

MDM2-p53 Interaction in Paediatric Solid Tumours: Preclinical Rationale, Biomarkers and Resistance

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Abstract: p53 is one of the main regulators of apoptosis, senescence, cell cycle arrest and DNA repair. The expression, function and stabilization of p53 are governed by a complex network of regulators including p14^{ARF} and MDM2. MDM2 is the main negative regulator of p53 activity and stability.

Unlike tumours in adults, which tend to overcome p53 regulation by p53 mutations, the paediatric tumours neuroblastoma and sarcoma frequently retain wild type p53. Nevertheless, in childhood cancer the p53 pathway is commonly impaired due to upstream MDM2-p14^{ARF}-p53 network aberrations. In contrast, aberrations of the p53 downstream pathway are very rare. In cancer cells with intact p53 downstream function MDM2 inhibition, and subsequent rapid increases in nuclear p53 levels, potentially “re-activate” dormant apoptotic pathways and rapidly induce apoptotic cell death. As a result MDM2-p53 interaction inhibitors, including *cis*-imidazolines analogs (Nutlins), are potentially very effective agents in neuroblastoma and sarcomas.

Predictive biomarkers are important as a lack of p53 mutations appears to reliably predict response to these inhibitors. Tumours should be screened for p53 mutations in children considered for MDM2-p53 interaction inhibitors. In addition, it is essential that other predictive biomarkers are investigated. The serum concentration of macrophage inhibitory cytokine-1 (MIC-1) may be a good pharmacodynamic biomarker based on recent findings.

In conclusion, targeting the interaction between p53 and its main negative regulator MDM2 represents a major new therapeutic approach in poor prognosis paediatric malignancies without p53 mutations.

Keywords: MDM2-p53 interaction, nutlin, neuroblastoma, paediatric oncology, retinoblastoma, sarcomas.

BACKGROUND

Today cancer in children is the most common cause of death after accident [1]. Despite encouraging improvements in outcome during the past decades, survival is dismal for a group of children with poor prognosis cancers who relapse (<25% overall 5 year survival at diagnosis) [2]. One of the biological hallmarks of cancer is its ability to continuously proliferate and escape tumour surveillance mechanisms like senescence and apoptosis [3]. The p53 protein is one of the key regulators of cell cycle arrest, apoptosis, DNA repair and senescence. It is activated and stabilised in the presence of stress events such as DNA damage from radiotherapy or chemotherapy, oncogenic mutations, hypoxia and other tumourigenic stimuli. p53 is a transcription factor upregulating many genes involved in cell cycle arrest and apoptosis and is usually expressed at low levels in the cell where it has a short half-life of only 30 minutes. The expression, function

and stabilization of p53 are governed by a complex network, which includes its regulators p14^{ARF} and MDM2 [4].

As a central modulator of numerous essential cellular processes, p53 is positioned at the nexus of many upstream regulators [5, 6]. Tumours that retain the wild-type p53 gene virtually always have defects in other components of the p53 pathway, resulting either in loss of p53 stabilization and activation following stress signals or in disruption of critical mediators of p53 transcriptional activity [4].

A typical example of non-mutational inactivation of p53 is over-expression of the E3-ubiquitin ligase MDM2. This nuclear protein is the main negative regulator of p53 activity and stability (see Fig. 1). MDM2 blocks the transactivation domain of p53, recruits co-repressor proteins to p53, stimulates p53 nuclear export where it promotes p53 ubiquitination and degradation [4, 7]. The expression of MDM2 is directly regulated by p53, resulting in a negative autoregulatory feedback loop that keeps p53 levels and activity low in unstressed cells and helps to switch off p53 at the end of a stress response [8]. MDM2 is in turn inhibited by the tumour suppressor p14^{ARF}, which prevents MDM2-mediated ubiqui-

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tion of p53, blocks nuclear export of MDM2 and p53, and sequesters MDM2 into the nucleolus (see Fig. 1) [4]. In cancer cells with intact p53 function (wild type p53 and a functional downstream pathway), MDM2 inhibition and subsequent rapid increases in nuclear p53 levels potentially “re-activate” dormant apoptotic pathways and rapidly induce apoptotic cell death [9].

It has been reported that most paediatric tumours tend to escape p53 control through inactivating upstream lesions in the p53 pathway rather than via *p53* mutations or downstream pathway aberrations, in contrast to what happens in adult cancers where *p53* mutations are described in almost 50% of all tumours at diagnosis [4, 10, 11]. A potential novel approach to cancer treatment in children is to re-establish the disrupted p53-related network in order to reinstate p53-dependent cell cycle arrest and apoptosis in tumours where p53 activity is still preserved and sometimes its expression even increased, such as in *MYCN* amplified neuroblastoma [12].

To date the best-characterized MDM2/p53 antagonist is the *cis*-imidazoline derivative, termed Nutlin-3a, discovered by high-throughput screening. Hoffmann-La Roche termed the imidazoline derivatives ‘Nutlins’ after the location of the Roche research site in Nutley, New Jersey [13]. High-resolution X-ray structure studies showed these *cis*-imidazolines bound to the p53-binding site of MDM2 by mimicking the interaction of the three critical amino acid residues.

