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Therapeutic considerations for Mdm2: not just a one trick pony

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Abstract

Background—The *mdm2* proto-oncogene is elevated in numerous late stage cancers. The Mdm2 protein manifests its oncogenic properties in part through inactivation of the tumor suppressor protein p53. Recent efforts in anti-cancer drug design have focused on the identification of small molecules that disrupt the Mdm2-p53 interaction, in hopes of re-engaging the p53 pathway.

Objective—In addition to binding p53, Mdm2 complexes with numerous proteins involved in DNA repair, translation, metabolic activities, tumor growth and apoptosis. Additional biochemical analysis is required to understand how Mdm2 integrates into all of these cellular processes. Post-translational modifications to Mdm2 can alter its ability to associate with numerous proteins. Changes in protein structure may also affect the ability of small molecule inhibitors to effectively antagonize Mdm2.

Conclusion—The complexity of Mdm2 modification has been largely neglected during the development of previous Mdm2 inhibitors. Future high-throughput or *in silico* screening efforts will need to recognize the importance of post-translational modifications to Mdm2. Furthermore, the identification of molecules that target other domains in Mdm2 may provide a tool to prevent other pivotal p53-independent functions of Mdm2. These aims provide a useful roadmap for the discovery of new Mdm2 binding compounds with therapeutic potency that may exceed its predecessors.

Keywords

DNA-dependent protein kinase; p53; Hdm2; Mdm2; Mdmx; Nutlin compounds; p53; RING domain; Small molecule inhibitors

1. Introduction

The murine double minute gene was discovered in murine cells containing 3 copies of the gene designated 1, 2, and, 3 on a double minute chromosome (1). The second cloned gene product designated *mdm2*, was the only copy that was able to cause cellular transformation, thereby characterizing it as an oncogene. Even though double minute chromosomes are not found in human cells, the nomenclature of the Mdm2 protein has been adapted to the human ortholog. For clarity in this review we will distinguish between the use of Mdm2 for mouse and Hdm2 for human versions of the protein.

The Mdm2 protein contains 489 amino acids, while Hdm2 possesses 491 amino acids. The predicted molecular weight of Hdm2 is 56 kDa and migrates on an SDS-PAGE gel around 85–95 kDa. The size discrepancy may be attributed to the fact that Mdm2 has an acidic isoelectric point and is a phosphoprotein. Mdm2 has several domains including: an amino terminus that contains a hydrophobic cleft, a bipartite nuclear localization sequence, a nuclear export sequence, an acidic domain, a Zinc finger, a RING Finger, a Walker A motif and a nucleolar signal sequence (Figure 1). The crystal structures of the RING/Zinc domains and the amino terminus have been solved (2,3). The crystal structure of the acidic domain has yet to be solved

due to disordering of the amino acid composition of this region. The RING finger domain may bind to RNA and nucleotides as well as functioning as an E3 ligase, catalyzing the transfer of ubiquitin or Nedd8 to substrates. The interesting aspect of the ability of Mdm2 to function as an E3 ubiquitin or Nedd8 ligase is that it requires either homodimerization or heterodimerization with its family member Mdmx (2). Mdm2 is a highly regulated protein through many types of post-translational modifications, all of which can influence its enzymatic activity.

2. Hdm2 in Human Cancer

Hdm2 is elevated in high-grade human cancers and can correlate with a poor prognosis (4). A seminal paper in the field showed that Hdm2 was frequently overexpressed in human bone and soft tissue sarcomas and was characterized as an oncogene (5). In addition to increased gene expression, *hdm2* mRNA can be alternatively spliced to create truncated protein products (6). These variants have largely spliced out the amino terminus and some have the acidic domain missing. These separate domains are capable of cellular transformation, suggesting that various domains of the Mdm2 protein may regulate numerous cellular proteins. The fact that multiple domains can contribute to transformation suggests that Mdm2 is a multi-faceted protein.

While the frequency of *mdm2* gene amplification is around 10% in all human cancers, detection in human cancer can range from 30–80% depending on the reagents used and the subjective scoring methods (4,7). The lower frequency of amplification suggests that the *mdm2* gene is induced. An extensive review of tumor types correlated with Mdm2 protein overexpression shows elevation in a number of cancer types including: glioblastoma, breast, and osteosarcoma (4). Later work revealed that growth factors could stimulate *mdm2* gene induction through the activation of transcription factors, NMYC, Ets and AP1 (7–9). Recent work shows that the promoter region of *hdm2* can have a single nucleotide polymorphism (SNP309), which may be regulated by the estrogen receptor or SP2, and can have a prognostic value depending on the type of cancer (9–11). Thus, the *hdm2* gene is regulated by numerous transcription factors, which may relate to the higher levels of Hdm2 protein.

