-associated protein in human sarcomas [see comments]. Nature 1992;358:80–3.

35. Jones SN, Hancock AR, Vogel H, Donehower LA, Bradley A. Over-expression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. Proc Natl Acad Sci U S A 1998;95:15608–12.

36. Bond GL, Hu W, Bond EE, et al. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 2004;119:591–602.

37. Bond GL, Hirshfield KM, Kirchhoff T, et al. MDM2 SNP309 accelerates tumor formation in a gender-specific and hormone-dependent manner. Cancer Res 2006;66:5104–10.

38. Atwal GS, Rabadan R, Lozano G, et al. An information-theoretic analysis of genetics, gender and age in cancer patients. PLoS ONE 2008;3:e1951.

39. Phelps M, Darley M, Primrose JN, Blaydes JP. p53-independent activation of the hdm2-P2 promoter through multiple transcription factor response elements results in elevated hdm2 expression in estrogen receptor α-positive breast cancer cells. Cancer Res 2003;63:2616–23.

40. Gilkes DM, Pan Y, Coppola D, Yeatman T, Reuther GW, Chen J. Regulation of MDMX expression by mitogenic signaling. Mol Cell Biol 2008; 28:1999–2010.

41. Riemenschneider MJ, Buschges R, Wolter M, et al. Amplification and overexpression of the MDM4 (MDMX) gene from 1q32 in a subset of malignant gliomas without TP53 mutation or MDM2 amplification. Cancer Res 1999;59: 6091–6.

42. Laurie NA, Donovan SL, Shih CS, et al. Inactivation of the p53 pathway in retinoblastoma. Nature 2006;444:61–6.

43. Bartel F, Schulz J, Bohnke A, et al. Significance of HDMX-S (or MDM4) mRNA splice variant overexpression and HDMX gene amplification on primary soft tissue sarcoma prognosis. Int J Cancer 2005;117:469–75.

44. Mendrysa SM, O'Leary KA, McElwee MK, et al. Tumor suppression and normal aging in mice with constitutively high p53 activity. Genes Dev 2006;20:16–21.

45. Alt JR, Greiner TC, Cleveland JL, Eischen CM. Mdm2 haplo-insufficiency profoundly inhibits Myc-induced lymphomagenesis. EMBO J 2003;22:1442–50.

46. de Fougerolles A, Vornlocher HP, Maraganore J, Lieberman J. Interfering with disease: a progress report on siRNA-based therapeutics. Nat Rev Drug Discov 2007;6:443–53.

47. Pan Y, Chen J. MDM2 promotes ubiquitination and degradation of MDMX. Mol Cell Biol 2003;23:5113–21.

48. Li M, Brooks CL, Kon N, Gu W. A dynamic role of HAUSP in the p53-Mdm2 pathway. Mol Cell 2004;13:879–86.

49. Kessler BM, Fortunati E, Melis M, Pals CE, Clevers H, Maurice MM. Proteome changes induced by knock-down of the deubiquitylating enzyme HAUSP/USP7. J Proteome Res 2007;6:4163–72.

50. Vassilev LT, Vu BT, Graves B, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004;303:844–8.

51. Wade M, Wong ET, Tang M, Stommel JM, Wahl GM. Hdmx modulates the outcome of p53 activation in human tumor cells. J Biol Chem 2006;281:33036–44.

52. Xia M, Knezevic D, Tovar C, Huang B, Heimbrook DC, Vassilev LT. Elevated MDM2 boosts the apoptotic activity of p53-MDM2 binding inhibitors by facilitating MDMX degradation. Cell Cycle 2008;7:1604–12.

53. Shvarts A, Steegenga WT, Riteco N, et al. MDMX: a novel p53-binding protein with some functional properties of MDM2. EMBO J 1996;15:5349–57.

54. Kussie PH, Gorina S, Marechal V, et al. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science 1996;274:948–53.

55. Kritzer JA, Lear JD, Hodsdon ME, Schepartz A. Helical β-peptide inhibitors of the p53-hDM2 interaction. J Am Chem Soc 2004;126:9468–9.

56. Bottger V, Bottger A, Howard SF, et al. Identification of novel mdm2 binding peptides by phage display. Oncogene 1996;13:2141–7.

57. Bernal F, Tyler AF, Korsmeyer SJ, Walensky LD, Verdine GL. Reactivation of the p53 tumor suppressor pathway by a stapled p53 peptide. J Am Chem Soc 2007;129:2456–7.

58. Chene P. Inhibition of the p53-MDM2 interaction: targeting a protein-protein interface. Mol Cancer Res 2004;2:20–8.

59. Shangary S, Qin D, McEachern D, et al. Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. Proc Natl Acad Sci U S A 2008;105:3933–8.

60. Lu Y, Nikolovska-Coleska Z, Fang X, et al. Discovery of a nanomolar inhibitor of the human murine double minute 2 (MDM2)-p53 interaction through an integrated, virtual database screening strategy. J Med Chem 2006;49:3759–62.