This review analyses the rationale for MDM2/p53 as a therapeutic target in paediatric solid tumours and the pre-clinical data of pharmacological inhibitors of the pathway that could potentially be used in the treatment strategy of several paediatric solid tumours such as neuroblastoma, sarcomas and retinoblastoma. The rationale for the use of MDM2/p53 inhibitors in paediatric leukaemias has been reviewed elsewhere [14] and falls outside the scope of this review.

NEUROBLASTOMA

Neuroblastoma is the most frequent solid extracranial tumour of childhood. More than 1200 new cases per year are diagnosed in USA and Europe [15]. This aggressive embryonal malignancy is one of the major causes of death in children from cancer and accounts for approximately 15% of all paediatric cancer deaths [16]. Fifty per cent of cases are considered high risk (e.g. tumours with *MYCN* amplification or metastatic) and for those, outcome after multimodal treatment (chemotherapy, surgery, high-dose chemotherapy with haematopoietic stem cell rescue, radiotherapy and minimal residual disease therapy) is still poor. Five-year overall survival has remained below 40% in larger multicentre trials without immunotherapy [17, 18]. Recently the addition of immunotherapy with an antibody targeting disialoganglioside (GD₂) Ch14.18 anti-GD₂, IL2 and GM-CSF to standard treatment has shown an increase in 2-year progression-free survival to 60% and 2-year overall survival to 80% [19]. A considerable proportion of patients eventually still die

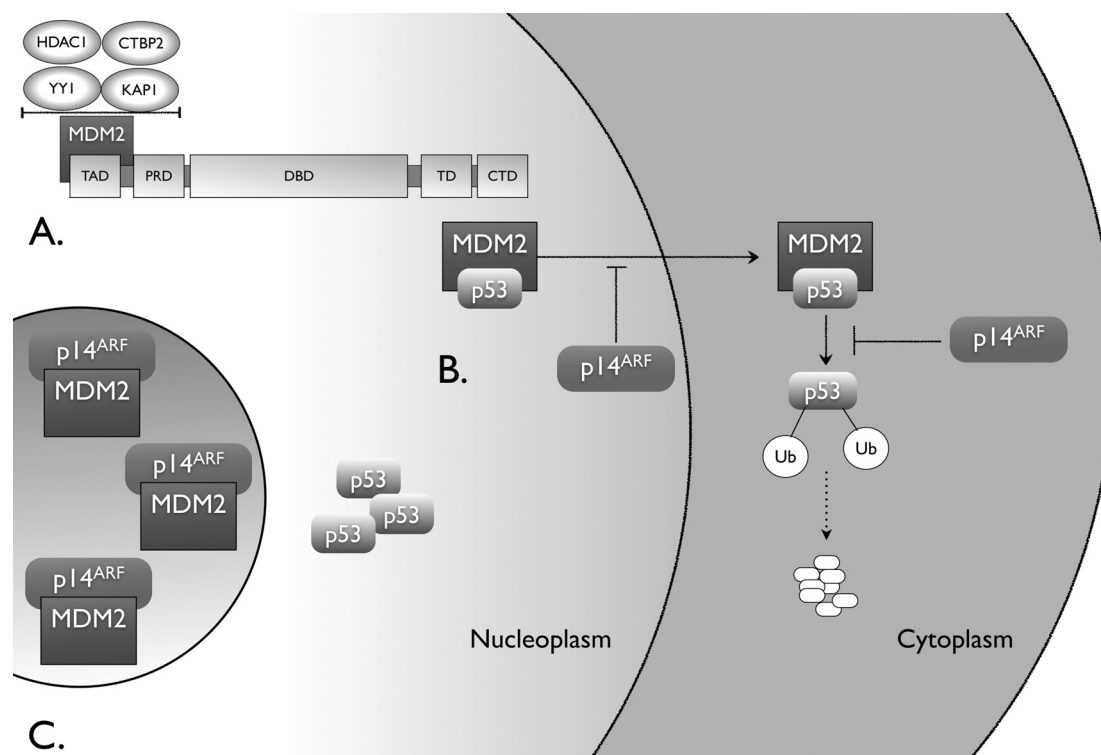


Fig. (1). Non mutational inactivation of p53 through MDM2 and p14^{ARF} inhibitor activity of MDM2. **A).** MDM2 blocks the transactivation domain of p53 and recruits co-repressor proteins to p53 such as HDAC1, CTBP2, YY1 and KAP1 (TAD, transactivation domain; PRD, proline-rich domain; DBD, DNA-binding domain; TD, tetramerization domain; CTD, C-terminal regulatory domain). **B).** MDM2 stimulates nuclear export where it promotes p53 ubiquitination and degradation. **C).** MDM2 is in turn inhibited by the tumour suppressor p14^{ARF}, which prevents MDM2-mediated ubiquitination of p53, blocks nuclear export of MDM2 and p53, and sequesters MDM2 into the nucleolus.

from their disease [20]. Patients who experience relapse or progression have an extremely poor outcome with long-term survival of 8% [21]. There is an unmet need to develop new anti-cancer drugs for these poor prognosis patients.

