

## Extra View

# The pre-clinical development of MDM2 inhibitors in chronic lymphocytic leukemia uncovers a central role for p53 status in sensitivity to MDM2 inhibitor-mediated apoptosis

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**Key words:** CLL, p53, MDM2 inhibitors, molecular response predictors

Inhibitors of the MDM2-p53 interaction are actively being developed as anti-cancer agents. Drug-induced interference with the MDM2 E3 ligase function or with MDM2 protein-protein interactions abrogates tonic suppression and destruction of the p53 protein; consequently, p53 steady state levels rise resulting in the induction of p53-dependent anti-proliferative and pro-apoptotic genes. Some cancerous cells harboring wild type *p53* respond to MDM2 inhibitor-induced elevated p53 protein levels with apoptotic cell death while non-malignant cells, for poorly understood reasons, appear relatively resistant. Deciphering the mechanisms of resistance or susceptibility to MDM2 inhibitor-induced cancer cell death is of significant importance for the clinical development and applications of MDM2 inhibitory compounds and serves to illuminate aspects of MDM2 and p53 biology. Using data from *ex vivo* MDM2 inhibitor treatment of a large cohort of molecularly highly characterized CLL cases, we were able to demonstrate the central role of p53 status as a determinant of resistance in this common leukemia. In the context of these experimental findings, we summarize pertinent knowledge of the biology of p53, MDM2, p53 target genes and MDM2 binding proteins. Finally, using data from a large SNP-array-based high-density genomic profiling study in CLL, we summarize the genomic copy number and allele status for important p53 effector genes as well as for MDM2 binding/target proteins, thus demonstrating the power of high resolution genomic analysis in support of targeted drug development.

## Selected Aspects of p53 Biology and Pathobiology

The p53 tumor suppressor protein is a critical regulator of cell cycle progression, apoptosis and senescence. Various cellular stress signals converge on p53 resulting in p53-mediated transcriptional activation

or repression of target genes.<sup>1</sup> p53 also possesses transcription-independent activities resulting in apoptosis, for which the mechanisms and degree of relative importance are less well understood.<sup>2-4</sup> In addition to p53's established role as a tumor suppressor, emerging data suggests it also has cellular metabolic activities involving glucose metabolism, autophagy and antioxidant properties with the overall goal of promoting survival.<sup>5,6</sup> p53 activity is tightly controlled through changes in steady-state levels and post-translational modifications. In unstressed cells, p53 protein levels are tonically suppressed by the E3 ligase activity of MDM2. Inhibition of the MDM2 E3 ligase function or interference with the MDM2-p53 protein-protein interaction results in elevated steady state p53 levels and in some cancer cells promotes apoptosis. Consequently, the MDM2-p53 interaction has been identified as a target for drug development in cancer. Given the strict dependence of MDM2 inhibitor-induced cell death on functional p53 as recently demonstrated, it is rational to assume that preexisting or acquired mutations in p53, or dysfunction of p53 and downstream p53 regulatory network components pose limits to MDM2 inhibitor efficacy in the clinical setting.<sup>7</sup>

The *p53* gene is located on the short arm of chromosome 17 at position 17p13.1 and mono allelic deletions of *p53* occur in human cancers at various frequencies. Furthermore, missense mutations in exons 5-8 of *p53* are common in sporadic human cancers albeit with substantially different frequencies depending on tumor type, stage and etiology.<sup>8</sup> Various other somatic mutations in *p53* including nonsense mutations and absent expression due to unidentified epigenetic molecular mechanisms have also been described. Germline mutations of *p53* are associated with Li-Fraumeni and Li-Fraumeni-like syndrome and predispose individuals to the development of multiple tumor types at an early age.<sup>9</sup>

In addition to p53 dysfunction intrinsic to *p53* gene defects, human cancers display p53 deregulation as a consequence of (i) abnormalities involving activation pathways of p53 and (ii) mutations/aberrations of downstream p53 effectors and upregulation of the expression of MDM2.<sup>2</sup>

## Incidence and Anatomy of p53 Mutations in Human Cancers

Somatic mutations of *p53* occur in over 50% of human cancers. Epithelial cancers, like ovarian, colorectal, esophageal, head and

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Submitted: 02/15/08; Accepted: 02/19/08

Previously published online as a *Cell Cycle* E-publication:  
<http://www.landesbioscience.com/journals/cc/article/5754>

neck, lung and skin cancers have the highest mutation rates, ranging anywhere from 35–50% (based on data from IARC TP53 database version R12, November 2007). Other cancer types, including leukemias and lymphomas, cancer of the cervix, bone or prostate and sarcomas have p53 mutation rates of less than 20%.<sup>10</sup>

p53 tumor-associated mutations are predominantly point mutations, resulting in single amino acid substitutions, thus commonly preserving the ability of p53 to express the mutant protein. Missense mutations account for ~75% of p53 mutations, with frameshift mutations, nonsense mutations, silent mutations and deletions occurring less frequently. The bulk of p53 mutations cluster within the DNA binding domain in exons 5 to 8 leading to disruption of p53's critical transcriptional activities.<sup>11</sup> There appear to be clear "hotspots" in particular tumors, whereby certain p53 codons show an unexpectedly high mutation frequency.<sup>10</sup> Mutations can also occur, albeit less frequently, in the amino-terminal transactivation and carboxy-terminal regions, that can impact degradation of p53, oligomerization and trafficking of p53 into and out of the nucleus.<sup>12,13</sup>

In cancer cells, p53 mutations can be associated with loss of heterozygosity (LOH) at 17p, resulting in cells with only one p53 (mutant) allele. Mono-allelic p53 states with absent p53 expression from the retained allele have been identified as well, resulting in cells with absent p53 protein.<sup>7,14</sup> In cells with two p53 alleles and heterozygous p53 mutations, mutant p53 can act as dominant negative inhibitors of the remaining wild-type protein.<sup>15</sup> In addition, p53 mutants have been described that interfere with ATM or p63/p73 function.<sup>16-18</sup> Importantly, mutations within the p53 gene do not necessarily equate to complete loss of p53 activity implying that the functional outcome from individual mutations may have a unique impact on the p53 regulatory network.<sup>19</sup> Additional investigations into the significance of partial loss of function or gain of function p53 mutants are important to understanding the biology of individual mutations.