61. Vassilev LT. MDM2 inhibitors for cancer therapy. Trends Mol Med 2007;13:23–31.

62. Rothweiler U, Czarna A, Krajewski M, et al. Isoquinolin-1-one inhibitors of the MDM2-p53 interaction. ChemMedChem 2008;3:1118–28.

63. Parks DJ, LaFrance LV, Calvo RR, et al. Enhanced pharmacokinetic properties of 1,4-benzodiazepine-2,5-dione antagonists of the HDM2-p53 protein-protein interaction through structure-based drug design. Bioorg Med Chem Lett 2006;16:3310–4.

64. Hardcastle IR, Ahmed SU, Atkins H, et al. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction based on an isoindolinone scaffold. J Med Chem 2006;49:6209–21.

65. Dudkina AS, Lindsley CW. Small molecule protein-protein inhibitors for the p53-MDM2 interaction. Curr Top Med Chem 2007;7:952–60.

66. Chen L, Yin H, Farooqi B, Sebti S, Hamilton AD, Chen J. p53 α-helix mimetics antagonize p53/MDM2 interaction and activate p53. Mol Cancer Ther 2005;4:1019–25.

67. Bowman AL, Nikolovska-Coleska Z, Zhong H, Wang S, Carlson HA. Small molecule inhibitors of the MDM2-p53 interaction discovered by ensemble-based receptor models. J Am Chem Soc 2007;129:12809–14.

68. Wade M, Rodewald LW, Espinosa JM, Wahl GM. BH3 activation blocks Hdmx suppression of apoptosis and co-operates with Nutlin to induce cell death. Cell Cycle 2008;7:1973–82.

69. Hu B, Gilkes DM, Farooqi B, Sebti SM, Chen J. MDMX overexpression prevents p53 activation by the MDM2 inhibitor Nutlin. J Biol Chem 2006;281:33030–5.

70. Rallapalli R, Strachan G, Tuan RS, Hall DJ. Identification of a domain within MDMX-S that is responsible for its high affinity interaction with p53 and high-level expression in mammalian cells. J Cell Biochem 2003;89:563–75.

71. Popowicz GM, Czarna A, Rothweiler U, et al. Molecular basis for the inhibition of p53 by Mdmx. Cell Cycle 2007;6:2386–92.

72. Popowicz GM, Czarna A, Holak TA. Structure of the human Mdmx protein bound to the p53 tumor suppressor transactivation domain. Cell Cycle 2008;7:2441–3.

73. Uhrinova S, Uhrin D, Powers H, et al. Structure of free MDM2 N-terminal domain reveals conformational adjustments that accompany p53-binding. J Mol Biol 2005;350:587–98.

74. McCoy MA, Gesell JJ, Senior MM, Wyss DF. Flexible lid to the p53-binding domain of human Mdm2: implications for p53 regulation. Proc Natl Acad Sci U S A 2003;100:1645–8.

75. Showalter SA, Bruschweiler-Li L, Johnson E, Zhang F, Bruschweiler R. Quantitative lid dynamics of MDM2 reveals differential ligand binding modes of the p53-binding cleft. J Am Chem Soc 2008;130:6472–8.

76. Hu B, Gilkes DM, Chen J. Efficient p53 activation and apoptosis by simultaneous disruption of binding to MDM2 and MDMX. Cancer Res 2007;67:8810–7.

77. Yang Y, Ludwig RL, Jensen JP, et al. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell 2005;7:547–59.

78. Murray MF, Jurewicz AJ, Martin JD, et al. A high-throughput screen measuring ubiquitination of p53 by human mdm2. J Biomol Screen 2007;12:1050–8.

79. Lai Z, Yang T, Kim YB, et al. Differentiation of Hdm2-mediated p53 ubiquitination and Hdm2 autoubiquitination activity by small molecular weight inhibitors. Proc Natl Acad Sci U S A 2002;99:14734–9.

80. Yang Y, Kitagaki J, Dai RM, et al. Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. Cancer Res 2007;67:9472–81.

81. Stad R, Ramos YF, Little N, et al. Hdmx stabilizes Mdm2 and p53. J Biol Chem 2000;275:28039–44.

82. Hajduk PJ. SAR by NMR: putting the pieces together. Mol Interv 2006;6:266–72.

83. House CM, Hancock NC, Moller A, et al. Elucidation of the substrate binding site of Siah ubiquitin ligase. Structure 2006;14:695–701.

84. Stuhmer T, Chatterjee M, Hildebrandt M, et al. Nongenotoxic activation of the p53 pathway as a therapeutic strategy for multiple myeloma. Blood 2005;106:3609–17.

85. Drakos E, Thomaides A, Medeiros LJ, et al. Inhibition of p53-murine double minute 2 interaction by nutlin-3A stabilizes p53 and induces cell cycle arrest and apoptosis in Hodgkin lymphoma. Clin Cancer Res 2007;13:3380–7.

