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REVIEW

Targeting the MDM2–p53 interaction as a therapeutic strategy for the treatment of cancer

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Abstract: The tumor suppressor p53 functions as an important defense against the development of cancer, and is negatively regulated by interaction with the oncogene and E3 ligase MDM2. In a tightly controlled system of feedback, MDM2 is, in turn, inhibited by the tumor suppressor $p14^{ARF}$. The inhibition of MDM2-p53 interaction is an appealing therapeutic strategy for the treatment of cancer, and significant advances have been made in the development of small-molecule inhibitors which block this interaction and reactivate wild-type p53. However, the p53 gene is frequently mutated or deleted in cancer, or the wild-type p53 function inhibited by high levels of MDM2. Neuroblastoma (NB) is one such cancer and has presented a major therapeutic challenge in pediatric oncology. Although most NB tumors have wild-type p53, the $p14^{ARF}$/MDM2/p53 pathway is often altered, leading to resistance to many mainstay chemotherapeutics and a high incidence of relapse. In preclinical studies, the MDM2/p53 interaction inhibitor nutlin-3a has shown effectiveness in the treatment of chemoresistant NB with wild-type, mutant or null-p53 status, indicating that nutlin-3a has potential for the treatment of a broad range of chemoresistant and relapse tumors.

Keywords: p53, MDM2, MDMX, TAp73, nutlin-3a

Introduction

The p53 protein was first identified in 1979.[1–3] It was initially thought to be an oncogene because of its antigenic association with SV40, a transforming tumor virus.[4] Thus for a number of years it was not recognized as a key regulator of cell cycle arrest, DNA repair, senescence, autophagy and apoptosis. Its role as a tumor suppressor was not defined until 1989.[5] The p53 gene, *TP53*, was cloned in 1983,[6] which led to the discovery that its mutation or deletion occurs in upwards of half of all cancers. In 1990, alteration of the gene was definitively linked to a rare familial syndrome, Li-Fraumeni, which is associated with various cancers including adrenocortical tumors, brain and breast cancers.[7] Tumor-associated p53 mutations are generally single base substitutions that lead to inhibition of normal function and sometimes confer new oncogenic properties (gain of function).[8]

As a transcription factor, p53 coordinates its network of actions primarily through its ability to transactivate target genes induced by the cellular stresses of DNA damage, ionizing radiation, UV exposure and a host of other internal and external events. Its levels are in large part regulated by the properties of the mouse double minute 2 protein, MDM2 (HDM2, human), an oncogene product and E3 ubiquitin ligase (an enzyme involved in coupling ubiquitin, a degradation tag, to lysine

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residues).[9] MDM2 is transcriptionally upregulated by p53 following p53 activation. In turn, MDM2 acts to inhibit p53 action by a, binding to the transactivation region of p53,[10] b, promoting nuclear export of the p53 protein[11] and c inducing ubiquitin-mediated proteosomal degradation.[12] Thus MDM2 inhibits both the p53-mediated transactivating function of the p53 protein and the stability of the p53 protein itself. Such autoregulatory feedback tightly controls p53 levels to maintain homeostasis in unstressed cells and to restore homeostasis following stress responses.[13]

In roughly half of human cancers, the p53 gene is normal, but wild-type p53 function is inhibited by either inactivation of the cell cycle inhibitor and tumor suppressor $p14^{ARF}$ or the amplification and overexpression of MDM2.[14] MDM2 overexpression is linked to non-Hodgkin's lymphomas and B cell chronic lymphocytic leukemias,[15] and a single nucleotide polymorphism at position 309 of intron 1 leads to several-fold increased levels of MDM2 in clusters of familial cancer.[16] A second protein, MDMX (MDM4, mouse), a homologue of MDM2, is now known to stabilize the interaction between p53 and MDM2, thus promoting the oncogenic properties of MDM2.[17]

Both MDM2 and MDMX have been identified as potential therapeutic targets for treatment of cancer. Small molecule antagonists of MDM2 have been developed which disrupt the interaction between MDM2 and p53, leading to p53 stabilization and activation.[18,19] The best known of these, nutlin-3a, is a nonpeptidic small-molecule inhibitor containing a *cis*-imidazoline core structure. It binds with high affinity to the N-terminal region of MDM2 in the p53 binding pocket. Importantly, it is nongenotoxic, in contrast to most chemotherapeutic compounds, a property that has led to its extensive preclinical study in models of human cancers.[20]