### Clinical Significance of p53 Mutations

The clinical course of cancer patients with and without p53 mutations differs in chemo therapy response rates, time to disease relapse and survival. The association between p53 mutational status and prognosis appears to be variable across the spectrum of affected tumor types. In hematologic malignancies, p53 is infrequently mutated at the time of diagnosis, although studies have shown an increase in p53 mutational prevalence with progression of disease.<sup>20-23</sup> Studies involving patients with chronic lymphocytic leukemia, aggressive B-cell lymphomas, myelodysplastic syndrome and acute myeloid leukemia have suggested an association between p53 mutations, resistance to therapy and shortened survival.<sup>24</sup> In chronic myeloid leukemia, T-cell acute lymphoblastic leukemia, and multiple myeloma, p53 mutations have been associated with disease progression and relapse.<sup>21,25,26</sup>

In solid tumors, the potential link between p53 mutations and prognosis appears to be more complex. Much of the difficulty in interpreting published prognostic studies stems from the limited study power due to small numbers of included patients with p53 mutations, differences in disease management and the varying methods by which p53 mutational status was determined (many of these studies relied upon p53 immunohistochemistry; a method that does not comprehensively interrogate p53 mutational status and functional activity).<sup>27</sup>

### p53 Activation and Destruction Pathways

The p53 network, depending on cellular context, can be triggered by a number of different pathways facilitating either repair or apoptosis of the damaged cell.<sup>1</sup> Several kinases serve as crucial DNA damage sensors signal in part through phosphorylation of the amino-terminal domain of p53, which prevents the binding of MDM2.<sup>1,28</sup> Moreover, increased expression of the p14<sup>ARF</sup> protein, which binds directly to MDM2 and inhibits its p53-degradation activity, has been observed in various tumors as a consequence of oncogene activation, and it has been proposed that ARF is responsible in part for p53's tumor suppressor activity.

p53 destruction occurs primarily through interactions with MDM2. In addition to MDM2, there have been several other E3 ligases identified that are thought to modulate p53 levels.<sup>29</sup>

### Covalent Modifications of p53

In addition to gene mutations, p53 is subject to a number of post-translational modifications that can up/downregulate p53 function; these include phosphorylation, acetylation, sumoylation and glycosylation and little is known about how these affect the p53 network. For example, phosphorylation of p53 on residue serine 46 is needed for p53-induced expression of certain pro-apoptotic genes, but is unnecessary for activation of cell cycle arrest mediators. Therefore, it has been proposed that phosphorylation of p53 at certain residues may be important in modulating the choice of response to p53 activation.<sup>30</sup> Acetylation of lysine residues at the carboxy terminus of p53 has been shown to occur, but there have been conflicting reports about the importance of these changes in affecting p53 cellular localization and transcriptional activation.<sup>31</sup>

### Mutations of Important Downstream Effectors of p53

The complexity of the p53 network is partly reflected in the many downstream effectors believed to be altered by p53. Over 4,000 putative p53 DNA binding sites have been identified via bioinformatics and microarray approaches, although it remains unknown how many of these sites actually are physiologically important.<sup>32</sup> In the context of cancer biology and cancer therapies, PUMA deficiency inhibits p53-induced apoptosis in various experimental systems, thus identifying this gene as an important p53 effector.<sup>33-36</sup> For the various other p53 targets, it appears more plausible that groups of target genes are integrally involved in executing specific functions, and the group of genes can vary based on tissue type, tissue insult and desired effect. Pertinent to the clinical setting, chronic changes in the p53 network that will occur with the ongoing administration of MDM2 inhibitors may thus gain importance and focused study of such changes warrants incorporation into clinical trial designs.

Additional mechanisms of resistance to MDM2-inhibitors in cancer cells with wt p53 is the deletion, mutation or epigenetic silencing of genes important for p53-mediated apoptosis. In the setting of work on MDM2 inhibitors in CLL, we analyzed the gene copy status of a selected group of p53 effectors and MDM2 binding proteins and have summarized the data in table 1. One important conclusion from this unique dataset is that most of these genes are unaltered in CLL. None of the genes are ever bi-allelically deleted, and in some instances they are present in three copies due to trisomies (usually trisomy 12).

**Table 1 Display of genomic copy number of selected genes with importance to p53 and MDM2 biology in CLL**

Gene	Chromosome	Physical position (megabases)	Range of informative SNPs	Gene copy number state in CLL based on SNP-arrays		
PUMA	19	52.415–52.427	rs8101091 (52.292)–rs2037735 (52.704)	2/178 (3N)	176/178 (2N)	
NOXA	18	55.718–55.721	rs952785 (55.624)–rs10515981 (55.744)	5/178 (3N)	173/178 (2N)	
BAX	19	54.150–54.157	rs4802507 (53.996)–rs10500301 (54.6)	2/178 (3N)	176/178 (2N)	
PIDD	11	0.79–0.794	rs217238 (1.947)	1/178 (3N)	177/178 (2N)	
GADD45A	1	67.863–67.866	rs4655769 (67.847)–rs2820473 (68.084)		178/178 (2N)	
14-3-3 $\sigma$	1	26.874–26.875	rs595725 (28.86)		177/178 (2N)	1/178 (1N)
REPRIMO	2	154.159–154.160	rs10497123 (154.122)–rs1369256 (154.196)		178/178 (2N)	
B99	22	45.013–45.050	rs136012 (44.473)–rs4823554 (45.084)		178/178 (2N)	
MDM2	12	67.488–67.520	rs10492299 (67.202)–rs3730504 (67.492)	35/178 (3N)	143/178 (2N)	
NANOG	12	7.833–7.840	rs7969455 (7.757)–rs2110067 (8.16)	1/178 (5N)	143/178 (2N)	
				34/178 (3N)		
MDMX	1	201.217–201.251	rs3911890 (201.208)–rs16776 (201.249)		178/178 (2N)	
TIGAR	12	4.3–4.34	rs3217824 (4.26)–rs7309402 (4.33)	1/178 (5N)	143/178 (2N)	
				34/178 (3N)		
DRAM	12	100.774–100.820	rs10492087 (100.748)–rs10507136 (100.854)	35/178 (3N)	142/178 (2N)	1/178 (1N)
NPM	5	170.747–170.770	rs10516082 (170.738)–rs1382902 (171.017)	1/178 (3N)	177/178 (2N)	
NUMB	14	72.812–72.995	rs1708809 (72.627)–rs10483861 (73.269)		169/178 (2N)	9/178 (1N)
DR5	8	23.105–23.139	rs10503721 (22.802)–rs7834641 (23.176)		175/178 (2N)	3/178 (1N)
SCOTIN	3	48.484–48.516	rs2034330 (48.0)–rs3905330 (48.510)		176/178 (2N)	2/178 (1N)
APAF1	12	97.542–97.632	rs1468832 (97.321)–rs1477237 (97.698)	35/178 (3N)	142/178 (2N)	1/178 (1N)
p53AIP1	11	128.310–128.318	rs10488706 (128.218)–rs2155177 (128.449)		177/178 (2N)	1/178 (1N)
PERP	6	138.454–138.470	rs9321637 (138.247)–rs2235561 (138.482)		174/178 (2N)	4/178 (1N)
CD95/FAS	10	90.740–90.766	rs951647 (90.657)–rs10509572 (90.784)		176/178 (2N)	2/178 (1N)
CDKN1A (p21 <sup>WAF1</sup> )	6	36.754–36.763	rs2007741 (36.531)–rs236374 (36.84)		176/178 (2N)	2/178 (1N)
BTG2	1	200.006–200.010	rs10494836 (200.007)–rs2185781 (200.195)		178/178 (2N)	
PCAF	3	20.057–20.171	rs1051497 (20.008)–rs10510499 (20.170)	2/178 (3N)	176/178 (2N)	
E2F1	20	31.727–31.737	rs10485498 (31.531)–rs10485499 (31.959)		178/178 (2N)	
p73	1	3.592–3.673	rs2072493 (3.596)–rs3935559 (3.737)		178/178 (2N)	

Genomic profiling of 178 CLL cases compared with paired buccal DNA was performed using 50K SNP-arrays as described.<sup>7</sup> DChipSNP-based heatmap displays were analyzed for SNP positions flanking genes of interest and copy numbers recorded. Tabulated are from left to right, gene name, chromosome on which the gene resides, physical span of the gene on the respective chromosome, informative SNP positions immediately flanking the gene of interest and location of those SNPs and gene copy state.