3. Mdm2 protein interactions

3.1 Binding to tumor suppressors

Studies examining the overexpression of Mdm2 (human and mouse) found that it transformed and immortalized rodent cells, and that this event was concluded to be dependent on inactivation of p53 (1,5). Mdm2 was initially determined to inhibit p53-dependent transactivation of a p53 responsive element by binding wild-type p53 (12). The induction of the *mdm2* gene by p53 is central to a DNA damage response and inactivation of p53 (13,14). Mdm2 protein functions as an inhibitor of p53 by binding to p53's transactivation domain. Mdm2 ubiquitinates p53, which leads to nuclear export followed by proteasomal degradation (15). A partial crystal structure was solved for the 109 residue N-terminal domain of Mdm2 (*Xenopus*) bound to a 15 residue transactivation domain peptide of p53 (3). The most important component of the Mdm2-p53 interface is the contribution of the aromatic and hydrophobic amino acids (Phe19, Trp23 and Leu26) on p53, which insert into a symmetrical secondary trough structure formed by Mdm2. Mutation of this triad contributes to a decrease in the ability of p53 to transactivate genes. A biochemical analysis of the Mdm2-p53 interaction has also been performed using an ELISA-based assay. The same triad of amino acids on p53 from the previous study were determined to be necessary for p53 to bind to Mdm2 and critical amino acids on Mdm2 were found to be L66, Y67 and E69 (Table 1) (16). p53 binding is not restricted to the N-terminus of Mdm2 as deletion of this region still allowed for binding to p53. It has been established that p53 will bind to residues 235–300 in the central domain of Mdm2 (Table

1) (17). This data is useful, since the crystal structure utilized only partial 3-dimensional representations of both p53 and Mdm2.

The generation of *mdm2* knockout mice leads to an embryonic lethality around the E5 (18, 19). Viable mice are generated when p53 was also knocked out, suggesting that during early mouse development Mdm2 regulates p53. In addition, Mdm2 was found to be important for maintaining the homeostatic levels of p53 (20). As a result of the loss of the *mdm2* gene, cells in specific tissues became sensitive to irradiation in adult mice. These models largely provide genetic inference that Mdm2 plays a role in regulating p53. The generation of *mdm2* transgenic mice shows a p53-independent role in transforming and promoting cancer development. Different approaches show that either by high copy number or tissue specific induction of *mdm2* transgene, tumor development in p53 null mice was seen (21,22). Work in p53^{-/-} mice has indicated that Mdm2 has additional roles in the regulation of DNA synthesis (22). Another study revealed that a cDNA for an Mdm2 splice variant actually selected for mutations in critical domains of Mdm2, and demonstrated the importance of sequencing inserts from transgenic founder mice for oncogenic proteins (23). Work in primary human cells showed that Hdm2 was able to cause transformation through regulation of other key proteins that govern cellular processes. The combination of adenovirus E1A, Ha-RasV12 and Hdm2 was able to transform normal human cells in the absence of telomerase activation (24). In support of this theory, early work examined the amount of Mdm2 bound to p53 in cell lines and found that a large portion of Mdm2 was free of p53 suggestive of p53-independent functions of Mdm2 (25). Altering the Mdm2 levels either by gene expression or amplification will have a dramatic impact on growth and survival, correlating with the aggressive nature of metastatic cancer.

The p53 family members p63 and p73, share some overlapping functions to act as tumor suppressors to induce cell cycle arrest and apoptosis, but have alternate roles in cell differentiation and embryonic development. All three members have splice variants that are missing the amino or carboxy termini. p73, but not p63, can bind to Mdm2. The amino terminus of p73, which is similar to p53, is required to bind to Mdm2. Deletion of residues 58–89 on Mdm2 also resulted in a loss of p73 binding (Table 1) (26). Mdm2 inhibited p73-mediated transcription of the apoptotic *bax* gene promoter to a similar fashion as p53. Similarly, p73 β transcriptional activity on a p53 response element was inhibited by Mdm2 (27). Both p73 α and β isoforms transcriptionally activate and cause Mdm2 protein expression and are known to bind to the N-terminal residues 1–150 (Table 1) of Mdm2 (28). Mdm2 is also able to conjugate ubiquitin to p73 and lead to its destabilization. This destabilization subverts p73 α and β mediated apoptosis. Thus p73 shares a degree of similarity with p53 with respect to *mdm2* gene upregulation and p73 destabilization, and the p73-Mdm2 autoregulatory feedback loop represents a valid chemotherapeutic target.

Another tumor suppressor linked to the interaction of the p53-Mdm2 autoregulatory loop is p19^{ARF}, which is a product of the *Ink4a* gene. Mouse p19^{ARF} was demonstrated to form a protein-protein interaction with Mdm2 *in vivo* and block the interaction of Mdm2 and p53. Mdm2 deletion mutants indicated binding of p19^{ARF} to Mdm2 resided within residues 155–221 (29). ARF overexpression led to a greater stability of p53 half-life and p53-dependent increase in transcription and cell cycle arrest (30). ARF was demonstrated to interact with Mdm2 and a deletion of the N-terminal 1–62 residues of ARF was found to be necessary for p53 binding and cell cycle arrest. ARF was shown to be capable of eliciting a p53 response with an increase in Mdm2 and p21 levels, with a partial ARF binding site mapped to residues 222–437 on Mdm2 (31). ARF ectopic expression also led to a G1 and G2/M mediated cell cycle arrest. The precise role of how ARF affects the ability of Mdm2 to function as an E3 ubiquitin ligase or be post-translationally modified by other proteins requires additional research.