p53 IN NEUROBLASTOMA

There are substantial pre-clinical data to suggest that MDM2/p53 inhibitors will be active in paediatric tumours such as neuroblastoma. p53 in neuroblastoma is usually nuclear and wild-type at diagnosis with intact intrinsic and extrinsic apoptotic mechanisms, including mitochondrial-mediated cytochrome c release and caspase activation, with less than 2% tumours having p53 mutations (reviewed by [22]). Even in the relapsed setting p53 mutations have been reported in only 15% of tumours in the only published series to date of paired diagnostic and relapsed neuroblastomas [23].

p53 has been reported to be a direct transcriptional target of MYCN in neuroblastoma and sensitises cells for MYCN driven apoptosis [12, 24]. More recent data suggests that MYCN-dependent sensitization to apoptosis requires activation of p53 and its phosphorylation at serine 46. The p53S46 kinase HIPK2 (Homeodomain Interacting Protein Kinase 2) accumulates on MYCN expression, and its depletion by RNA interference impairs p53S46 phosphorylation and apoptosis [25].

MYCN and p53 are both expressed in the normal embryonic nervous system, during the phase of precursor cell expansion prior to the onset of differentiation [26]. In the context of normal embryonic development, MYCN driven p53-dependent apoptosis may be important in eliminating any rapidly proliferating neuroblasts exposed to potential teratogens, to prevent deregulated proliferation and aberrations during development [27].

However, the various selective pressures on MYCN amplified tumours either spontaneously or by chemotherapy may lead to selection for those cells that can evade apoptosis by various mechanisms which result in MYCN amplified neuroblastoma progressing to a more aggressive phenotype.

There is evidence from *in vitro* studies that MYCN amplified cell lines may circumvent MYCN driven p53-dependent apoptosis by selecting for cells with aberrations in the p53/MDM2/p14^{ARF} pathway (reviewed in [4]). Analysis of neuroblastoma cell lines reported to date with aberrations in the p53/MDM2/p14^{ARF} pathway demonstrated that 25/34 (74%) cell lines are MYCN amplified and predominantly established following previous therapy at relapse [12, 23].

In particular, suppression of the MDM2 inhibitor p14^{ARF} through multiple mechanisms (i.e., deletions or epigenetic silencing) [23, 28], amplification of MDM2 [29], and elevated expression of p14^{ARF} inhibitors BMI-1 [30] and TWIST-1 [31] are all found in subsets of primary and relapsed neuroblastoma samples. The presence of wild-type p53 could explain why neuroblastoma may be initially quite chemosensitive, particularly MYCN amplified cases, however despite an initial response, more than half of all cases relapse with chemoresistant disease confirming that MYCN amplified tumours eventually develop mechanisms to evade MYCN driven apoptosis [27].

There is also evidence that some of the most frequent segmental chromosomal aberrations in neuroblastoma result in increased activity of MDM2 or suppression of p14^{ARF}. The most common and prognostically most unfavourable genomic alteration in neuroblastoma, after MYCN amplification, is unbalanced gain of chromosome 17q [32, 33]. The PPM1D (WIP1) gene at 17q23.2, which encodes a phosphatase that stabilizes MDM2 and increases the affinity of MDM2 for p53 [34], might be one of the targets of 17q gain in neuroblastoma [35]. Another important chromosomal abnormality is 1p deletion, which occurs in 25 to 35% of primary neuroblastoma tumours [36]. Detailed analysis of the different genes located in the smallest region of deletion at 1p36.31 points to CHD5 as the strongest candidate tumour suppressor gene in this region [37, 38]. The CHD5 protein has been shown to be required for transcriptional activation of p19^{ARF} in mouse strains with rearrangements corresponding to the human 1p36 region and to control proliferation, senescence, and apoptosis *via* the p19^{ARF}-p53 pathway [39].

Inhibition of the expression or function of MDM2 in neuroblastoma cells leads to nuclear accumulation of functional p53 [40-42], indicating that targeting of MDM2 may offer therapeutic benefit. Further illustrating the complexity of MYCN paradoxical role in proliferation and apoptosis, MDM2 has also been reported to be a direct transcriptional target of MYCN [8] and very recently MDM2 has been reported to stabilise MYCN mRNA and its translation in a p53 independent manner so forming a positive feedback loop to promote MYCN amplified neuroblastoma growth and survival [43].

Fig. (2) summarises the p53 upstream control network and its relationship with MYCN.

MYCN amplified neuroblasts proliferate and transform despite the presence of wild-type p53 and the strong differentiation programme driven by, and the apoptotic stimuli within, the microenvironment [44, 45]. Recent data from a transgenic mouse model demonstrate that this process is markedly restrained by MDM2 haploinsufficiency, suggesting that aberrant regulation of MDM2 by the MYCN transgene is an important component of overall suppression of p53 in neuroblastoma [44].

Aggressive neuroblastoma tumours that lack MYCN amplification typically show markers of increased c-MYC activity [24]. It is conceivable that these tumours also rely on MDM2 to inhibit p53-mediated apoptosis, as has been shown for other c-MYC driven malignancies [46].