## MDM2 Function and Biology

**MDM2 and p53.** MDM2 (mouse double minute 2), and its human homologue human double-minute 2 oncoprotein (HDM2) (Ch 12q13-14), have been identified as potentially the most critical regulators of p53.<sup>37-39</sup> The critical balance of these two proteins is reflected by the fact that a knockout of the *MDM2* gene is embryonically lethal in mice, but that the phenotype can be rescued by concurrent deletion of *p53*.<sup>40,41</sup>

The HDM2 protein is comprised of several conserved domains, including an N-terminal protein binding domain, nuclear import and export sequences, an acidic domain, a zinc-finger region, and a RING finger region containing the E3-ligase activity.<sup>42,43</sup> The crystal structure of the amino-terminus of MDM2 in complex with a short peptide from the N-terminus of p53 revealed the key structural interactions between these two proteins.<sup>44</sup> Amino acids Phe<sub>10</sub>, Trp<sub>23</sub> and Leu<sub>26</sub> form the core element of p53 that interacts with the hydrophobic binding pocket of MDM2.<sup>42,45</sup>

Numerous studies have established a delicately balanced MDM2-p53 autoregulatory system where MDM2 and p53 closely regulate the activity of each other in response to cellular stressors. In fact, binding of MDM2 to p53 affects the transcriptional activity, stability and subcellular localization of p53.<sup>46</sup> After binding to p53, MDM2 shuttles the heterodimeric complex to the cytoplasm for ubiquitin-mediated proteosomal degradation.<sup>47,48</sup> In addition, MDM2 binding to p53 sterically blocks the transactivation domain of p53.<sup>39,42</sup>

MDM2 is prevented from interacting with p53 through two principle and distinct mechanisms. First, p53 is phosphorylated by a number of serine/threonine kinases within its N-terminus, thus preventing intercalation of key residues between p53 and MDM2.<sup>49,50</sup> Alternatively, p14<sup>ARF</sup> binds MDM2 and prevents interactions with p53.<sup>51,52</sup>

While MDM2 principally functions to repress the activity of p53, the transcription of *MDM2* is partially activated by p53.<sup>53</sup> Thus in response to environmental cues, p53 is activated and stabilized, and the resultant accumulation allows it to bind to the P2 promoter

of *MDM2*, enhancing the expression of *MDM2*.<sup>53,54</sup> The accumulation of *MDM2* subsequently suppresses p53, completing the negative regulatory loop.

### Protein Variables in MDM2 Function with Theoretical Importance to MDM2 Inhibitor Mechanism of Action and Clinical Development

**MDMX.** MDMX (Ch 1q32) was identified through a screen of p53 binding proteins with a strong sequence homology to *MDM2*.<sup>55,56</sup> Many of the N-terminal amino acids in *MDM2* responsible for p53 binding are conserved in *MDMX* and the C-terminal Zinc finger and RING finger motifs are also identified in *MDMX*, suggesting a similar structural makeup.<sup>56,57</sup> It has been demonstrated that *MDMX* is capable of binding to the transactivation domain within p53, thus inhibiting p53 transactivation activity.<sup>55,58,59</sup> Importantly however, *MDMX* is incapable of inducing the ubiquitination of p53 and unlike *MDM2*, transcription of *MDMX* is not induced by p53, due to a lack of p53 responsive elements within its promoter region.<sup>59</sup> Despite their similar sequences, *MDM2* and *MDMX* appear to function as non-redundant proteins, reflected by the inability of either protein to compensate for the embryonic lethality of the loss of the other protein, yet both losses may be functionally rescued by the knockout of p53.<sup>59,60</sup>

Despite similar p53 interactive domains, there is conflicting data on the effects of small molecule *MDM2* inhibitors on *MDMX* binding to p53. Laurie and colleagues demonstrated that although Nutlin-3 had an approximately 40-fold higher  $K_i$  for inhibiting the p53/*MDMX* interaction compared with p53/*MDM2*, the small molecule inhibitor was still able to block co-immunoprecipitation of p53 and *MDMX*.<sup>61</sup> However, Hu et al., was unable to confirm the inhibition of *MDMX* and p53 interactions with Nutlin. Furthermore, anticipating the cellular effects of small molecule inhibitors on *MDMX*/p53 interactions is complicated by highly variable levels of cellular *MDMX*, as the level of *MDMX* appears to affect the responsiveness of the *MDM2*/p53 complex to small molecule inhibitors.<sup>62,63</sup> Ultimately given the disparity of Nutlin concentrations required for *MDM2* and *MDMX* inhibition, there may be little *MDMX* inhibition seen in-vivo. However, newer *MDM2* inhibitors may have altered binding affinities for these two proteins, potentially making effects on the p53/*MDMX* complex more important.<sup>64</sup>

### Non-p53 Mediated MDM2 Protein Interactions

*MDM2* also contributes to various non-p53 mediated cellular signaling events.<sup>65</sup> The critical *MDM2* N-terminal domain involved in interactions with p53 is also important for binding several additional proteins and these interactions may be subject to perturbations by small-molecule based *MDM2* inhibitors.

**E2F1/DP1.** E2F1/DP1 (Ch 20a11.2 / Ch 13q34) is a heterodimeric transcription factor involved in both the regulation of S-phase cell cycle progression as well as apoptosis.<sup>66-68</sup> *MDM2* increases the transcription of *E2F1* and potentiates the transactivation capabilities of E2F1, possibly through the binding of the N-terminus of *MDM2* to the transactivation domain of the E2F1 protein as well as the C-terminus of DP1 and participates as a co-activator of transcription in a trimeric complex.<sup>69</sup> Currently available data imply that the

pro-growth versus pro-apoptotic nature of the E2F and DP families of proteins may be dependent on the specific family member involved, gene dosage, as well as p53 and pRb status.<sup>70,71</sup> Recently, Ambrosini et al., demonstrated that in p53 expressing cell lines, treatment with Nutlin-3 resulted in decreased levels of E2F1.<sup>72</sup> Interestingly, even in cells lacking p53, Nutlin-3 enhanced apoptotic activity of genotoxic chemotherapy potentially by stabilizing E2F1 levels resulting in elevations of the levels of the proapoptotic genes p73 and NOXA. In summary, current data do not allow for definitive conclusions regarding the anticipated cellular effects of inhibiting *MDM2* interactions with the E2F/ DP heterodimeric family.