The retinoblastoma gene product (Rb) is involved in multiple cellular processes such as the DNA damage response, replication and apoptosis. Rb is a primary cell cycle regulator as it interacts with the E2F transcription family members and blocks their transcriptional activity. Upon DNA damage, the Rb-E2F1 complex is disrupted leading to an E2F1 induction of apoptotic genes through p73 activation (32). A protein-protein interaction between Mdm2 and Rb have been mapped to amino acids 254–264 (Table 1) in the acidic domain of Mdm2 (33). Mdm2 interacts with Rb at residues 785–803 which disrupts binding of Rb-E2F1 complexes both *in vitro* and *in vivo* (33,34). Overexpression of Mdm2 in cells blocked Rb-mediated suppression of E2F activity. Another study has found that Mdm2 stabilizes the protein levels of E2F1, which is accomplished through formation of an E2F1-Mdm2 complex and displacement of an E2F1 specific E3 ubiquitin ligase, SCF^{SKP2} (35). A very broad interface between E2F1 and Mdm2 was mapped to residues 1–179 and 299–491 (Table 1) on Mdm2. Rb was shown to be ubiquitinated by Mdm2 as other E3 ubiquitin ligases were not found to modify Rb (36). Since, decreased levels of E2F1 were dependent on Mdm2 and not p53 status and Rb is a negative regulator of E2F1 activity; a specific Mdm2 inhibitor for these interactions would serve a dual effect.

Another protein that is involved in the retinoblastoma pathway is gankyrin, which is an ankyrin repeat oncoprotein that is specifically overexpressed in hepatocellular carcinomas. Gankyrin has been demonstrated to bind to Rb and the S6 ATPase subunit of the 26S proteasome (37). Overexpression of gankyrin decreased p53 levels by proteasomal degradation and was shown to physically interact with Mdm2 on residues 412–437 (Table 1) (37). The likely role for gankyrin is to assist Mdm2 in targeting p53 specifically for degradation by the proteasome and is supported by the physical binding of gankyrin to part of the proteasome complex. Thus, decreasing gankyrin-mediated Mdm2 activity might increase the half-life of p53.

3.2. Mdm2 interaction with Mdmx

Mdmx (also referred to as Mdm4) is a related Mdm2 family member. While their domain architecture is similar, the amino acid composition is not well conserved (38). A mouse model demonstrated that Mdmx, like Mdm2, was embryonically lethal albeit in a later developmental stage. When crossed in a p53-null background the *mdmx*^{-/-} phenotype was rescued suggesting Mdmx operated as an *in vivo* regulator of p53 (39). As opposed to Mdm2, Mdmx has limited ubiquitin ligase activity towards p53 *in vivo* (40). It is not clear if this is due to limitations related to forced overexpression, or if specific conditions are necessary to activate Mdmx, such as recruitment of other proteins or post-translational modifications. An examination of the role of Mdmx in relation to Mdm2 and p53 function has revealed that Mdmx stabilizes both of these proteins (41). Mdm2 and Mdmx heterodimerize through their RING finger domains (42). Mdm2 and Mdmx share the same positions of cysteines and histidines in their RING domain in a C2H2C4 arrangement, which allow for metal ion binding and heterodimerization (43). Mdmx was found to localize to the cytoplasm and require to be shuttled into the nucleus by Mdm2. Mdmx was also shown to block p53-mediated transcriptional activity (44). Mdmx may be destabilized by Mdm2, yet how this occurs is not clear biochemically. Mdmx may be regulated by post-translational modifications in response to DNA damage and this facilitates destabilization (45). An examination of Mdm2 and Mdm4 loss in mouse embryonic fibroblasts null for p53 where a temperature-sensitive p53 mutant was reintroduced has revealed that p53 induced specific apoptotic genes in Mdm2-null cells. Cell cycle arrest was observed in Mdmx null cells, underscoring the fact that it has unique cellular roles (46). How Mdmx interacts with Mdm2 and influences the p53 autoregulatory feedback loop through protein-protein interactions, adds a tier of complexity to how DNA damage is signaled, tolerated, and repaired.

The question remains how Mdm2 can possess its reported auto-ubiquitination activity, as well as targeting Mdmx for degradation (47,48). It is plausible that E1/E2(Ubc5) enzyme complex

transfers ubiquitin to MDM2, and can continue to load ubiquitin onto Mdm2, if not transferred to a substrate. Mdmx and Mdm2 can occur as heterodimers in the cell and may be why both are subject to destabilization. A precise demonstration of an auto-ubiquitination activity or a continued activity of the E2 enzyme loading ubiquitin to the RING domain or another E3 ubiquitin enzyme contribution from a complex like the Cul4 complex needs to be further examined with respect to Mdm2 ubiquitination (49). One report suggests that Mdm2's E3 ligase activity is not required for *in vivo* Mdm2 degradation (50). A clear interrelationship between Mdm2 and Mdmx, poly-versus monoubiquitination activity, nuclear export and p53 regulation needs to be sorted out.

3.3. Mdm2 and transcriptional regulation

Since p53 has a role as a transcription factor, Mdm2 might logically regulate transcriptional proteins, such as co-regulators and repressors. p300 is a coactivator for p53-mediated transcription and can regulate Mdm2 activity. PCAF (p300/CREB-binding protein-associated factor), is a histone acetyltransferase enzyme which has been shown to activate p53 acetylation after DNA damage (51). A logical link between PCAF and Mdm2 required further examination. PCAF binding occurred at amino acids 50–384 of Mdm2 and was found to inhibit p53 acetylation (52). Recently, the biochemical regulation of Hdm2 has been investigated with respect to enzymatic activity of PCAF. PCAF can ubiquitinate Hdm2 and control its stability (53). Post-translational modification of Hdm2 by a novel PCAF ubiquitin ligase activity allows for more active p53 pools to transcribe downstream target genes.