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood, and arises from the neural crest element of the sympathetic nervous system.[21] NB displays a wild-type p53 genotype at diagnosis and generally responds to initial therapy. However, relapse is common, in which the p53/MDM2 pathway is deregulated, or, less commonly, in which p53 is directly mutated, resulting in chemoresistance.[22–24] Nutlin-3a is effective in the treatment of both chemosensitive and chemoresistant NB, including tumors with either wild-type or mutant p53.[25,26] In this review, we will discuss the key functional characteristics of p53 and MDM2, and the molecular mechanisms that underlie the effectiveness of MDM2-p53 targeted therapeutics for cancer, using NB as the model system.

p53 structure, activation, and function

p53 is a 393 amino acid nuclear phosphoprotein consisting of four functionally critical domains. The first 92 residues contain a transactivation (TA) and proline-rich domain, which is responsible for binding basal transcription factors including TFIID/TAF[27] and which responds to DNA damage, UV or ionizing radiation. In response to such signals, Ser15 and Ser20 are rapidly phosphorylated.[28] These phosphoryations are carried out by the protein kinases ATM/ATR (ataxia telangiectasia mutated and Rad3 associated), DNA-PK and Chk1/Chk2 (checkpoint kinase 1and 2), which function to mediate cell cycle checkpoints and DNA repair.[29,30] The initial outcome, which occurs rapidly after stress, is to inhibit interaction between p53 and MDM2, inducing the immediate stabilization of p53; however, studies with knockin mouse models have shown that p53 stabilization *in vivo* also occurs in the absence of phosphorylation,[31] implying a greater extent of complexity in the activation of p53.

The highly conserved core DNA binding domain (DNABD) lies between p53 residues 102 and 292. It is folded into a β sheet containing short α helices, and contains two inverted pentameric sequences which act to bind to a consensus DNA sequence (response element, RE) in or near promoters of target genes.[4,7] The p53 protein functions transcriptionally as a tetramer, and the residues 324–355 are necessary for this oligomerization.[32] The C-terminal basic domain (amino acids 356–393) contains a nuclear export signal as well as six lysines which contribute to ubiquitination by MDM2, leading to p53 proteasomal degradation. When acetylated by the histone acetyltransferase CBP/p300 (CREB-binding protein) in response to stress, they are no longer targeted by MDM2, and the stability of p53 is increased. p53 stability is also increased by the binding and sequestration of MDM2 to the nucleolus by the tumor suppressor $p14^{ARF}$. $p14^{ARF}$ is present at low levels in unstressed cells, and its levels rise dramatically in response to stress signals. In addition to binding and sequestering MDM2, $p14^{ARF}$ also inhibits MDM2 ligase activity.[33]

The effects of p53 stabilization and activation include cell cycle arrest, DNA repair, senescence, apoptosis and autophagy. Some of these effects may be mediated by transcription-independent means, but most are mediated directly by p53 transactivation of target genes.[34] Cell cycle and growth arrest result from p53 induction of p21 and Gadd45 (growth arrest and DNA-damage protein), and partial p53 acetylation is needed for this. Gadd45 functions as a stress sensor of genotoxic and other stresses; the Gadd45 protein interacts with p21

to halt G1/S and G2/M progression.[35] p21 is a cyclin-dependent kinase (CDK) inhibitor, and its continued transcription eventually leads to cell senescence. p53 promotes DNA repair by a transcription-dependent means through upregulation of proteins such as p53R2 (a p53-inducible small subunit of ribonucleotide reductase). It also acts more directly by a transcription-independent mechanism, by interaction with DNA repair proteins such as 53BP1 (a DNA double-strand break repair protein).[36]

The best known role for p53 is its transcriptional activation of genes involved in both intrinsic apoptosis (Bax, Puma, Noxa) and extrinsic apoptosis (Fas, DR4/DR5, TRAIL-R2).[37] Intrinsic pathways induce signals initiated by DNA damage and other stresses, resulting in release of cytochrome c from the mitochondria, whereas extrinsic pathways induce apoptosis by the stimulation of death receptors located on the plasma membrane. Both pathways ultimately involve the activity of a group of cysteine proteases, the caspases, which share a common effector caspase, caspase 3. In contrast to the activation of genes involved in cell cycle arrest and DNA repair, full p53 acetylation is required for the activation of these apoptotic genes; thus, the extent of p53 acetylation, and especially C-terminal acetylation, is a function of irreversible cellular damage.[38] The importance of p53 acetylation is underscored by the presence of CBP/p300 mutations in several cancers. Moreover, following stress responses, high cellular levels of deacetylases are induced to quickly disable p53 function.[39]