**NPM.** Nucleophosmin (NPM) (Ch 5q35) is a nucleolar phosphoprotein with multiple cellular activities.<sup>73</sup> Recently, evidence has been advanced that after cellular stress or genotoxic chemotherapy exposure, NPM undergoes nucleoplasmic redistribution, allowing NPM to interact with p53 as well as *MDM2*.<sup>74,75</sup> Binding of NPM to *MDM2* occurs at the N-terminus of *MDM2* (amino acid residues 1–110) as well as the C-terminal RING domain, preventing *MDM2* interaction with p53, and stabilizing p53 levels.<sup>74,76</sup> Importantly, a high frequency of NPM exon-12 mutations resulting in aberrant cytoplasmic localization of NPM have been described in AML, although the exact mechanism whereby the altered localization of NPM contributes to the malignant phenotype has yet to be fully elucidated.<sup>77</sup> Given that multiple domains are involved in *MDM2* binding to NPM, it is unclear how small molecule inhibitors directed only at the N-terminal NPM1/*MDM2* interactive domain will affect various NPM function. Therefore, results of ongoing pre-clinical experiments on *MDM2* inhibitor treatment of well-characterized AML cases (with and without NPM mutations) are eagerly awaited and expected to shed light on this important question.

**PCAF.** The p300/CREB-binding protein-associated factor (PCAF) (Ch 3p24), a histone acetyltransferase (HAT), functions as a transcriptional coactivator. After genotoxic stress, PCAF can acetylate p53, which increases its cellular activity.<sup>78</sup> Recently, Kobet and colleagues demonstrated that *MDM2* prevented p53 acetylation by p300, thus limiting p53 activity.<sup>79</sup> Subsequently, Jin et al., demonstrated that *MDM2* also induced PCAF ubiquitination and subsequent degradation.<sup>80</sup> *MDM2* appears to interact with PCAF through its N-terminal domain. However, PCAF expression is highly tissue-dependent and therefore, the physiologic effects of inhibiting *MDM2*/PCAF interactions may be cell specific.<sup>65</sup>

**NUMB.** NUMB (Ch 14q24.3) is a lineage specific protein that acts to antagonize Notch signaling in stem cells and somatic cells and is critical in cell fate determination.<sup>81,82</sup> Previous research has demonstrated that the N-terminus of *MDM2* can bind NUMB.<sup>83</sup> Changes in cellular levels of *MDM2* may affect the ability of NUMB to alter cell fate decisions.<sup>84</sup> NUMB also interacts with p53, preventing *MDM2*-mediated ubiquitination and degradation, and reductions in NUMB levels have been shown to reduce steady-state levels of p53 thus resulting in lower levels of apoptosis.

Recently, Colaluca and colleagues demonstrated that Nutlin failed to interfere with the NUMB/*MDM2* as well as NUMB/p53 interactions, thus reflecting subtle differences in the interactive domains compared with *MDM2*/p53.<sup>85</sup> However, the combination of cytotoxic chemotherapy combined with Nutlin diminished NUMB/*MDM2* interactions.

**p73.** p73 (Ch 1p36.33) belongs to the p53 superfamily of proteins.<sup>86,87</sup> Each family member shares both structural as well as functional similarities, including transcriptional activation capabilities. Multiple isoforms of p73 are known, and the N-terminal truncated isoform of p73 ( $\Delta$ Np73) can inhibit wild-type p73 (TAp73) dependant p53-mediated apoptosis.<sup>88</sup> The N-terminus of MDM2 binds to the N-terminus of p73 resulting in the inhibition of p73-dependent transcription, potentially by abrogating p300/CBP/p73 interactions and reducing the transactivational capabilities of the complex.<sup>89</sup> Recently, Lau and colleagues demonstrated that Nutlin-3 interfered with p73/MDM2 binding in a number of cancer cell lines, resulting in increased p73 transcriptional activity and increased levels of apoptosis in a p53 independant fashion.<sup>90</sup> Similar to the effects of Nutlin inhibition on MDMX/p53 interactions, at least 20-fold higher concentrations of Nutlin are required for inhibition of p73/MDM2 interactions compared with p53/MDM2. Therefore, the clinical effect, if any, of small molecule MDM2 inhibitors on p73-dependant apoptosis remains unpredictable.

### Preclinical Application of MDM2 Inhibitors

**Proteins and peptides.** Initial preclinical experiments evaluating the functional importance of the MDM2-p53 interaction relied on antibodies or peptides that interfered with the intermolecular interactions between these two proteins.<sup>91,92</sup> By blocking the critical interactions between these proteins, researchers established that p53 can be relieved of MDM2-mediated repression. Chen and colleagues developed an *MDM2* sequence-based antisense deoxyoligonucleotide that inhibited *MDM2* expression, resulting in increased p53- induced signaling and subsequent apoptosis in a number of cancer cell lines. This work has been substantiated by a number of additional observations.<sup>93-95</sup>

### Small Molecule E3-Ligase Inhibitors

Lai and colleagues identified 3 different chemical structures that were able to inhibit the E3 ligase activity of MDM2.<sup>96</sup> Interestingly, these compounds did not inhibit other ubiquitinating enzymes or autoubiquitination of MDM2 itself. Yang and colleagues have also identified a class of small molecule E3 ligase inhibitors.<sup>97</sup> These HLI98-based compounds have a relatively restricted activity toward the MDM2 E3 ligase activity, but do demonstrate activity against the HECT E3 ligase as well. Exposure of cell lines to these inhibitors resulted in an increase in p53 levels, the upregulation of the biomarker p21<sup>WAF1/CIP1</sup> and the pro-apoptotic protein PUMA leading to an increased rate of apoptosis. Further modifications to improve the specificity and affinity of these compounds will be required before further clinical evaluations can proceed.

### Small Molecule Inhibitors of MDM2/p53 Binding

Inhibiting protein/protein intermolecular interactions with small molecules are typically limited due to the large surface areas involved in the binding.<sup>98</sup> However, X-ray crystallography has demonstrated that three hydrophobic amino acid residues in the N-terminus of p53 (Phe<sub>19</sub>, Trp<sub>23</sub> and Leu<sub>26</sub>) critically interact with the N-terminus of the MDM2 protein.<sup>44,99</sup> Because MDM2 is unable to modulate p53 activity if it is prevented from binding p53, researchers have begun development of small molecule inhibitors to disrupt the MDM2/p53 interface.