PRECLINICAL USE OF MDM2/P53 INHIBITORS IN NEUROBLASTOMA

Nutlin-3a inhibits the MDM2-p53 interaction and restores the p53 pathway *in vitro* and *in vivo* [13]. Nutlin-3 has been shown to be effective against a variety of cancer cells with wild-type p53, including neuroblastoma, retinoblastoma [47], osteosarcoma [13] and leukaemia [48-50]. Several studies have shown that Nutlin-3 is less toxic to normal cells than to neoplastic cells. Although transient p53 activation by blockade of the MDM2-p53 interaction leads to cell cycle arrest in both normal cells and neoplastic cells, normal cells are more resistant to apoptosis than neoplastic cells [51-53].

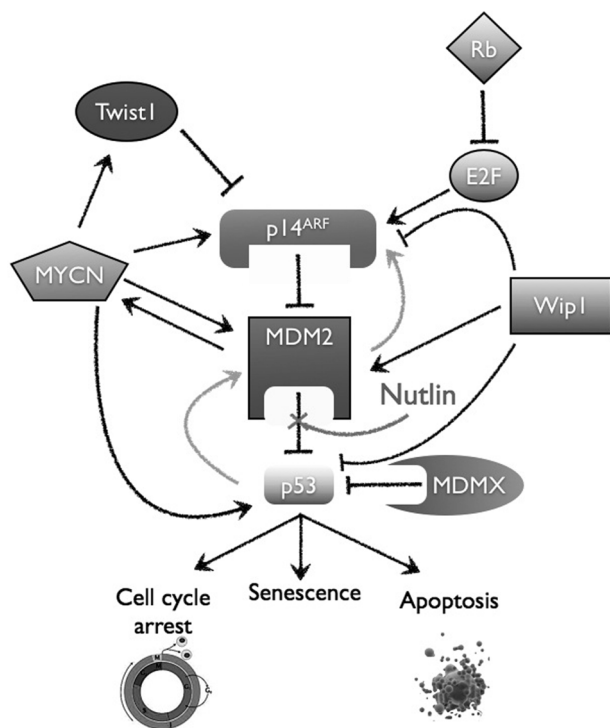


Fig. (2). Upstream p53 regulatory network. The expression, function and stabilization of p53 are governed by a complex network, which includes p14^{ARF} and MDM2. MDM2 is the main negative regulator of p53 activity and stability. Expression of MDM2 is regulated by p53 itself resulting in a negative auto-regulatory feedback loop. MDM2 is in turn inhibited by p14^{ARF}. In neuroblastoma there is a close functional interaction between MYCN and MDM2. MDM2 is found to be a direct transcriptional factor of MYCN. Moreover, suppression of the MDM2 inhibitor p14^{ARF} by TWIST1 and BMI-1 is found in both primary and relapsed neuroblastoma. Wip1 stabilizes MDM2 and increases its affinity for p53. *WIP1* gene is located on chromosome 17q whose gain is associated with poor prognosis neuroblastoma. p53 has also been reported to be a direct transcriptional factor of MYCN in neuroblastoma. MDM2 has been reported to stabilize MYCN mRNA and its translation in a p53 independent manner so forming a positive feedback loop to promote MYCN amplified neuroblastoma growth and survival. In the normal retina Rb suppresses E2F and prevents transcriptional activation of p14^{ARF}. When Rb activity is lost E2F activates transcription of p14^{ARF}, which leads to p53 activation by suppression of MDM2. In retinoblastoma increased expression of MDMX has been reported in 65% of human sample and this represents another mechanism of p53 inactivation.

Several studies have been published showing pre-clinical activity of the MDM2/p53 antagonist Nutlin-3 in neuroblastoma cell lines alone and in combination with conventional chemotherapy (such as cisplatin, etoposide and camptothecin) [54, 55], and in neuroblastoma xenograft models including multidrug resistant p53 wild-type cell line xenografts [56].

It has been recently shown that Nutlin-3, and a structurally unrelated MDM2/p53 antagonist, a spirooxindole MI-63, are more active against MYCN amplified neuroblastoma cell lines [57]. Gamble *et al.* showed this by an increased p53 transcriptional response, increased apoptosis and en-

hanced growth inhibition in MDM2 amplified neuroblastoma cell lines compared with non-MYCN amplified neuroblastoma cell lines and in the regulable MYCN expressing neuroblastoma cell line (SHEP-Tet21N) in the presence of MYCN [57]. The hypothesis was that this greater sensitivity was at least in part because p53 is a direct transcriptional target of MYCN [12, 24] and because the p53 pathway is already activated in MYCN amplified neuroblastoma. It was hypothesised that MDM2/p53 antagonists will be more active against MYCN amplified neuroblastoma. These results have not been confirmed by other authors [58] who showed a MYCN-independent activity of Nutlin-3, although this study did not use the isogenic SHEP-Tet21N cell line, and this issue therefore needs clarification. Other authors [54] showed a trend toward increased sensitivity to Nutlin-3a and to cisplatin plus Nutlin-3a in the MYCN-induced version of the SHEP-Tet21N but not in the MYCN inducible SHEP MYCN3 cell line.