An interaction between Mdm2 and a transcriptional regulatory protein for p53 identified to interact with p300, JMY (junction-mediating and regulatory protein) has been investigated. JMY interacted with Mdm2 in the amino terminus (58–89) and the Ring domain (441–491) and 58–89 (Table 1), and it was found to be targeted for degradation by Mdm2 after DNA damage (54). This illustrates how Mdm2 alters transcriptional control of p53 by degradation of a p300-associated cofactor. Mdm2 has also been described to monoubiquitylate the histone H2B protein *in vivo* dependent on the RING domain (55). This ubiquitination event on H2B appears to be favored within actively transcribed areas of human genes (56). However, this has not been directly associated with Hdm2 E3 ligase activity. Thus, multiple ties to transcriptional regulation and either an enhancement or inhibition of Hdm2 E3 ligase activity.

3.4 Mdm2 and regulation of protein translation

The process of translation of mRNA into protein intimately involves the ribosomal machinery within the cell. An association between Mdm2 and the protein translational machinery was found between the ribosomal protein L5, 5S RNA, Mdm2 and p53 (57). A link to p53 with sensing ribosomal stress was indicated by studies that inhibit ribosomal biogenesis (58,59). The L11 ribosomal protein was the first identified partner of Mdm2 in the regulation of the p53-Mdm2 autoregulatory loop (60,61). A report of a multiprotein complex of Mdm2, L5, L11 and L23 in the absence of the 80S ribosome or polysomes has been reported (62). The L5 protein has been shown to inhibit the ability of Mdm2 to ubiquitinate p53. This led to the induction of p21 through p53 (63). L5 binding to Mdm2 mapped to the central acidic domain (residues 153–294) (64). Amino acids 284–374 of Mdm2 were bound by L11, and L23 binding was determined to bind Mdm2 at residues 150–300 (Table 1) (62). Furthermore, Mdm2 zinc finger mutants have been shown to disrupt the L5/L11 interaction with Mdm2 and have decreased nuclear export demonstrating the importance of these interactions (65).

Nucleophosmin is a nucleolar protein involved in ribosomal biogenesis, that interacts with Hdm2 in the p53 interaction domain residues 1–110 (Table 1) and the C-terminus in the RING domain *in vitro* and *in vivo* (66). UV damage regulates nucleophosmin and inhibits the negative regulatory effect of Hdm2 on p53. Mdm2 was recently shown to interact with nucleostemin,

by binding to the acidic domain of Mdm2 (210–284) (Table 1) (67). The interaction between Mdm2 and nucleostemin leads to regulation of p53 activity. Regulation of p53 appears to be finely regulated through nucleostemin because high levels of the protein will bind and inhibit Mdm2, while low levels will cause an L5/L11 complex to bind and inhibit Mdm2 through nucleolar stress (68).

Proteomic approaches using electrospray ionization tandem mass spectrometry have identified Hdm2-interacting proteins including previously reported Mdm2-interacting proteins, such as p14^{ARF} and the ribosomal proteins L5 and L11 (69). The most novel identification was EF1- α , a translational elongation protein, which colocalized with Hdm2 in the cytoplasm (69). An EF1- α interaction site was mapped to the N-terminal residues 1–58 and within residues 220–325 in Hdm2's central domain (Table 1). A functional link between Hdm2 and an alteration in protein synthesis was not discovered in this study leaving the purpose of an Hdm2-EF1- α interaction subject to further investigation. Since L5 and L11 proteins were also identified in the screen and are intimately involved in protein translation, they could be playing an antagonistic role with EF1- α in sensing and signaling ribosomal stress through interactions with p53 and Mdm2. This unique interaction might also tie in to the ability of Mdm2 to bind RNA and imply a role in translational regulation of specific RNA species (64). This could be a pivotal event in the regulation of metabolism and proliferation of cancer. While these suppositions need to be investigated and integrated into a coherent story it may yet provide another point for therapeutic intervention.

3.5 Mdm2 and DNA repair proteins

The promyelocytic leukemia tumor suppressor, PML, is an abundant nuclear phosphoprotein, which forms a multiprotein complex characterized as nuclear bodies in response to genotoxic stress. PML has been described to have a protective effect on p53 upon DNA damage and subvert the activity of p53 away from Mdm2 (70). This involves a post-DNA damage mechanism whereby PML and Mdm2 are sequestered to the nucleolus through an unknown mechanism (71). PML forms a complex with Mdm2 both *in vitro* and *in vivo* independent of p53 (72). Residues 222–272 within the central acidic domain of Mdm2 interacted with the C-terminal portion of PML (Table 1). PML nuclear bodies contain numerous DNA repair proteins, and recent work show that Mdm2 binds to some components of the DNA repair machinery. Mdm2 was found to bind to Nbs1, which is part of the MRE11/RAD50/Nbs1 (MRN) DNA repair complex partially responsible for repair of DNA double strand breaks (DSBs) (73). Experiments with Mdm2 overexpressed in MEFs show a delay in DSBs over 1 hour, yet it remains unclear if this delay has a biological outcome. An interaction between Hdm2 and Nbs1 was demonstrated to occur at residues 198–228 (74). The kinetics of DNA repair with human epithelial cells needs to be performed to demonstrate that Hdm2 is playing a similar role. An interaction of Hdm2 with DNA polymerase ϵ has been described to stimulate its enzymatic activity (75). When the N-terminal 1–166 residues of Mdm2 are deleted, a loss of polymerase ϵ activity towards DNA substrates occurs (Table 1). In tumorigenesis, an Mdm2-dependent event could be envisioned to delay the repair process leading to faulty DNA replication and translocations or additional mutations to DNA.