Vassilev and colleagues were the first to identify a potent class of small molecules able to disrupt the MDM2/p53 interaction.<sup>100</sup> Three chemical derivatives termed the Nutlins were characterized and were found to efficiently displace p53 from MDM2 in the nanomolar range. Crystallography demonstrated that the Nutlins bind to the hydrophobic pocket of the N-terminus of MDM2 in a reminiscent manner to the three hydrophobic amino acid residues in the N-terminus of p53. These researchers have demonstrated that the Nutlins stabilize p53, leading to the activation p53 dependent signaling pathways, cell cycle arrest and apoptosis of a number of cancer cell lines.<sup>100</sup> Importantly, cells with mutated or deleted p53 were insensitive to the antiproliferative effects of the Nutlins.<sup>100,101</sup> Xenographic models have also demonstrated tumor control with Nutlin-3 and Nutlin-3a, the active stereoisomer.<sup>100-102</sup>

Recently, a second class of MDM2 antagonists representing a Benzodiazepinedion-based series of compounds has been developed and has demonstrated an ability to disrupt the MDM2/p53 interaction.<sup>103,104</sup> Further chemical modifications enhanced the IC<sub>50</sub> of the compound with submicromolar efficacy in vitro and demonstrated inhibitory activity in a xenographic melanoma model.<sup>105</sup>

Ding et al., published a series of spiro(oxindole-3,3'-pyrrolidine) based small molecules that can inhibit MDM2-p53 binding in the nanomolar range.<sup>106</sup> In-vitro studies demonstrated submicromolar antiproliferative IC<sub>50</sub> values for a number of cancer cell lines with an approximately 30-fold selectivity for cells expressing wild-type p53.<sup>107,108</sup> Hardcastle and colleagues published a series of isoindolinone-based chemical compounds that demonstrated an increase in p21<sup>WAF1/CIP1</sup> levels using micromolar concentrations of the compounds.<sup>109</sup> Recently, Bowman and colleagues identified five unique chemical moieties with MDM2 binding properties using a computer algorithm to identify small molecule interactions with MDM2 while allowing for multiple protein conformations.<sup>110</sup> However, little in-vitro data exists on the activity of these compounds.

### Potential Pitfalls Towards the Efficacy of Small Molecule Inhibitors in Human Tumors Inferred from Data Using Various Experimental Systems

Signaling cascades rarely function in isolation, with cross-talk occurring at multiple levels. Additionally, redundancy in critical pathways is often seen. As previously described, MDMX is also a known antagonist of p53 activity.<sup>55,59</sup> Optimal p53 activation might require inhibition of both MDM2 and MDMX and recent studies have shown that failure to inhibit MDMX binding can limit the apoptotic response of cancer cell lines to Nutlin-3.<sup>63</sup> Likewise, numerous additional proteins including p73 interact with both MDM2 as well as MDMX. The effect of small molecule MDM2 inhibitors on the interplay of pathways involving these molecules remains essentially unknown.<sup>111,112</sup>

Despite wild-type p53 expression, the capacity of cancer cells to efficiently undergo apoptosis may still be limited. After exposing a series of 10 cell lines with wild-type p53 to Nutlin-3a, Tovar and colleagues noted a varied level of apoptosis after 72 hours.<sup>102</sup> Utilizing a gene array-based expression analysis, they revealed an up regulation of early p53 induced genes, including p21<sup>Waf1/Cip1</sup> in all cell lines, but numerous downstream apoptotic proteins showed a marked differential expression. Fourteen of the differentially regulated genes in cells with altered apoptotic potential to Nutlin-3a

were associated with downstream p53 signaling, including PUMA and BAX.

### Toxicity of p53 Activation to Normal Tissues

p53 levels are tightly regulated to limit activation only after cellular recognition of stressors which trigger cell cycle arrest and potentially apoptosis. Given the embryonic lethality of unregulated p53 in MDM2 deficient animals, one concern over MDM2 inhibitors is their potential toxicity, especially with chronic utilizations. This may be especially relevant in tissues with a high sensitivity to p53 mediated activity, including hematopoietic tissues.<sup>113</sup> However, early indications are that normal human fibroblasts as well as mice treated with small molecule MDM2 inhibitors do not display the degree of apoptotic induction that malignant cells do.<sup>100,113</sup> The fibroblasts enter cell cycle arrest but retain their viability for prolonged periods despite persistent exposure. Interestingly, studies by Coll-Mulet and Secchiero indicate a relative selectivity of p53-mediated apoptosis to B-CLL samples over patient derived T cells or even clonogenic bone marrow progenitor cells.<sup>114,115</sup> Bone marrow stem cells undergo morphologic changes ex-vivo after exposure to Nutlin, but do not display increased levels of apoptosis and the effects are reversible after discontinuation of the drug.<sup>116</sup>

Recent evidence indicates that the embryonic lethality seen in *MDM2* knockout animals may not extend into adulthood. Mendrysa and colleagues created a conditional hypomorphic *MDM2* mouse under the control of the Cre-LoxP system.<sup>117,118</sup> In this system, the heterozygous animals, expressing *MDM2* from the loxP containing allele, expressed only 30% of the *MDM2* compared with their wild-type littermates. Lifespan was apparently unaffected in these mice.<sup>118</sup> These mice also demonstrated a marginal anemia and lymphopenia as well as an increased level of apoptosis seen in the small bowel. There was an approximately threefold decrease in spontaneous tumors in the *MDM2* under expressing animals, and when crossed into an adenomatous polyposis coli (*Apc*) background, the hypomorphic *MDM2* mice had a lower level of polyps and subsequent malignancies.<sup>118</sup>

### Lessons Learned from ex vivo Treatment of Primary Human Tumor Cells with MDM2 Inhibitors

The pre-clinical and early phase clinical development of targeted therapeutic compounds is greatly aided through studies of the compound effects on primary human cancer cells. It is in this setting that hypotheses formulated based on in vitro or cell line data are tested, and subsequently validated or refuted. One of the limitations to streamlined pre-clinical to clinical compound transitions is access to well-annotated and well-characterized primary human tumor specimens. Given these constraints, hematological malignancies offer easy access to tumor tissue and tumor cells can be purified to almost homogeneity thus reducing confounding effects of non-malignant cells.

Chronic lymphocytic leukemia (CLL), the most common leukemia in the Western world, has been studied extensively and is very suitable for translational research analysis. Our group has characterized a large cohort of CLL patients for all clinically relevant biomarkers, including ZAP70 expression, immunoglobulin heavy chain variable region mutation status, p53 mutations, CD38 expression and interphase FISH. Further, we have obtained high density

genomic profiling data of sub chromosomal copy number and allele status for 178 patients using SNP-array technology.