Very recently Gamble *et al.* have investigated the effect of p14^{ARF} inactivation on the response to MDM2/p53 antagonists and have shown that p14^{ARF} inactivation results in a preferential G₁ arrest and lower levels of apoptosis following treatment with MDM2/p53 antagonists (Gamble *et al.*, personal communication). These findings are in line with another study that has identified a potentiating effect of p14^{ARF} expression on the response of neuroblastoma cells to Nutlin-3 [59].

An interesting observation is that Nutlin-3 does not only induce G₁ cell cycle arrest and apoptosis in neuroblastoma cells with wild-type p53, but also premature cellular senescence and neuronal differentiation depending on the cellular background [58]. SK-N-SH cells that survived Nutlin-3 treatment acquired a senescence-like morphology and stained positive for senescence-associated β -galactosidase. Nutlin-3 induced a polar morphology and neurite outgrowth in surviving NGP and CLB-GA cells. These observations indicate that Nutlin-3 has pleiotropic activities to counteract the malignant phenotype of neuroblastoma cells and suggest that selective MDM2/p53 inhibitors are well suited for treating tumours that are arrested in their differentiation, such as neuroblastoma.

Of note, Nutlin-3 may revert multidrug resistance of p53 mutant neuroblastoma and rhabdomyosarcoma cells by inhibiting the drug efflux activity of P-glycoprotein and MRP-1 [60]. In another study it was shown that neuroblastoma cells with acquired multidrug resistance to conventional chemotherapeutics drugs remain sensitive to Nutlin-3, provided that wild-type p53 is retained [56]. Nutlin-3 treatment of mice bearing chemoresistant neuroblastoma xenografts with wild-type p53 induced a reduction in the tumour growth rate, whereas this was not the case for chemoresistant xenografts with mutant p53. Nutlin-3 was also able to induce anti-tumour activity against metastatic neuroblastoma lesions, as MYCN DNA content, MYCN mRNA levels and the amount of metastatic foci in liver and lungs were lower in mice treated with Nutlin-3 [56].

NUTLIN-3 AND ANGIOGENESIS IN NEUROBLASTOMA

Neuroblastoma has been recently seen as a potential target for anti-angiogenesis [61]. Patterson *et al.* studied the

effects of Nutlin-3a treatment on tumour angiogenesis with or without bevacizumab [62]. Tumour growth and angiogenesis following MDM2 inhibition and anti-VEGF antibody therapy were assessed in an orthotopic (renal capsule injection) xenograft model of neuroblastoma, which recapitulates the highly vascular and invasive growth pattern seen in patients. Nutlin-3 was shown to suppress HIF-1 α , to downregulate VEGF expression and to inhibit tumour angiogenesis in neuroblastoma, independent of its pro-apoptotic activity mediated through p53 [62]. By adding upstream repression of HIF-1 α with Nutlin-3a, the efficacy of bevacizumab was increased [62].

The combination of Nutlin-3a and bevacizumab more effectively inhibited xenograft tumour growth than either agent alone. Compared to control, Nutlin-3a and bevacizumab partially suppressed tumour growth by 57% and 44%, respectively, whereas the combination treatment reduced tumour growth by 79% [62].

Interestingly, compared to control tumours, there was significant suppression of microvessel density, which was more pronounced in the combination group compared to either bevacizumab or Nutlin-3a as single agent. While Nutlin-3a and bevacizumab both demonstrated significant anti-angiogenic effect, combining the two agents significantly suppressed CD-31 staining (a marker for endothelial cells) compared to either treatment alone [62]. This data suggests that combination therapy cooperatively inhibits endothelial cell development in neuroblastoma.

The authors also investigated for possible effects of the combination treatment on metastases. Total metastatic burden was lower in mice treated with Nutlin-3a plus bevacizumab compared to each drug alone. Nutlin-3a alone was more effective than bevacizumab in reducing metastatic disease [62].

BONE AND SOFT TISSUE SARCOMAS

Bone and soft tissue sarcomas are amongst the more frequent solid tumours in children [63]. Currently used chemotherapy is frequently inadequate, with 50-80% long-term survival depending on tumour subgroup, and is associated with severe toxicity [63].

Rhabdomyosarcoma (RMS) is the most common paediatric soft tissue sarcoma with a yearly incidence of approximately 4.6 cases per million [64, 65]. The most frequent histological subtypes of RMS are embryonal rhabdomyosarcoma (eRMS) and alveolar rhabdomyosarcoma (aRMS) [66]. IRS-III outcome data indicate that, with multimodal therapy, cure rates have improved to nearly 70%; however, survival of children with aggressive subtypes (i.e., aRMS) or with metastatic disease continues to be poor [65, 67, 68]. Attempts to improve survival have not been successful with different combinations of known chemotherapeutic agents [69].