86. Kojima K, Konopleva M, McQueen T, O'Brien S, Plunkett W, Andreeff M. Mdm2 inhibitor Nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia. Blood 2006;108:993–1000.

87. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005;435:677–81.

88. Kojima K, Konopleva M, Samudio IJ, Schober WD, Bornmann WG, Andreeff M. Concomitant inhibition of MDM2 and Bcl-2 protein function synergistically induce mitochondrial apoptosis in AML. Cell Cycle 2006;5:2778–86.

89. Wang X, Arooz T, Siu WY, et al. MDM2 and MDMX can interact differently with ARF and members of the p53 family. FEBS Lett 2001;490:202–8.

90. Ries S, Biederer C, Woods D, et al. Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. Cell 2000;103:321–30.

91. Wallace M, Worrall E, Pettersson S, Hupp TR, Ball KL. Dual-site regulation of MDM2 E3-ubiquitin ligase activity. Mol Cell 2006;23:251–63.

92. Ambrosini G, Sambol EB, Carvajal D, Vassilev LT, Singer S, Schwartz GK. Mouse double minute antagonist Nutlin-3a enhances chemotherapy-induced apoptosis in cancer cells with mutant p53 by activating E2F1. Oncogene 2007;26:3473–81.

93. Varfolomeev E, Blankenship JW, Wayson SM, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-κB activation, and TNFα-dependent apoptosis. Cell 2007;131:669–81.

94. Tovar C, Rosinski J, Filipovic Z, et al. Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. Proc Natl Acad Sci U S A 2006;103:1888–93.

95. Paris R, Henry RE, Stephens SJ, McBryde M, Espinosa JM. Multiple p53-independent gene silencing mechanisms define the cellular response to p53 activation. Cell Cycle 2008;7:2427–33.

96. Kitagawa M, Aonuma M, Lee SH, Fukutake S, McCormick F. E2F-1

transcriptional activity is a critical determinant of Mdm2 antagonist-induced apoptosis in human tumor cell lines. Oncogene 2008;27:5303–14.

97. Ringshausen I, O'Shea CC, Finch AJ, Swigart LB, Evan GI. Mdm2 is critically and continuously required to suppress lethal p53 activity *in vivo*. Cancer Cell 2006;10:501–14.

98. Brummelkamp TR, Fabius AW, Mullenders J, et al. An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. Nat Chem Biol 2006;2:202–6.

99. Gorgoulis VG, Vassiliou LV, Karakaidos P, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005;434:907–13.

100. DiTullio RA, Jr., Mochan TA, Venere M, et al. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. Nat Cell Biol 2002;4:998–1002.

101. Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434:864–70.

102. Terzian T, Suh YA, Iwakuma T, et al. The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. Genes Dev 2008;22:1337–44.

103. Lukashchuk N, Vousden KH. Ubiquitination and degradation of mutant p53. Mol Cell Biol 2007;27:8284–95.

104. Schlaeger C, Longerich T, Schiller C, et al. Etiology-dependent molecular mechanisms in human hepatocarcinogenesis. Hepatology 2008;47:511–20.

105. Valentin-Vega YA, Barboza JA, Chau GP, El-Naggar AK, Lozano G. High levels of the p53 inhibitor MDM4 in head and neck squamous carcinomas. Hum Pathol 2007;38:1553–62.

106. Veerakumarasivam A, Scott HE, Chin SF, et al. High-resolution array-based comparative genomic hybridization of bladder cancers identifies mouse double minute 4 (MDM4) as an amplification target exclusive of MDM2 and TP53. Clin Cancer Res 2008;14:2527–34.

107. Ramos YF, Stad R, Attema J, Peltenburg LT, van der Eb AJ, Jochemsen AG. Aberrant expression of HDMX proteins in tumor cells correlates with wild-type p53. Cancer Res 2001;61:1839–42.

108. de Graaf P, Little NA, Ramos YF, Meulmeester E, Letteboer SJ, Jochemsen AG. Hdmx protein stability is regulated by the ubiquitin ligase activity of Mdm2. J Biol Chem 2003;278:38315–24.

109. Giglio S, Mancini F, Gentiletti F, et al. Identification of an aberrantly spliced form of HDMX in human tumors: a new mechanism for HDM2 stabilization. Cancer Res 2005;65:9687–94.

110. Rallapalli R, Strachan G, Cho B, Mercer WE, Hall DJ. A novel MDMX transcript expressed in a variety of transformed cell lines encodes a truncated protein with potent p53 repressive activity. J Biol Chem 1999;274:8299–308.

111. Chandler DS, Singh RK, Caldwell LC, Bitler JL, Lozano G. Genotoxic stress induces coordinately regulated alternative splicing of the p53 modulators MDM2 and MDM4. Cancer Res 2006;66:9502–8.

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