To understand determinants of sensitivity or resistance to MDM2 inhibitors in CLL cells and to assess target (*MDM2*-p53) specificity, we analyzed apoptosis induction after treatment of highly pure CLL tumor cells ex vivo with 2 MDM2 inhibitors, Nutlin-3 and MI-63. Upon comparative analysis of MDM2-inhibitor  $IC_{50}$  kill data of primary CLL cells with p53 mutational status and p53 immunoblotting data both pre- and post-MDM2 inhibitor treatment, it became evident that p53 status was the overriding molecular determinant of response; all 18 CLL cases with aberrant p53 (mutant or absent expression) displayed relative resistance to MDM2 inhibitor-mediated apoptosis when compared with results from 88 CLL cases with wild type p53 function. Only very few (~3%) of CLL cases with wild type p53 displayed relative resistance to MDM2 inhibitor-induced apoptosis that was comparable in magnitude to cases with p53 aberrations.

This data, which represents by far the largest collection of experiments using MDM2 inhibitors on primary human tumors, allowed us to conclude that the effect of MDM2 inhibitors on MDM2 binding proteins other than p53 is not sufficient for cell death induction in this tumor type. Further, defects in p53 transcriptional and non-transcriptional targets are decidedly uncommon in CLL, a statement supported through genomic copy number analysis of various important p53 target genes (Table 1).

Within the group of CLL cases with wild type p53 (88 cases), the vast majority displayed a narrow range of  $IC_{50}$  values for MDM2 inhibitor-mediated apoptosis. This data suggest that molecular variables other than p53 status (*MDM2* levels, *MDMX* levels, others) do not have a strong quantitative influence on MDM2 inhibitor-mediated cell kill in CLL.

Using our clinical database, we detected an inverse correlation between ex vivo sensitivity to MDM2 inhibitor treatment and disease progression in CLL with the cases displaying high ex vivo sensitivity needing therapy sooner than relatively less sensitive cases. This analysis therefore uncovers a CLL target group with wt p53 that may benefit most from MDM2 inhibitor therapy. Interestingly, no association between the allele status of *MDM2*-SNP309 (GG, TG or TT) and ex vivo sensitivity to MDM2 inhibitors was found suggesting that the differences in disease progression may not be due to differences in baseline *MDM2* levels.

### Selected Aspects of the Design of Early Phase Clinical Trials of MDM2 Inhibitors in Humans

Despite animal data suggesting acceptable toxicity of MDM2 inhibitors in vivo, caution is advised during planned early phase clinical trials of these compounds in cancer patients. In particular, the interplay of intermittent or low level cellular stressors due to environmental factors and p53 elevations due to therapeutic MDM2 blockade may induce unanticipated acute or chronic toxicities.

Given the importance of intact p53 to the apoptotic cellular response to MDM2 inhibitors, it may prove necessary from an ethical standpoint to interrogate p53 status pre trial enrollment for prospective patients. Such analysis may need coordination between various specialists to secure access to primary tumor tissue or analysis of previously stored and archived tumor biopsies. Data from our work on CLL suggest that the most comprehensive assessment (100% sensitivity

and 100% specificity) of p53 status is achieved using p53 immunoblotting pre- and post Mdm2 inhibitor treatment of tumor cells.

Given the strong dependence of MDM2 inhibitor-mediated cell kill on wild type p53 status, the emergence of resistance due to acquired p53 mutations is of significant concern. This potential problem is worsened by the clinical observation that the salvage of patients with acquired p53 mutations may be more difficult or impossible using conventional genotoxic drugs. It is therefore of great importance to monitor p53 mutation status serially in patients treated with MDM2 inhibitors in early phase clinical trials and to devise rational combination therapy approaches aimed at suppression of emergence of p53 mutants. It may also become important to measure p53 mutations in subclones of tumors prior to MDM2 inhibitor treatment and methods may need to be devised to detect mutations occurring in fractions of a percent of tumor cells or less. Finally, in the setting of CLL, combinations using MDM2 inhibitors with drugs less sensitive to p53 mutation status, like alemtuzumab (anti-CD52 directed antibody) or flavipiridol, may be advanced early in the disease-specific development of these compounds. In other cancer types, additional non-genotoxic drugs may be utilized.

**Acknowledgements**

Supported by a Leukemia and Lymphoma Society of America Special Fellow Award (SM), a Leukemia Research Foundation New Investigator Award (SM) a Leukemia and Lymphoma Society Translational Research Grant (SW and SM) and supported by the National Institutes of Health through (1 R21 CA124420-01A1; SM).

This research is supported (in part) by the National Institutes of Health through the University of Michigan's Cancer Center Support Grant (5 P30 CA46592).

**Conflict of interest**

Shaomeng Wang is a shareholder and founder of Ascenta Therapeutics Incorporated. He is a consultant to Ascenta Therapeutics Incorporated and the principal investigator of a research contract between Ascenta Therapeutics Incorporated and the University of Michigan.

**References**

1. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000; 408:307-10.
2. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nature Reviews Cancer* 2002; 2:594-604.
3. Bennett M, Macdonald K, Chan SW, Luzio JB, Simari R, Weissberg P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis.[see comment]. *Science* 1998; 282:290-3.
4. Ding HF, Lin YL, McGill G, et al. Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. *Journal of Biological Chemistry* 2000; 275:38905-11.
5. Vousden KH, Lane DP. p53 in health and disease. *Nature Reviews Molecular Cell Biology* 2007; 8:275-83.
6. Green DR, Chipuk JE. p53 and metabolism: Inside the TIGAR.[comment]. *Cell* 2006; 126:30-2.
7. Saddler C OP, Kujawski L, Shangary S, Talpaz M, Kaminski M, Erba H, Shedden K, Wang S, Malek SN. Comprehensive biomarker and genomic analysis identifies p53 status as the major determinant of response to MDM2 inhibitors in chronic lymphocytic leukemia. *Blood* 2008; 111:1584-93.
8. Hainaut P, Hollstein M. p53 and human cancer: the first ten thousand mutations. *Advances in Cancer Research* 2000; 77:81-137.
9. Malkin D, Li FP, Strong LC, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms.[see comment][erratum appears in *Science* 1993; 259:878; PMID: 8438145]. *Science* 1990; 250:1233-8.
10. Petitjean A, Mathe E, Kato S, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Human Mutation* 2007; 28:622-9.