3.6 Hypoxia and Mdm2

Hypoxia inducible factor-1 alpha (HIF1 α) is a transcription factor that is stabilized by a series of biochemical events that prevents the von Hippel-Lindau (VHL) tumor suppressor acting as a recruitment factor for an E3 ubiquitin ligase complex. Elevated HIF1 α heterodimerizes with HIF1 β in the nucleus and this complex activates target genes. HIF1- α has been demonstrated to bind Mdm2 and Hdm2, both *in vitro* and *in vivo* (76–78). Furthermore, VHL has been found to inhibit Mdm2-dependent ubiquitination and nuclear export of p53 (79). HIF1 α shields p53 from Mdm2-mediated degradation and blocks p53 nuclear export by Mdm2 (78). *In vivo*

HIF1 α , and HIF2 α interact with Mdm2 under hypoxic conditions and is necessary for induction of target genes (76,77,80). Thus, Mdm2/Hdm2 antagonizes p53 ability to induces anti-angiogenic genes, yet also augments HIF1 α pro-angiogenic genes that are intimately linked to the tumor microenvironment. How Hdm2 plays a dual role in such activities is currently under investigation and future work should reveal the pivotal event necessary to regulate both proteins. Should further work support involvement of Hdm2 in regulating angiogenic factors, this would implicate Hdm2 in the angiogenic switch and thereby provide a target for therapeutic intervention.

4. Post-translational modifications of Mdm2

4.1 Phosphorylation by DNA damage signaling kinases

DNA damage is central in activating the p53 pathway to promote apoptosis. Four major DNA damage-induced kinases that transmit early signals upon DNA damage are: ataxia-telangiectasia mutated (ATM) kinase, ATM and Rad3-related kinase (ATR), the DNA-dependent kinase (DNA-PK) and Abelson murine leukemia viral oncogene homolog1 (c-Abl). Activation of p53 requires that it must be free of inhibitory proteins like Hdm2. In response to genotoxic stress, Mdm2/Hdm2 is phosphorylated at numerous sites and other post-translational modifications have been identified (Figure 1). In response to DNA damage ATM activates many substrates and is capable of phosphorylating S395 of MDM2 both *in vitro* and *in vivo*, and mutation of this site led to a decrease in p53 cytoplasmic movement (81). c-Abl is a non-receptor tyrosine kinase that is activated upon DNA damage. It was observed that c-Abl could enhance p53's ability to drive p53-responsive promoters and that p53 protein levels were stabilized with the addition of c-Abl (82). In response to genotoxic stress, c-Abl phosphorylates Mdm2 on Y394, both *in vitro* and *in vivo* (Figure 1). (83). The phosphorylation of the Y394 site also turns off the ubiquitin ligase activity of Mdm2. A recent examination of Mdm2 phosphorylation by Abl revealed another tyrosine phosphorylation site at Y276 (Figure 1) (84). Y276 was phosphorylated weakly by c-Abl *in vivo* upon DNA damage. The biological relevance of the Y276 phosphorylation appears to enhance binding of Mdm2 to ARF and consequently prevents p53 from degradation (84). The DNA-dependent protein kinase (DNA-PK) was demonstrated to phosphorylate Mdm2 *in vitro*. This occurred at S17 adjacent to the p53 binding domain and was found to prevent p53-MDM2 binding *in vitro* (Figure 1) (85). Additional work using NMR demonstrated that S17 was contained within a flexible lid and when phosphorylated, caused the lid to bind to the pocket domain and block the binding of p53 (86). Regulation of MDM2 by phosphorylation after DNA damage is crucial to the stability and activity of p53. Exploiting known sites of phosphorylation that render MDM2 less active to target p53 for proteasomal degradation remains an attractive therapeutic route for drug discovery, yet may have limitations on specificity.

4.2 Phosphorylation by receptor-activated kinases

A group of kinases not necessarily directly involved in perpetuating DNA damage signals can phosphorylate and modulate some function of Mdm2 in the cell. Casein kinase 2 (CK2) is a serine/threonine kinase that regulates multiple proteins involved in a variety of cellular functions, including cell cycle control. CK2 was demonstrated to phosphorylate Mdm2 *in vitro* (87). Cellular stress activates the MAPK pathway, specifically p38 MAPK. A downstream substrate of p38 MAPK is MAPKAP kinase 2 (MK2). MK2 phosphorylates Mdm2 on S157 and S166 both *in vitro* and *in vivo* (Figure 1) (88). Deletion of MK2 led to a greater stabilization of p53, while mutation of Mdm2 at two sites to mimic phosphorylation resulted in enhanced degradation of p53. Incubation of CK1 δ with Mdm2 causes phosphorylation within the central domain of Mdm2 enhanced binding to p53. An Mdm2 mutant for S246, S253, S256, S260 and S262, where residues were changed to aspartic acid to mimic phosphorylation, promoted

greater binding between Mdm2 and p53 (17). This greater binding increased the destabilization of p53.