Ewing Sarcoma (ES) is one of the small round cell neoplasms of the neuroectodermal origin, with ES of bone representing the least differentiated form [70]. Most patients with localized disease survive with current aggressive treatment but up to 80% of patients with metastases die because of disease progression [71]. Despite intensive protocols the chance of rescuing a patient with relapsed ES is low

and the probability of survival at 5 years is less than 20% [72, 73].

PRECLINICAL USE OF MDM2 INHIBITORS IN RHABDOMYOSARCOMA

Taylor *et al.* demonstrated the presence of wild-type p53 in 19 out of 20 eRMS and aRMS tissue samples obtained either at the time of diagnosis or after chemotherapy [11]. Moreover, reports identify approximately one-third of RMS with MDM2 overexpression and thus an interruption of p53 activity [74]. Therefore, MDM2-p53 interaction inhibitor may also have an important role in this paediatric tumour since p53 and its downstream pathway are intact in most of RMS cases.

A preclinical study in RMS showed that exposure to MI-63, another MDM2-p53 interaction inhibitor, decreased the viability of eRMS and aRMS cells with wild-type p53 compared to p53 mutated cell lines where there were only minimal changes [75]. MDM2 inhibition induced increased p53 levels and apoptosis markers (Bax, cleaved caspase-3 and cleaved PARP) as well as indicators of cell cycle arrest [75]. No changes were recorded in the mutated p53 RMS cell lines, regardless of the histological subtype, clearly indicating that MI-63 selectively induced apoptosis in RMS cells expressing wild-type p53 but not in RMS cells expressing mutated p53. It is noteworthy that no damage to normal human skeletal muscle cells was found (minimal presence of p53 and p21 but no induction of apoptotic proteins) [75]. When added to doxorubicin, synergism was observed [75]. Doxorubicin binds and intercalates DNA, inhibiting macromolecular synthesis and replication by blocking the action of DNA topoisomerase II. As doxorubicin may have p53-independent activities, a combination treatment with an MDM2 inhibitor would potentiate each drug's anti-proliferative effects.

Miyachi *et al.* [76] studied the restoration of the p53 pathway by Nutlin-3a in RMS cell lines with different p53 and MDM2 status. They found Nutlin-3a induced increases of p53 protein through a post-transcriptional mechanism in RMS cell lines with wild-type p53 as well as a dose-dependent increase in the mRNA levels and protein levels of p21 and MDM2. On the other hand, Nutlin-3 had little effect on the mRNA and protein levels of p53, p21, and MDM2 in the RMS cell lines with mutated p53. Accordingly the MDM2 inhibitor dose-dependently inhibited the growth of wild-type p53 RMS cell lines, regardless of the MDM2 status [76]. p53 activation led to cell cycle arrest in RMS cell lines with wild-type p53. In contrast, Nutlin-3 did not have any effect on the cell cycle progression of cell lines with mutant p53. Apoptosis markers, such as Annexin V, caspase-3, and mRNA levels of PUMA, BAX and NOXA were increased in p53 wild-type cell lines. In contrast, only slight differences in the mRNA levels of PUMA, BAX, and NOXA were found in RMS cell lines with mutant p53 [76].

Interestingly Nutlin-3 enhanced the cytotoxicity of chemotherapeutic agents in RMS cell lines with wild-type p53 [77]. Drugs currently used in RMS treatment, vincristine and actinomycin D, were found to have additive and synergistic combination effects with low dose Nutlin-3a when used in RMS cell lines with wild-type p53 and amplified or normal

MDM2, leading to an enhanced anti-tumour effect [77]. Variably increased levels of Annexin V were found after combination treatment in wild-type *p53* cell lines regardless of *MDM2* status [76]. Combination treatment with Nutlin-3 and vincristine or actinomycin D led to activation of the *p53* protein and was more effective at inducing apoptosis than treatment with either agent alone [76]. These observations suggest that a combination of an *MDM2* antagonist and a currently used chemotherapeutic agent might be a useful approach to enhance anti-tumour efficacy in RMS.

PRECLINICAL USE OF MDM2 INHIBITORS IN EWING'S SARCOMA

Mutations of *p53* are rare in Ewing's sarcoma (ES): approximately 90% of ES retain a functional wild-type *p53*, suggesting that targeted activation of wild-type *p53* may be an effective therapeutic strategy for ES. Sonnemann *et al.* replicated in ES similar results to what had been found in other tumour entities. They found that treatment with Nutlin-3 increased *p53* levels and induced *p53* target gene expression (*MDM2*, *p21*, *PUMA*) in ES cells with wild-type *p53*, but not in ES cells with mutated *p53*. Consistently, Nutlin-3 elicited apoptosis only in wild-type *p53* cells [78].

PRECLINICAL USE OF MDM2 INHIBITORS IN OTHER SARCOMAS

Ohnstad *et al.* [77] recently demonstrated that liposarcoma and osteosarcoma cell lines harbouring wild-type *p53* and amplified *MDM2* are very sensitive to Nutlin-3a. Good sensitivity was observed also when Nutlin-3a was used in a wild-type *p53* osteosarcoma cell line with non-amplified *MDM2*. No response was detectable in osteosarcoma and rhabdomyosarcoma cell lines where *p53* was mutated or deleted. In this setting, adding Nutlin-3a could be used to reduce the genotoxic burden of conventional chemotherapy. Indeed methotrexate, cisplatin and doxorubicin showed various degrees of additivity or synergism, allowing up to 10-fold dose reduction of conventional chemotherapeutic drugs when combined with Nutlin-3a, in line with other publications [60, 79, 80].