11. Petitjean A, Achatz MI, Borresen Dale AL, Hainaut P, Olivier M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene* 2007; 26:2157-65.
12. Zhang Y, Xiong Y. A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation.[see comment]. *Science* 2001; 292:1910-5.
13. Liang SH, Clarke MF. The nuclear import of p53 is determined by the presence of a basic domain and its relative position to the nuclear localization signal. *Oncogene* 1999; 18:2163-6.
14. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Research* 1994; 54:4855-78.
15. de Vries A, Flores ER, Miranda B, et al. Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function. *Proceedings of the National Academy of Sciences of the USA* 2002; 99:2948-53.
16. Song H, Hollstein M, Xu Y. p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM.[see comment]. *Nature Cell Biology* 2007; 9:573-80.
17. Strano S, Munarriz E, Rossi M, et al. Physical and functional interaction between p53 mutants and different isoforms of p73. *Journal of Biological Chemistry* 2000; 275:29503-12.
18. Strano S, Fontemaggi G, Costanzo A, et al. Physical interaction with human tumor-derived p53 mutants inhibits p63 activities. *Journal of Biological Chemistry* 2002; 277:18817-26.
19. Soussi T, Lozano G. p53 mutation heterogeneity in cancer. *Biochemical & Biophysical Research Communications* 2005; 331:834-42.
20. Peller S, Rotter V. TP53 in hematological cancer: low incidence of mutations with significant clinical relevance. *Human Mutation* 2003; 21:277-84.
21. Neri A, Baldini L, Trecca D, Cro L, Polli E, Maiolo AT. p53 gene mutations in multiple myeloma are associated with advanced forms of malignancy. *Blood* 1993; 81:128-35.
22. Cordone I, Masi S, Mauro FR, et al. p53 expression in B-cell chronic lymphocytic leukemia: a marker of disease progression and poor prognosis. *Blood* 1998; 91:4342-9.
23. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. *Journal of Clinical Oncology* 2001; 19:1405-13.
24. Wattel E, Preudhomme C, Hecquet B, et al. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. *Blood* 1994; 84:3148-57.
25. Beck Z, Kiss A, Toth FD, et al. Alterations of P53 and RB genes and the evolution of the accelerated phase of chronic myeloid leukemia. *Leukemia & Lymphoma* 2000; 38:587-97.
26. Imamura J, Miyoshi I, Koeffler HP. p53 in hematologic malignancies. *Blood* 1994; 84:2412-21.
27. Kirsch DG, Kastan MB. Tumor-suppressor p53: implications for tumor development and prognosis. *Journal of Clinical Oncology* 1998; 16:3158-68.
28. Meek DW. Mechanisms of switching on p53: a role for covalent modification? *Oncogene* 1999; 18:7666-75.
29. Dey A VC, Lane DP. Updates on p53: modulation of p53 degradation as a therapeutic approach. *British Journal of Cancer* 2008; 98:4-8.
30. Oda K, Arakawa H, Tanaka T, et al. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 2000; 102:849-62.
31. Prives C, Manley JL. Why is p53 acetylated? *Cell* 2001; 107:815-8.
32. Wang L, Wu Q, Qiu P, et al. Analyses of p53 target genes in the human genome by bioinformatic and microarray approaches. *Journal of Biological Chemistry* 2001; 276:43604-10.
33. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Molecular Cell* 2001; 7:683-94.
34. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. *Molecular Cell* 2001; 7:673-82.
35. Jeffers JR, Parganas E, Lee Y, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* 2003; 4:321-8.
36. Villunger A, Michalak EM, Coultas L, et al. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003; 302:1036-8.
37. Cahilly-Snyder L, Yang-Feng T, Francke U, George DL. Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somat Cell Mol Genet* 1987; 13:235-44.
38. 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.
39. Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* 1993; 362:857-60.
40. Jones SN, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 1995; 378:206-8.
41. 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.
42. Chen J, Marechal V, Levine AJ. Mapping of the p53 and mdm-2 interaction domains. *Mol Cell Biol* 1993; 13:4107-14.
43. Piette J, Neel H, Marechal V. Mdm2: keeping p53 under control. *Oncogene* 1997; 15:1001-10.
44. 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.