An intercellular phosphatidylinositol 3 kinase (PI3K) link to the Mdm2 pathway regulates HIF-1 α activity and p53. The PI3K/Akt/Mdm2 pathway was originally discovered by examining the signaling pathways of IGF1, insulin, and EGF and determined how Mdm2 nuclear entry regulates p53 activity (89,90). Mdm2 is a direct substrate of Akt and the phosphorylation sites fall within a bipartite nuclear localization sequence. A second signaling pathway emanating from cell surface receptor signaling activates the MAPK/p90Rsk pathway, which signals Mdm2 to export itself and p53 out of the nucleus (91). Thus, Mdm2 moving in and out of the nucleus leads to the destabilization of p53 and the time required for the activation of the signaling pathways to sign such shuttling corresponds to the half-life of Mdm2 and p53. Since a tumor will try and engage pro-survival and growth advantage, it is not uncommon to find high levels of growth factor secretion or gain in function of receptors.

4.3 Phosphatase regulation of Mdm2

Mdm2 dephosphorylation has been recently linked to a stress-activated phosphatase that may also act as an oncogene. Wip1 (or PPM1D) is a serine-threonine phosphatase that was identified to activate p53 transcription after DNA damage (92). Wip1 endogenously interacted with Mdm2 and dephosphorylated, S395, the ATM phosphorylation site. Tumor cells may select for alterations in the pathway that governs survival or proliferation, such as the tumor suppressor, PTEN. PTEN is a dual specificity phosphatase that dephosphorylates phosphatidyl inositol 3,4,5 phosphate that activates Akt (93). Thus, PTEN alters Akt activation, which prevents Mdm2 nuclear localization. Cytoplasmic Mdm2 is unstable and is degraded. The lack of nuclear Mdm2, frees p53 transcriptional constraints, which thereby induces cell cycle arrest genes (94). While the signals involved are still under investigation, it appears that serine 46 phosphorylation of p53, which is catalyzed by HIPK2 is important for at least targeting PTEN gene for expression over Mdm2 gene expression (95,96). The elevated expression of PTEN would prevent the present Mdm2 protein from gaining nuclear entry, but PTEN also undergoes nuclear localization and binds to p53. The p53-PTEN complex requires acetylation of p53 by p300 (97). Thus, the activation of pro-death p53 will activate a futile cell death cycle that involves PTEN, p300 and likely other components, in addition to not transcriptionally targeting *mdm2* expression and prevention of MDM2 access to p53.

5. Targeting MDM2 therapeutically

The development of molecules that prevent the p53-Mdm2 interaction has garnered a significant amount of interest. Recent data suggest that such molecules may also be useful in preventing other Mdm2 interactions, and thus may even have efficacy in tumors lacking p53. The first inhibition of the p53-Mdm2 protein-protein interaction utilized an antibody through cellular microinjection that recognized the p53 binding motif in Mdm2 and increased the transcriptional activities of p53 (98). The therapeutic value of such an antibody is less attractive, as large macromolecules are incapable of penetrating the cell membrane without conjugation that increases lipid permeability. Development of peptidomimetic inhibitors that successfully imitated the p53 alpha-helical region binding to Mdm2 has been performed, yet their structure and size render their cellular permeability rather low (99).

Naturally-derived molecules have been found to inhibit the p53-Mdm2 interaction (Table 2). Chlorofusin, derived from fermentations of *Microdochium caespitosum*, was shown to bind the amino terminus of Mdm2 and prevent the formation of the p53-Mdm2 complex (100). A chalcone derivative, demonstrated by ELISA to interfere with p53-Mdm2 complex formation, bound an unexpected area of Mdm2, at residues Phe55 and Tyr56, which reside downstream of the p53 binding pocket. Interestingly, two of the chalcone derivatives also influenced p53

in such a way that rendered it incapable of binding DNA (101). These natural product molecules were modified in hopes of enhancing their efficacy, but were met with limited success.

The first representative of SMIs, Syc-7, was shown to disrupt the p53-Mdm2 interaction and affect the viability of several tumor cell lines, all of which expressed wild-type p53 (102). However, the ability of these groups to remain fixed in space was seen as negative for Syc-7, as linkages to the benzyl functional groups were rather flexible. A 3-dimensional search program of the NCI chemical database revealed a sulfonamide-based compound that was capable of hindering p53-Mdm2 complex formation. This compound was also shown to modestly increase transcriptional p53 activity in cells overexpressing Mdm2 (103). This may have been due to a low cell permeability, which could be redesigned by using different functional groups to increase solubility.

Nutlins were the first small molecules developed at Roche that could selectively inhibit the p53-Mdm2 complex formation (104). Initial high-throughput screens identified several structures and eventually Nutlin-3a was developed. NMR spectroscopy demonstrated that Nutlin-3 (Table 2) could prevent the formation of the p53-Mdm2 (105). Recent findings have implicated this compound in several anti-cancer pathways. Nutlin-3a was found to decrease the rate of tumor growth in nude mice xenografts (104). Although some have suggested that Nutlin-3a has no efficacy on tumors expressing mutant p53, it has recently been shown to suppress angiogenesis independent of p53 (76). Furthermore, it was recently demonstrated that Nutlin-3a can upregulate apoptotic genes independent of p53. It was shown to do so by blocking the p73-Mdm2 interaction (106). This compound warrants further investigation, as it has been the first Mdm2 inhibitor to show substantial effects independent of wild-type p53.