Bax, a key apoptotic protein, is a member of the large Bcl-2 family, members of which share four conserved domains, BH1-4 (Bcl-2 Homology 1–4). The Bcl-2 family contains both antiapoptotic and proapoptotic members. Bax is a direct initiator of cell death and is localized to the mitochondria where it homodimerizes and ultimately induces the release of cytochrome c.[40] Puma and Noxa, in contrast, contain a single BH3 domain and function more indirectly by binding to and inhibiting prosurvival Bcl-2 family members.[40] Fas, DR4/DR5 and TRAIL-R2 are members of the tumor necrosis factor (TNF) family, and function as either cell surface receptors or ligands in the induction of apoptosis.[41]

It is now known that the p53 protein can itself play a direct apoptotic role by binding to the mitochondrial membrane and Bcl-2 family members. p53 has been shown to localize to the mitochondria and interact with the prosurvival proteins Bcl-XL and Bcl-2, allowing Bax and Bak (another proapoptotic member) to oligomerize and induce pore formation, releasing cytochrome c. p53 has also been shown to interact directly with Bak, freeing it from interaction with Mcl1, a third prosurvival member.[42]

MDM2 structure and function

The gene for MDM2 encodes a 489 amino acid protein, which includes 12 exons, two p53-response elements (RE) and two promoters. Transcription from these promoters generates two MDM2 proteins: the full-length and fully functional p90, and a p76 protein. This shorter protein lacks p53-binding ability and acts as a dominant negative inhibitor of p90.[43] Genetic studies in mice initially revealed that MDM2 is required as inhibitor of p53 function, as the embryonic lethality of MDM2-null mice is reversed by p53 knockout.[44] To function as a negative regulator of p53 transcriptional activity, the N-terminus of the full-length MDM2 must bind to the N-terminus of p53. The first approximately 120 amino acids of MDM2 contain a short, hydrophobic surface pocket and four amino acids in p53 mediate binding to this region: Phe19, Leu22, Trp23 and Leu26.[45] MDMX (MDM4) also acts as a negative regulator of p53, again by inhibition of p53 transactivation and the promotion of MDM2 function, but it does not mediate p53 degradation.[17](Figure1)

Initially, it was thought that MDM2 interacted only with the p53 N-terminus. It is now known that MDM2 binds to both the DNABD and C-terminal domains of p53. Inhibition of this binding, in combination with interplay between posttranslational modifications such as phosphorylation and acetylation, releases p53 from repression.[46] Interestingly, it has recently been shown that TAFII250 (a component of TFIID, a general transcription factor), has multiple functions in MDM2-mediated regulation of p53. It binds and stimulates the TA domain of p53; conversely, it also promotes MDM2-dependent turnover of p53 by downregulating MDM2 autoubiquitination and promoting p53-MDM2 interaction through interaction with the acidic domain of MDM2. Thus, while playing an essential role in basal p53 transcription, TAFII250 also stimulates p53 ubiquitination and degradation.[47]

The three major roles of MDM2 are: (1) the inhibition of p53 transactivation by binding to the TA region of p53; (2) the promotion of nuclear export and cytoplasmic accumulation of p53 by monoubiquitination and; (3) the induction of p53 proteosomal degradation by polyubiquitination of six key lysines in the p53 C-terminus. However, polyubiquitination of these lysines is not a universal requirement for MDM2-mediated degradation. In some cases, it requires other lysines, as shown by the substitution of these six lysines with arginine, or by their deletion. In such cases, MDM2 can

still promote the degradation of p53.[48] Early work proposed that the C-terminal p53 domain functioned only as a target of regulation by MDM2. However, it has since become apparent that this domain interacts with the DNABD in the activation of p53-specific genes; acetylation of the C-terminal region positively affects the ability of p53 to bind DNA. The revised view is that the DNABD provides sequence-specificity, the C-terminal domain recognizes structural features of target DNA, and both are necessary for the activation of p53 target genes.[38]