Nutlin-3a induced accumulation of *p53* protein in the sarcoma cell lines with wild-type *p53*, followed by activation and accumulation of *p53* targets *p21* and *MDM2*. In cell lines where *p53* was not functional, the downstream targets were not affected by any of the treatments [77].

Very recently Ray-Coquard *et al.* [81] reported their experience in using RG7112 in a selected population of patients with liposarcoma. RG7112 is a Nutlin-like potent inhibitor of *MDM2*-*p53* interaction, that effectively stabilises *p53* protein, activates *p53* signalling and inhibits cancer cell growth. In their proof-of-mechanism study the authors showed that RG7112 inhibited *MDM2* and activated the *p53* pathway in adults with *MDM2*-amplified liposarcoma. One patient out of 20 had a confirmed partial response and 14 had stable disease. Unfortunately this study was not designed to evaluate the impact of the drug on survival, hence these results are not suitable for an efficacy evaluation of this new class of drugs and conclusions cannot be drawn. Of note is that one patient had non-functional mutated *p53* and one patient progressed during the first cycle.

RETINOBLASTOMA

Retinoblastoma is the third most common form of cancer in infants. It is due in part to mutations of both copies of *RB1* gene. Forty per cent of patients carry a germ line mutation in *RB1* and are therefore at risk for developing multiple tumours in both eyes as well as monocular tumours [82, 83].

Enucleation is an option for unilateral disease but bilateral disease still remains a challenge as preservation of the eyes and vision becomes a priority. Unfortunately even with aggressive therapy, in 50% of cases both eyes are lost in patients with advanced disease. Patients with tumour invasion of the cut end of the optic nerve are uniformly considered to be at high risk for relapse [84]. The reported survival rate is as low as 40% [85]. Therapy intensification with higher-dose chemotherapy and autologous stem cell rescue has been explored, but its effectiveness remains unclear. Even with this treatment very few patients survive [86]. Despite the intensity of the treatment administered and the documented responses in cases of intracranial disease, patients usually succumb to disease, and survivors are anecdotal. The prognosis of patients with trilateral retinoblastoma is dismal; patients usually die of disseminated neuroaxis disease in less than 9 months [87].

MDM2/p53/RB INTERACTIONS IN RETINOBLASTOMA

It has been proposed that both *Rb* and *p53* pathways must be inactivated during cancer progression [88-90]. The *Rb* and *p53* pathways may be inactivated by mutations in the *RB1* and *p53* tumour suppressor genes themselves or through genetic alterations of other genes in the pathway. In the normal retina *RB1* suppresses *E2F3a/b* and thereby prevents transcriptional activation of *p14^{ARF}*. When *RB1* activity is lost, *E2F3a/b* activates transcription of *p14^{ARF}*, which leads to *p53* activation by suppression of *MDM2* and the initiation of cell cycle arrest and apoptosis. In order to induce tumourigenesis, loss of *Rb* has to be associated with a *p53* pathway disruption [91, 92].

However, retinoblastoma tumours that arise from cells that have lost *RB1* do not contain genetic lesions in the *p53* gene [93] or downstream pathway [94]. It has been reported that there are extra copies of *MDMX* and *MDM2* in 65% and 10% of human retinoblastomas, respectively [47]. Hence, the *p53* gene is intact in retinoblastoma, and the pathway is silenced mainly by increased expression of the upstream regulator *MDMX*.

MDMX does not have ubiquitin ligase activity, but it effectively inhibits *p53* activity by binding to its transactivation domain [95]. In retinoblastoma with *MDMX* gene amplification, the levels of *MDMX* mRNA and protein are also increased [47]. This correlates with a decrease in *p53* and *p21* protein levels, as previously shown in breast tumours with *MDMX* amplification [95].

These data suggest that in retinoblastoma amplification of *MDMX* and, to a lesser extent, *MDM2* suppresses the *p53* response to increased *p14^{ARF}* levels that results from activation of the oncogenic stress pathway following *RB1* inactivation.

Nork *et al.* [94] were the first to show that specific up-regulation of p53 induces retinoblastoma cell death. They transiently transfected retinoblastoma cells with a p53 expression vector and observed elevated p53 levels and cell death. Harbour *et al.* [96] provided evidence that it is possible to activate p53 and achieve retinoblastoma cell death without overexpressing the gene. They developed transducible peptides to interfere with MDM2, which allowed p53 to accumulate in a more physiologic range. They observed effective killing of retinoblastoma cells in the 200- μ M dose range in association with an up-regulation of the p53 target *Bax*. Their data was the first to suggest that inhibition of the MDM2-p53 interaction could be a potential therapeutic option in retinoblastoma.