In 2005 a group from Johnson & Johnson Pharmaceuticals reported a co-crystal structure of a benzodiazepinedione Mdm2 antagonist (Table 2). This molecule successfully mimicked the p53 alpha-helix that projects into the amino-terminus of Mdm2 (107). These potent compounds, TDP521252 and TDP665759, were shown to decrease proliferation in JAR cells with wild-type p53, but were shown to have little effect on a breast cancer line MDA-MB-231 with mutant p53. These compounds modestly induced p53 target genes, but were shown to be most potent when used in conjunction with the chemosensitizing agent, doxorubicin (108). This brought about an interesting question with regard to Mdm2 inhibitors. Even though these compounds were protecting p53 from Mdm2 mediated degradation, there appeared to be a requirement for a stimulus to activate p53. Some have suggested that the ability of these compounds to induce apoptosis in a p53-dependent manner suggests that phosphorylation of key serines on p53 may not be entirely necessary for apoptosis (109).

Structure-based methods examining the p53-Mdm2 co-crystal led to spiro-oxindole compounds (Table 2). MI-219 was shown to be selectively toxic to tumor cells (110). Analysis showed that MI-219 caused the upregulation of p21 and Mdm2, but only caused transcription-independent accumulation of p53. MI-219 was shown to cause apoptosis in cells with wild-type p53 to a greater extent than Nutlin-3, yet did not have good efficacy in mutant p53 lines.

An isoindolinone compound was developed and shown to inhibit the formation of the p53-Mdm2 complex (111). This compound was shown to disrupt binding in Mdm2 overexpressing SJSA cells that contain wild-type p53, as well as, cause induction of p21 and Mdm2 (112). Although this drug shows promise, due to its induction of apoptotic proteins, further investigations are necessary to show the gross biological effect this compound has on tumors. Through *in silico* modeling and NMR, several iso-1-quinolinones (Table 2) were designed that successfully interfered with Mdm2 binding to p53 (113). One specific compound (NXN-11) induced Noxa, an apoptotic effector protein induced by p53 and p73, to a greater extent than

Nutlin-3, and overall the compounds were effective in apoptotic induction in cells wild-type for p53.

An inhibitor of Mdm2 ubiquitin ligase activity was discovered using high-throughput screening. The HLI98 compounds (Table 2) showed some specificity towards Mdm2 *in vitro*, however, at higher concentrations the drug was also shown to interact with other RING and HECT domains of E3 ubiquitin ligases. These drugs were shown to stabilize p53 and Mdm2 in cells and induce the p53 target genes p21 and PUMA. According to the authors, the drugs would be of little therapeutic use due to their lack of solubility (114). A recent report has revealed critical details on how p53 translation is regulated by Mdm2. The RING domain of Mdm2 was found to bind to the p53 mRNA region where Mdm2 is known to bind. This causes a reduction in Mdm2 ubiquitination activity which leads to enhanced p53 mRNA translation (115). This may represent a point of argument to why an inhibitor specific for the RING domain of Mdm2 would not be ideal in cells with wild-type p53.

An alternative approach to preventing the p53-Mdm2 protein-protein interaction would be to develop a compound that binds p53 in such a way that prevents its association with Mdm2. A compound termed, RITA for (re)activation of p53 and induction of tumor cell apoptosis) was discovered through a cell-based assay that measured proliferation in response to various compounds. RITA promoted the induction of several p53 target genes in cell lines with wild-type p53 status (116). NMR studies using a portion of the Mdm2 protein have demonstrated that RITA can not block the p53-Mdm2 interaction (117). However, a two-site ELISA and pulldown assay have demonstrated that the p53-Mdm2 interaction is blocked by RITA (116). Collectively, small molecules that target Mdm2 are an evolving field and new series of compounds will surely continue to emerge.

6. Conclusion

The latter drug design approaches have provided more insight into the p53-Hdm2 interaction. However, the former mentioned compounds (those which directly bind Mdm2 to prevent the p53-Mdm2 complex) have proven most useful therapeutically. In the Hdm2 field, the vast majority of work has focused on genotoxic stress as it related to Hdm2 regulation of p53. New therapeutic drug design could capitalize on the novel p53-independent interactions of Hdm2. For instance, p73 may take over pro-apoptotic functions in the cell if p53 has been inactivated. Targeting this interaction might enhance cancer chemotherapy for individuals with non-functional p53. This has certainly been the case with the Nutlin-3a compound, where it decreased cell proliferation in the background of p53-null or mutated cell lines to examine the disruption of the p73-Hdm2 interaction (106). Another great avenue to pursue for drug discovery is the link between DNA repair and Hdm2. Emerging evidence suggests that Mdm2 is located in PML bodies at sites of DNA damage with little understanding of post-translational modifications or the regulation of repair components. Targeting both Hdm2 and proteins associated with DNA repair could allow for more efficient apoptosis and death of cancer cells.