Other MDM2-interacting proteins also regulate MDM2 function. The phosphorylation of MDM2 contributes to both the stabilization and inhibition of p53 function: phosphorylation of Tyr394 by c-Abl inhibits MDM2 ligase activity,[49] whereas phosphorylation of Ser166 and Ser186 by Akt/PKB promote its E3-ligase activity.[50] MDM2 mediates its own ubiquitination and degradation and is also ubiquitinated by other E3-ligases. MDM2 is deubiquitinated by Hausp, with which it forms a complex with Daxx (death-domain associated protein). Daxx stabilizes MDM2, and in a complex crosstalk of regulation, Daxx is deubiquitinated by Hausp, but ubiquitinated by MDM2. Thus, the interaction of MDM2 with Daxx may play a critical role in p53 activation.[51,52]

Alternative model for p53 activation

The traditional model of p53 transactivation was premised on a three-step process: The phosphorylation of key serines by ATM/ATR following a stress response, the stabilization and activation of p53 following release from MDM2, and the transcriptional targeting of response elements within or proximal to key genes by p53. A recently proposed alternative model suggests that p53 is, instead, constitutively and intrinsically activated.[31] According to this model, activated p53 binds p21 and MDM2 genes in unstressed cells, but is repressed by the actions of MDMX and its homologue, MDM2.[38,51] MDMX appears to play a more important role in this repression, as evidenced by the lethality of MDMX knockout mice containing functional MDM2.[53] Thus, MDMX/MDM2 forms a complex with activated p53, which requires antirepression for transcriptional function.[31] This alternate version for inducing p53 transcriptional activity suggests the following sequence of events: p53 is stabilized in response to stress via a number of mechanisms, many of which inhibit MDM2 and ubiquitination. This is followed by acetylation of p53 lysines, leading to antirepression and release of p53 from MDM2/MDMX and the phosphorylation of key serines on p53. Full activation necessitates the interaction of p53 with cofactors, some of which modify p53 as well as target histones and other transcriptional activators.

Therapeutic targeting of MDM2-p53 interaction

X-ray crystallography has been used to map the site of interaction between p53 and MDM2. This site has been identified as the N-terminus of p53 which contains four hydrophobic residues which interacts with a deep hydrophobic pocket within the first approximately 120 amino acids of MDM2. Three of these p53 amino acids are critical for this binding: Phe19, Trp23 and Leu26.[9,54] Many experimental approaches have been used to demonstrate that the antagonism of MDM2 results in activation of p53 tumor suppression. These approaches include the inhibition of MDM2 expression, the inhibition of MDM2 ubiquitin ligase activity, and, most clinically relevant, the use of nonpeptidic small-molecule inhibitors to block interaction between MDM2 and p53.[55,56] Beginning in the 1990s, studies of MDM2 downregulation

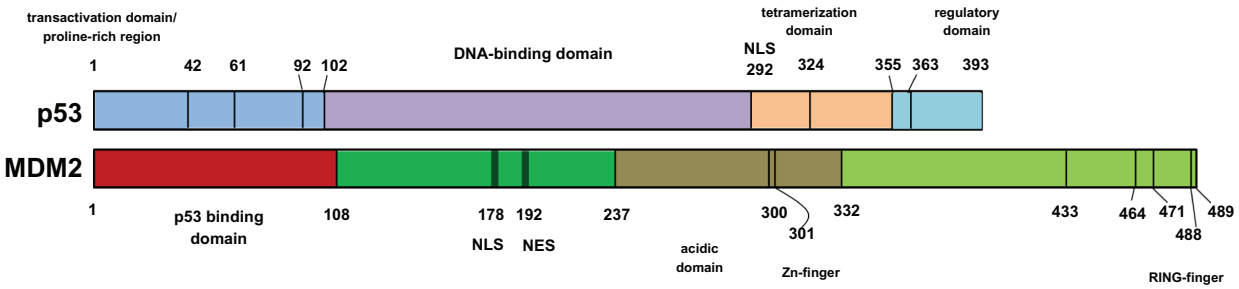

Figure 1 Schematic of the functional domains of p53 (top) and MDM2 (bottom). In unstressed cells, three N-terminal p53 residues (F19, W23 and L26) are indispensible for binding to a small, deep hydrophobic pocket in the N-terminus of MDM2 to inhibit p53 function. The transactivation/proline-rich domain of p53 responds to stress signals of DNA damage/radiation/UV by site-specific phosphorylation. The C-terminus ("regulatory domain") of p53 contains six lysine residues which respond to stress by acetylation, inhibiting MDM2-mediated ubiquination, carried out within the RING-finger motif of MDM2. Zinc fingers generally act as motifs for protein-protein or protein-nucleic acid interactions, but the exact role in MDM2 is unclear. The acidic domain of MDM2 contains residues essential for phosphorylation and regulation, and residues 464–471 contain a nucleolar localization signal.