45. Lin J, Chen J, Elenbaas B, Levine AJ. Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev* 1994; 8:1235-46.
46. Momand J, Wu HH, Dasgupta G. MDM2—master regulator of the p53 tumor suppressor protein. *Gene* 2000; 242:15-29.
47. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997; 387:296-99.
48. Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 1997; 420:25-7.
49. Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* 1997; 91:325-34.
50. Unger T, Juven Gershon T, Moallem E, et al. Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *Embo J* 1999; 18:1805-14.
51. Pomerantz J, Schreiber Agus N, Liegeois NJ, et al. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* 1998; 92:713-23.
52. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* 1998; 92:725-34.
53. Zauberman A, Flusberg D, Haupt Y, Barak Y, Oren M. A functional p53-responsive intronic promoter is contained within the human mdm2 gene. *Nucleic Acids Res* 1995; 23:2584-92.
54. Juven T, Barak Y, Zauberman A, George DL, Oren M. Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene. *Oncogene* 1993; 8:3411-6.
55. 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.
56. Shvarts A, Bazuine M, Dekker P, et al. Isolation and identification of the human homolog of a new p53-binding protein, Mdmx. *Genomics* 1997; 43:34-42.
57. Parant JM, Reinke V, Mims B, Lozano G. Organization, expression, and localization of the murine mdmx gene and pseudogene. *Gene* 2001; 270:277-83.
58. Bottger V, Bottger A, Garcia-Echeverria C, et al. Comparative study of the p53-mdm2 and p53-MDMX interfaces. *Oncogene* 1999; 18:189-99.
59. Marine JC, Jochemsen AG. Mdmx as an essential regulator of p53 activity. *Biochem Biophys Res Commun* 2005; 331:750-60.
60. Toledo F, Krummel KA, Lee CJ, et al. A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. *Cancer Cell* 2006; 9:273-85.
61. Laurie NA, Donovan SL, Shih CS, et al. Inactivation of the p53 pathway in retinoblastoma. *Nature* 2006; 444:61-6.
62. 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.
63. 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.
64. 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.
65. Zhang Z, Zhang R. p53-independent activities of MDM2 and their relevance to cancer therapy. *Curr Cancer Drug Targets* 2005; 5:9-20.
66. Bandara LR, Buck VM, Zamanian M, Johnston LH, La Thangue NB. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. *Embo J* 1993; 12:4317-24.
67. Field SJ, Tsai FY, Kuo F, et al. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* 1996; 85:549-61.
68. Johnson DG, Schwarz JK, Cress WD, Nevins JR. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* 1993; 365:349-52.
69. Martin K, Trouche D, Hagemeier C, Sorensen TS, La Thangue NB, Kouzarides T. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature* 1995; 375:691-4.
70. Kowalik TF, DeGregori J, Leone G, Jakoi L, Nevins JR. E2F1-specific induction of apoptosis and p53 accumulation, which is blocked by Mdm2. *Cell Growth Differ* 1998; 9:113-8.
71. Wunderlich M, Berberich SJ. Mdm2 inhibition of p53 induces E2F1 transactivation via p21. *Oncogene* 2002; 21:4414-21.
72. 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.
73. Falini B, Nicoletti I, Bolli N, et al. Translocations and mutations involving the nucleophosmin (NPM1) gene in lymphomas and leukemias. *Haematologica* 2007; 92:519-32.
74. Kurki S, Peltonen K, Latonen L, et al. Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. *Cancer Cell* 2004; 5:465-75.
75. Kurki S, Peltonen K, Laiho M. Nucleophosmin, HDM2 and p53: players in UV damage incited nucleolar stress response. *Cell Cycle* 2004; 3:976-9.
76. Kurki S, Latonen L, Laiho M. Cellular stress and DNA damage invoke temporally distinct Mdm2, p53 and PML complexes and damage-specific nuclear relocalization. *J Cell Sci* 2003; 116:3917-25.
77. Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* 2005; 352:254-66.
78. Liu L, Scolnick DM, Trievel RC, et al. p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. *Mol Cell Biol* 1999; 19:1202-9.
79. Kobet E, Zeng X, Zhu Y, Keller D, Lu H. MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins. *Proc Natl Acad Sci USA* 2000; 97:12547-52.
80. Jin Y, Zeng SX, Lee H, Lu H. MDM2 mediates p300/CREB-binding protein-associated factor ubiquitination and degradation. *J Biol Chem* 2004; 279:20035-43.
81. Knoblich JA, Jan LY, Jan YN. Asymmetric segregation of Numb and Prospero during cell division. *Nature* 1995; 377:624-7.
82. Verdi JM, Schmandt R, Bashirullah A, et al. Mammalian NUMB is an evolutionarily conserved signaling adapter protein that specifies cell fate. *Curr Biol* 1996; 6:1134-45.
83. Juven Gershon T, Shifman O, Unger T, Elkeles A, Haupt Y, Oren M. The Mdm2 oncoprotein interacts with the cell fate regulator Numb. *Mol Cell Biol* 1998; 18:3974-82.
84. Conboy IM, Rando TA. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell* 2002; 3:397-409.
85. Colaluca IN, Tosoni D, Nuciforo P, et al. NUMB controls p53 tumour suppressor activity. *Nature* 2008; 451:76-80.
86. Kaghad M, Bonnet H, Yang A, et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 1997; 90:809-19.
87. Melino G, De Laurenzi V, Vousden KH. p73: Friend or foe in tumorigenesis. *Nat Rev Cancer* 2002; 2:605-15.
88. Ishimoto O, Kawahara C, Enjo K, Obinata M, Nukiwa T, Ikawa S. Possible oncogenic potential of DeltaNp73: a newly identified isoform of human p73. *Cancer Res* 2002; 62:636-41.
89. Zeng X, Chen L, Jost CA, et al. MDM2 suppresses p73 function without promoting p73 degradation. *Mol Cell Biol* 1999; 19:3257-66.
90. Lau LM, Nugent JK, Zhao X, Irwin MS. HDM2 antagonist Nutlin-3 disrupts p73-HDM2 binding and enhances p73 function. *Oncogene* 2008; 27:997-1003.
91. Bottger A, Bottger V, Sparks A, Liu WL, Howard SE, Lane DP. Design of a synthetic Mdm2-binding mini protein that activates the p53 response in vivo. *Curr Biol* 1997; 7:860-9.
92. Garcia-Echeverria C, Chene P, Blommers MJ, Furet P. Discovery of potent antagonists of the interaction between human double minute 2 and tumor suppressor p53. *J Med Chem* 2000; 43:3205-8.
93. Chen L, Lu W, Agrawal S, Zhou W, Zhang R, Chen J. Ubiquitous induction of p53 in tumor cells by antisense inhibition of MDM2 expression. *Mol Med* 1999; 5:21-34.
94. Wang H, Nan L, Yu D, Agrawal S, Zhang R. Antisense anti-MDM2 oligonucleotides as a novel therapeutic approach to human breast cancer: in vitro and in vivo activities and mechanisms. *Clin Cancer Res* 2001; 7:3613-24.
95. Wang H, Yu D, Agrawal S, Zhang R. Experimental therapy of human prostate cancer by inhibiting MDM2 expression with novel mixed-backbone antisense oligonucleotides: in vitro and in vivo activities and mechanisms. *Prostate* 2003; 54:194-205.
96. 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 USA* 2002; 99:14734-9.
97. 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.
98. Fry DC, Vassilev LT. Targeting protein-protein interactions for cancer therapy. *J Mol Med* 2005; 83:955-63.
99. Bottger A, Bottger V, Garcia Echeverria C, et al. Molecular characterization of the hdm2-p53 interaction. *J Mol Biol* 1997; 269:744-56.
100. 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.
101. Barbieri E, Mehta P, Chen Z, et al. MDM2 inhibition sensitizes neuroblastoma to chemotherapy-induced apoptotic cell death. *Mol Cancer Ther* 2006; 5:2358-65.
102. 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 USA* 2006; 103:1888-93.
103. Grasberger BL, Lu T, Schubert C, et al. Discovery and cocrystal structure of benzodiazepinedione HDM2 antagonists that activate p53 in cells. *J Med Chem* 2005; 48:909-12.
104. Parks DJ, LaFrance LV, Calvo RR, et al. 1,4-Benzodiazepine-2,5-diones as small molecule antagonists of the HDM2-p53 interaction: discovery and SAR. *Bioorg Med Chem Lett* 2005; 15:765-70.
105. Koblisch HK, Zhao S, Franks CF, et al. Benzodiazepinedione inhibitors of the Hdm2:p53 complex suppress human tumor cell proliferation in vitro and sensitize tumors to doxorubicin in vivo. *Mol Cancer Ther* 2006; 5:160-9.
106. Ding K, Lu Y, Nikolovska-Coleska Z, et al. Structure-based design of potent non-peptide MDM2 inhibitors. *J Am Chem Soc* 2005; 127:10130-1.
107. Ding K, Lu Y, Nikolovska-Coleska Z, et al. Structure-based design of spiro-oxindoles as potent, specific small-molecule inhibitors of the MDM2-p53 interaction. *J Med Chem* 2006; 49:3432-5.
108. 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.
109. 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.

110. 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.
111. Kadakia M, Slader C, Berberich SJ. Regulation of p63 function by Mdm2 and MdmX. *DNA Cell Biol* 2001; 20:321-30.
112. Schon O, Friedler A, Bycroft M, Freund SM, Fersht AR. Molecular mechanism of the interaction between MDM2 and p53. *J Mol Biol* 2002; 323:491-501.
113. Vassilev LT. Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics. *Cell Cycle* 2004; 3:419-21.
114. Coll Mulet L, Iglesias Serret D, Santidrian AF, et al. MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. *Blood* 2006; 107:4109-14.
115. Secchiero P, Barbarotto E, Tiribelli M, et al. Functional integrity of the p53-mediated apoptotic pathway induced by the nongenotoxic agent nutlin-3 in B-cell chronic lymphocytic leukemia (B-CLL). *Blood* 2006; 107:4122-9.
116. 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.
117. Mendrysa SM, McElwee MK, Michalowski J, O'Leary KA, Young KM, Perry ME. mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. *Mol Cell Biol* 2003; 23:462-72.
118. 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.

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