7. Expert opinion

The Hdm2/Mdm2 field is slowly moving into a more biochemical-based analysis of Hdm2 to attempt to further understand the complexity of this protein's activity and regulation. The complexity of Hdm2 post-translational modifications has limited the investigation into various aspects of protein-protein interactions and compartmentalization. The importance of understanding the localization of Mdm2 within the cell, relative to post-translational modifications and the protein complexes formed with Mdm2 in the absence of forced overexpression, may provide a greater understanding of selective associations. Hdm2/Mdm2 is more complex than initially thought with the discovery of post-translational modifications,

alternative splice variants and numerous transcription factors responsible for elevating *mdm2* gene expression. Structurally, compounds have been discovered using various approaches targeting the amino or carboxy terminus. The investigation of Hdm2/Mdm2 protein has led to the identification of several proteins involved in DNA repair and transcription pathways that bind to and are modified by Hdm2/Mdm2. Post-translational modifications to Mdm2 could drastically change the conformation of Mdm2 leading to neutralization or the inability of SMIs to bind. A much broader scope must be taken when approaching the development of SMIs to alter Hdm2's activity. For example, if DNA-PK is activated in the cell in response to DNA double strand breaks, Mdm2 will be phosphorylated on serine 17. This event will cause the amino terminus to fold back into the hydrophobic cleft, thereby preventing the association with proteins that bind this domain. This would also hold true for small molecules that were designed to bind this domain, so genotoxic stress that activates kinases to phosphorylate the amino terminus may have the same effect as a small molecule inhibitor. Thus, investigators designing small molecules *in silico* or with high-throughput screening should consider how specific post-translational modifications and even combinations of these modifications could cause specific conformational changes in the various domains of Hdm2.

The discovery of several small molecules that may target Hdm2/Mdm2 is very encouraging. While still in development, it seems that the majority of compound screenings examine only the information generated from crystal structures. This is a very important aspect of drug design, yet crystal structures, solution studies or *in silico* predictions with post-translational modifications may provide more realistic modeling for drug discovery. Selecting Hdm2 inhibitors that target other domains outside of the p53-binding region may be the more beneficial approach to neutralize its ability to bind and regulate target proteins. One domain that seems to play the biggest role in regulating ubiquitination of substrates is the acidic domain. It seems that many proteins are able to bind this domain and it is required for the conjugation of ubiquitin. While the crystal structure of this domain is not available due to the disordered structure of the amino acids, it is not limited to other types of screening. It may become important to incorporate the phosphorylation of the acidic domain into the screening as well as surrounding domains to deduce the best candidate to alter Hdm2's activity. This may become important as the field begins to examine other facets of Mdm2 role in regulating DNA repair or translational machinery. Identifying small molecules that selectively target the acidic domain in conjunction with the inhibitors that target the amino and carboxy terminus would most likely be the most effective for neutralizing Mdm2 activity and having a stronger efficacy for cancer treatment.

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Figure 1. Post-translational modifications on Mdm2

Numbered tick marks per every 100 amino acids are depicted for residues 1–491 of Mdm2. Mdm2 domains are represented as the hydrophobic cleft (p53 binding) colored in yellow, nuclear localization sequence colored in blue, nuclear export sequence (NES) colored in aqua, the acidic domain in orange, the zinc finger colored in brown and the RING domain colored in red. Phosphorylation sites are depicted with green circles for phosphoserine or magenta circles for phosphotyrosine. Specific numbered phosphorylation sites are discussed and referenced within the text. This figure only depicts a partial representation of post-translational modifications that can occur to Hdm2/Mdm2.

Table 1

A survey of MDM2 protein-protein interactions

MDM2-binding partners	Interaction residues	Pathway	References
p53	L66, Y67, E69 235–500	Tumor suppressor	(16) (17)
p73	58–89 1–150	Tumor suppressor	(26) (28)
p19 ^{ARF}	155–221 222–437	Tumor suppressor	(29) (31)
Rb	254–264	Tumor suppressor	(33)
gankyrin	412–437	Tumor suppressor	(37)
MDMX	RING domain	Tumor suppressor	(41)
E2F1	1–179, 299–491	Transcription	(36)
PCAF	50–384	Transcription	(51)
JMY	58–89, 441–491	Transcription	(54)
Histone H2B	?	Transcription	(55)
L5	153–294	Translation	(63)
L11	284–374	Translation	(61)
L23	150–300	Translation	(61)
nucleophosmin	1–110, 276–491	Translation	(66)
nucleostemin	210–284	Translation	(68)
EF1-a	1–58, 220–325	Translation	(69)
PML	227–272	DNA repair	(72)
Nbs1	198–228	DNA repair	(74)
DNA polymerase ϵ	1–166	DNA repair	(75)
HIF1- α	?	Hypoxia	(78)

Table 2

Small molecule inhibitors of MDM2

Drug	Type	Predicted MDM2 binding site	Ability to antagonize other MDM2 interactions	References
Chalcone	Natural Derivative	Phe55, Tyr56	Not determined	(101)
Chlorofusin	Natural Derivative	Amino terminus	Not determined	(100,118)
Hexylitaconic Acid	Natural Derivative	Unknown	Not determined	(119)
Syc-7	Synthetic small molecule	P53 binding pocket	Not determined	(102)
Sulfonamide	Synthetic small molecule	P53 binding pocket	Not determined	(103)
Nutlins	Synthetic small molecule	P53 binding pocket	Yes	(76,104–106)
Benzodiazepinediones	Synthetic small molecule	P53 binding pocket	Not determined	(108)
Spiro-oxindoles	Synthetic small molecule	P53 binding pocket	Not determined	(110,120,121)
Isoindolinones	Synthetic small molecule	P53 binding pocket	Not determined	(111)
Isoquinolin-1-one	Synthetic small molecule	P53 binding pocket	Not determined	(122)