Abbreviations: NLS, nuclear localization signal; NES, nuclear export signal; Zn-finger, Zinc finger.

using antisense oligonucleotides have demonstrated p53 activation.[57,58] Although these antisense oligonucleotides suppressed tumor growth in mouse xenograft models, they have been difficult to develop for clinical applications. A second generation of MDM2 antagonists targeted the E3 ligase activity of MDM2 but lacked potency and specificity and demonstrated p53-independent off-target effects.[59]

More recently, the design of nonpeptidic, small-molecule MDM2 inhibitors has provided the proof-of-concept needed to advance the development of a number of small molecule inhibitors now in use in many preclinical studies. Small molecule MDM2 inhibitors were developed with three essential therapeutic properties in mind: 1) high binding specificity and affinity to MDM2, 2) potent anticancer efficacy especially in cancers with p53 wild-type (wt) status, and 3) favorable pharmacological and pharmacokinetic properties.

Several classes of these inhibitors have now been reported. These include analogs of spiro-oxindole, benzodiazepine, terphenyl, quilinol, chalone and sulfonamide, among others. Two of the most potent nonpeptidic MDM2 inhibitors (MI-63 and MI-219) are based on a spiro-oxindole structure, and one (MI-63) was found to have an extraordinarily high K_i of 3 nm; however, MI-63 displayed unfavorable pharmacological properties *in vivo* in mouse studies. MI-219 (Ascenta Therapeutics, Malvern, PA, USA) was developed as an analog of MI-63 to overcome these properties.[19] Preliminary data in human cell lines have shown that MI-219 is a promising therapeutic for treatment of cancer with wild-type p53 and lacks toxicity to normal cells.[60] Two benzodiazepines (BDPs) have been identified that disrupt the MDM2-p53 interaction, but *in vitro* studies are incomplete.[19]

The most widely reported of the MDM2 inhibitors are analogs of *cis*-imidazoline, which includes nutlin-3a (Hoffman-La Roche, Nutley, NJ, USA). To date, 160+ preclinical cancer therapy studies using nutlin-3a have been published. The nutlins were isolated from a racemic mixture in 2004 by Vassilev et al.[20,61] Nutlin-3a, an active enantiomer of nutlin-3, binds to MDM2 with high affinity (K_i = 36 nm). It blocks intracellular interaction of p53 with MDM2 by binding the N-terminal region of MDM2, inducing accumulation and activation of p53. In contrast to the vast majority of chemotherapeutics, nutlin-3a is nongenotoxic and does not typically induce apoptosis. Instead, it acts primarily by promoting cell cycle arrest,[20,55,62] cell senescence[63] and differentiation.[64] The stabilization, activation or antirepression of p53 by nutlin-3a are sufficient to activate genes involved in blocking cell cycle progression. However, these effects are insufficient to induce apoptosis, which requires additional p53 posttranslational modifications.[65] Thus, in preclinical trials, to promote apoptosis, nutlin-3a is usually used in combination with other chemotherapeutics. In tumors containing wt p53, it synergizes with the genotoxic agent doxorubicin,[66] and it has also been shown to be effective in inducing proteins of the extrinsic apoptotic pathway, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[41] It has shown particular effectiveness when used in combination with drugs that target cell cycle progression, by inducing cell cycle arrest in G1-S and G2-M while protecting noncancerous cells. Pretreatment with nutlin-3a before treatment with taxanes, which kill cells in M phase, protects normal cells. Likewise, pretreatment before gemcitabine and Ara-C therapy (both of which kill cells in S-phase) block normal cells in G1 and G2, respectively. Thus, activation of p53 in the absence of DNA damage protects normal tissues. This lack of genotoxicity to normal tissues is important therapeutically, although the regulatory mechanisms are not fully understood.[60,61] (Figure 2)

Both nutlin-3a and MI-219 bind to MDM2 with a much higher affinity than to MDMX. However, MDMX modulates the activity of MDM2 inhibitors; tumors which overexpress MDMX are known to abrogate the action of MDM2 inhibitors, and cell lines with lower MDMX levels are more sensitive to nutlin-3a treatment.[53] Similarly, the tumors most responsive to MDM2 inhibitors are those overexpressing MDM2 with low MDMX expression. These findings suggest that small molecule inhibitors should be developed that target both MDM2 and MDMX.

The retinoblastoma protein (Rb) is a tumor suppressor protein inactivated in many cancers. One of its best known functions is cell-cycle regulation. One recent insight into nutlin-3a action comes from studies of the Rb tumor suppressor.[67] p53 is required for transcription of the Rb gene,[68] and the Rb protein interacts with E2F1 (a member of the E2F transcription factor family with apoptotic properties) to induce assembly of transcription repressor functions;[69,70] these inhibit expression of E2F target genes usually involved in cell cycle progression. In this manner, Rb represses cell cycle progression genes. Hypophosphorylation of Rb leads to suppression of constitutive growth,[71] and p21 is responsible for accumulation of hypophosphorylated Rb. In most cells, treatment with nutlin-3a induces both increased levels of Rb hypophosphorylation and cell cycle arrest, while in a small subset of cells, lower levels of hypophosphorylated Rb are induced and cells enter apoptosis.[72] Thus, it appears that high levels of hypophosphorylated Rb inhibit apoptosis

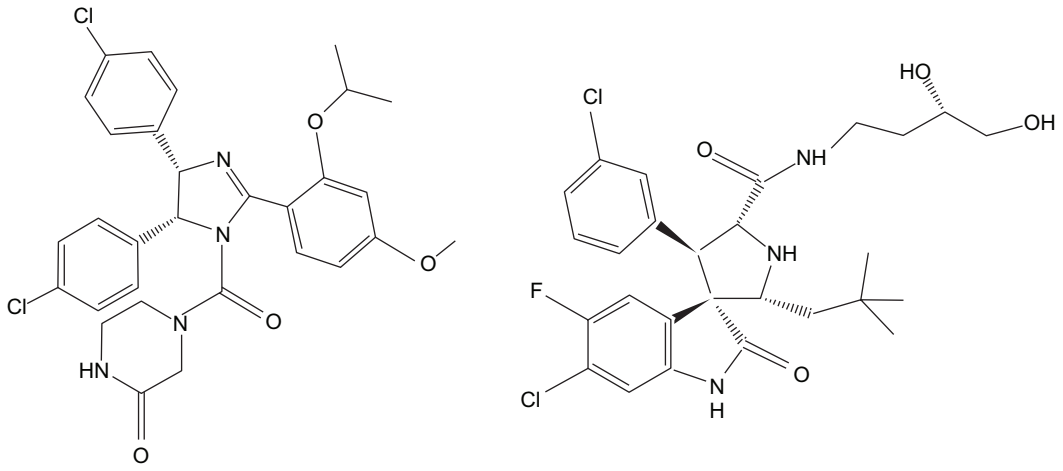

Figure 2 Chemical structures of nutlin-3a (**a**) and MI-219 (**b**), two small-molecule inhibitors of the MDM2-p53 interaction in advanced preclinical development for the treatment of cancer. Nutlin-3a is a *cis*-imidazoline analog, and MI-219 is a spiro-oxindole analog.

induced by nutlin-3a, and instead promote cell-cycle arrest, effects that are context-dependent.[67]

E2F1 plays an important role in the response of cells and tumor tissues to nutlin-3a treatment. E2F1 binds to MDM2 and nutlin-3a potentiates chemotherapy by inhibiting this interaction and inducing overexpression of E2F1. Overexpression of E2F1 is known to increase Bax translocation from the cytosol to the mitochondria,[73] and expression of the apoptotic protein Puma is transcriptionally upregulated by E2F1, which contains six E2F1 binding sites in its promoter.

Nutlin-3a has an inhibitory effect on angiogenesis. Hypoxia, a typical feature of advanced tumor growth, promotes angiogenesis by upregulating vascular endothelial growth factor (VEGF), a key angiogenic factor. Under hypoxic conditions, HIF-1alpha and its transcriptional target VEGF are both upregulated. An earlier study indicated that the inhibition of MDM2 interaction with HIF-1-alpha promotes its degradation, leading to suppression of VEGF expression.[75] Recently, it was shown that nutlin-3a directly inactivates the C-terminal transactivation domain of HIF-1alpha, leading to an inhibition of the expression of VEGF.[74]

Nutlin-3 is effective in the absence of functional p53

p53 is mutated or absent in many cancers, a feature which has major therapeutic implications. p53 is a member of a large family of proteins of which p63 is the ancestral gene. From genetic analyses, it has been found that p73 and then p53 evolved from this ancestral form.[76] Unlike p53, p73 family members are rarely mutated in cancer. TAp73 (transactivating p73, functionally similar to p53) and ΔNp73 (TA-truncated p73) act with p53 to maintain cellular equilibrium. ΔNp73 occurs as one of 4 isoforms, all of which act in a dominant-negative manner to suppress p53 and TAp73, while TAp73 can be induced by chemotherapeutic treatment (including camptothecin, etoposide, cisplatinum, doxorubicin, and Taxol) at low concentrations in cells with mutant or deleted p53.[77] TAp73 is an important determinant of chemosensitivity in many human cancers; however, mutant p53 often contains polymorphisms that function to inhibit TAp73, thus inducing drug resistance.[77,78] Mutant p53 and ΔNp73 are able to inhibit p53 and TAp73 by direct neutralization of TAp73 function, heterocomplex formation and possibly by promoter competition.[78,79] Many human cancers express upregulated ΔNp73, and, even in the presence of wild-type p53, drug resistance may be present.[80] ΔNp73 is also known to cooperate with the oncogenic Ras protein to induce high levels of phosphorylated Rb,[81] and ΔNp73 expression has a role in neuroblastoma oncogenicity.[82]

In model systems, nutlin-3 is effective in the absence of wild-type p53, a feature attributable to the presence of TAp73.[83,84] In addition to its interaction with p53, the N-terminus of MDM2 interacts with TAp73 as well as E2F1. The three key p53 amino acids (Phe 19, Trp23 and Leu26) that bind MDM2 also function to bind TAp73 to MDM2.[85] Thus, nutlin-3a treatment upregulates TAp73 function in the same manner in which it upregulates p53: by disrupting the interaction between MDM2 and TAp73, leading to TAp73

stabilization. However, markedly higher concentrations of nutlin-3a are required for TAp73-dependent effects and E2F1 mediated apoptosis.[86] Recently, cycloxygenase (COX) inhibitors have been shown to downregulate the multiple isoforms of ΔNp73 in NB;[85] combination treatment of COX inhibitors with nutlin-3a may represent a novel approach to the treatment of NB containing high levels of ΔNp73 in the absence of wild-type p53.

MDM2 inhibition for treatment of neuroblastoma

Neuroblastoma (NB) is a tumor derived from precursor cells of the sympathetic nervous system and the second most common solid tumor in children. Mutations of p53 are absent at diagnosis, although wild-type p53 is frequently hyperubiquitinated and sequestered in the cytoplasm.[87] Most neuroblastomas respond to initial treatment, and complete regression without treatment can occur in infancy. Doxorubicin is a common first-line treatment. In addition to its role as an intercalator of DNA, it also functions as an inhibitor of MDM2 enzymatic activity, blocking MDM2 E3 ligase activity and hence the ubiquitination of p53.[88] However, relapse with chemoresistant disease is common and is often associated with MDM2 overexpression, suppressed $p14^{ARF}$ function, and less commonly, p53 mutations.[22,23] High-risk NB remains one of the most problematic childhood cancers, and children with high-risk disease have a long-term survival rate of around 30%.[21] The specific targeting of the $p14^{ARF}$/MDM2/p53 axis in NB has become a research area of keen interest.

MycN oncogene amplification occurs in approximately 25% of neuroblastomas and is a highly significant prognostic marker for poor survival, with many relapse cases of NB expressing the MycN protein at high levels. MycN is a member of the basic-helix-loop-helix zipper family of transcription factors and binds to an E-box motif consensus sequence within the promoter of target genes, including, most importantly, MDM2. Thus, *MycN* amplification and overexpression is linked to MDM2 overexpression.[89,90] MycN has also been shown to mediate centrosome amplification in NB, leading to karyotypic instability.[91]

Recent work provides evidence that, paradoxically, p53 is directly targeted for transcription by MycN, which binds to an E-box motif within the p53 promoter. In these and other studies, fully functional p53 displays nuclear accumulation in neuroblastomas expressing high levels of MycN. Because neuroblastoma derives from undifferentiated neuroblasts, it is conjectured that MycN and p53 function together in balance during early embryonic development, as MycN plays a role in p53-driven apoptosis. However, both MycN and p53 are frequently overexpressed in primary neuroblastomas. Studies of a MycN-transgenic mouse model have shown that targeted expression of MycN in neural crest lineage cells leads to hyperproliferation.[12] MycN-overexpressing cells develop mechanisms for evading p53-induced apoptosis; this MycN phenotype is generally associated with an aberrant $p14^{ARF}$/MDM2/p53 pathway.[92] For example, MycN-amplified and overexpressing neuroblastomas are often resistant to first line drugs (including doxorubicin, cyclophosphamide, vincristine, cisplatin and etoposide, among others) for treatment of NB. Therapy-resistant and relapse tumors also often display alterations including MDM2 overexpression and deletion or methylation of $p14^{ARF}$, as well as downstream p53 mutations.[23]

In experimental settings, treatment with nutlin-3a results in rapid and dramatic accumulation of p53 in wt-p53 NB cells one to two hours following combination treatment with cisplatin or etoposide.[26,93] This response to nutlin-3a response is independent of *MycN* amplification status. Recently, nutlin-3a treatment in combination with vincristine was found to be highly effective against p53-mutated multidrug resistant NB cells *in vitro*.[94] Very recently, it was found that nutlin-3a is effective against chemoresistant NB containing both ΔNp73 and wt-p53, and is able to suppress NB cell growth in a subcutaneous xenograft model system.[95] In some NB cell lines, nutlin-3a promotes a senescence-like phenotype, while in others, it drives differentiation.[95] In culture, endogenous TAp73 levels, but not p53 levels, induce differentiation of NB cells by both neurite outgrowth and by the downregulation of N-Myc and upregulation of Rb.[96] The therapeutic upregulation of TAp73 in NB may therefore be of clinical importance.

In our laboratory, nutlin-3a was used to sensitize a p53-null and doxorubicin-resistant NB cell line to doxorubicin. We found that nutlin-3a treatment upregulated TAp73 and E2F1 protein levels, while also potentiating the ability of doxorubicin to block cell proliferation and activate apoptosis.[84] Based on our results, we postulated a mechanism for the interaction of TAp73 and E2F1 in p53-null NB cells treated with combined nutlin-3a and doxorubicin. In untreated cells, TAp73 and E2F1 are bound to MDM2. Combination treatment leads both to nutlin-3a mediated dislocation of E2F1/TAp73 from MDM2 and doxorubicin-mediated DNA damage. Without TAp73 dislocation from MDM2, induction of downstream apoptotic proteins will occur at only low levels. DNA damage induces the checkpoint kinases Chk1/Chk2

which subsequently phosphorylate both E2F1 and TAp73, stabilize E2F1 and promote TAp73 transcription. Thus, without concurrent DNA damage, we hypothesize that p53-null tumors will be resistant to nutlin-3a.

Conclusions

Activation of the p53 tumor suppressor pathway by MDM2 inhibition has been studied as a therapeutic strategy for the treatment of malignancies for over a decade. Although considerable progress has been made in the development of p53-MDM2 interaction inhibitors for the treatment of cancer, few have demonstrated the necessary specificity, potency or pharmacokinetic properties for *in vivo* evaluation. Nutlin-3a, a *cis*-imidazoline, is the most clinically promising and is currently in advanced preclinical development for treatment of tumors with wild-type p53. However, much less is known about the mechanisms of nutlin-3a action in systems where p53 is directly mutated or its function is inhibited. This would include chemoresistant cancers such as NB in which MDM2 is frequently overexpressed or the $p14^{ARF}$/p53 pathway is altered. Recent data showing that nutlin-3a is inhibitory against p53-mutant and p53-null NB cells suggests that this agent may have a wide spectrum of activity in tumors regardless of their p53 status. This attribute, in combination with its low toxicity, should provide impetus for clinical trials of nutlin-3a in NB and other tumors characterized by an aberrant p53/MDM2/$p14^{ARF}$ pathway.

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Disclosure

The authors report no conflicts of interest in this work.

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