PRECLINICAL USE OF MDM2 INHIBITORS IN RETINOBLASTOMA

Elison *et al.* [97] have shown that Nutlin-3a induces a p53 response in Wer1 and Y79 human retinoblastoma cell lines, as indicated by an increase in p53 protein levels and the downstream targets MDM2 and p21. Apoptosis, cell cycle arrest and decrease in cell viability were all p53-dependent because retinoblastoma cells expressing a siRNA to p53 were less sensitive to Nutlin-3a [97].

Nutlin-3 binds MDMX with a much higher inhibition constant than MDM2 (28 μ M vs 0.7 μ M) [47]. As MDMX seems to be the primary roadblock to p53-mediated apoptosis, this implies that high drug concentrations in the vitreous humor are required for efficient Nutlin-3 treatment of retinoblastoma. Achieving high vitreous concentrations following systemic Nutlin-3 administration is however hampered by poor penetration of the drug across the blood-ocular barrier. To circumvent this problem, Brennan *et al.* [98] developed an ocular formulation of Nutlin-3a that can be administered subconjunctivally. They demonstrated that this ocular Nutlin-3a formulation selectively activates the p53 pathway and that combining subconjunctival delivery of Nutlin-3a with systemic topotecan results in higher antitumor activity compared to currently used chemotherapy in an orthotopic xenograft model of retinoblastoma [98]. This data indicates that it is feasible to achieve the intraocular concentration of Nutlin-3a needed to efficiently inactivate both MDM2 and MDMX.

BIOMARKERS

Patient selection represents an important element for the successful use of MDM2/p53 inhibitor in clinic. Table 1 summarizes the MDM2-p53 interaction inhibitors and its position in the early clinical development. Based on preclinical studies it seems that wild type p53 is the strongest predictive biomarker for response to MDM2-p53 interaction inhibitor. Nevertheless recent evidence suggests that types of mutations do not impair the p53 pathway which remains fully or at least partially functional [81]. Therefore the presence of p53 mutations should be followed by an evaluation of the function of the p53 pathway before excluding patients from entering clinical trials with MDM2-p53 interaction inhibitor. New predictive biomarkers exploring p53 function such as downstream pathway activation through mRNA expression could be explored in future trials and may be more reliable than p53 mutation status alone.

Pharmacodynamic biomarkers for MDM2-p53 interaction inhibitors have been extensively studied in a number of clinical studies of the Nutlin derivatives (RG-7112 and RG-7388) [81, 99-101]. A proof-of-mechanism study using RG7112, one of the most potent drugs of the Nutlin family [81], was specifically designed to confirm the target inhibition of this new class of drugs in humans. For this purpose adult patients with liposarcoma (where *MDM2* 12q14-15 amplification is present in virtually all cases) were selected [102]. Several biomarkers were analysed, such as MDM2 inhibition and p53 reactivation markers (p53, p21, MDM2, Ki-67, and apoptotic markers) were measured in tumour biopsies and macrophage inhibitory cytokine-1 (MIC-1) in blood.

Changes in p53 and p21 expression levels in the tumour samples were found but they did not correlate with drug exposure. Similar results were reported for change from baseline in Ki-67 positive cells and TUNEL concentration. In contrast, serum MIC-1 percentage changes from baseline, as well as TUNEL staining, correlated with drug exposure, as was reported for the first-in-man studies of RG7112 [81, 99-101]. The significant correlations between MIC-1 and RG7112 exposure and between MIC-1 and tumour cell apoptosis suggest that serum MIC-1 concentration could be an interesting pharmacodynamic biomarker for monitoring drug response. Moreover this clearly showed that current MDM2-p53 interaction inhibitors achieve a good target inhibition in humans.

In children it is important to limit invasive procedures in children and it is therefore essential to validate potential biomarkers on alternative sources of tumour material, such as circulating cancer cells, free circulating DNA or cancer cells isolated from the bone marrow.

RESISTANCE

Despite the novel and non-genotoxic effect of Nutlin treatment, it has been shown that continuous exposure to Nutlin 3 can lead to the acquisition of somatic mutations in p53 and select for p53-mutated cells both in neuroblastoma and in other solid tumours such as osteosarcoma, *in vitro* [103, 104].

The exact mechanism by which cells acquired p53 mutations in the absence of DNA damage is yet to be completely understood. According to some authors p53-mutated cells are constantly emerging in cell culture at low rate, perhaps because of the deficiencies in DNA repair or replication fidelity. In this case repeated exposure to Nutlin would then select or enrich for p53-mutated cells [105].

A second possibility is that mutations in p53 arising after Nutlin treatment could result from misrepair of DNA breaks in cell that have initiated but not fully executed apoptosis, as already shown for TRAIL (tumour necrosis factor-related apoptosis inducing ligand) treatment [104].

Because MDM2-p53 interaction inhibitors have not been extensively used in humans this event has not yet been confirmed *in vivo* but preclinical evidence warrants careful monitoring for the appearance of *de novo* p53 mutations in tumours from patients treated with this new class of drugs.

Table 1. MDM2-p53 Interaction Inhibitors in Clinical Development.

Drug	Company	Mechanism of action	Study	Tumour Type	Status	ClinicalTrials.gov
RO5045337 (RG7112)	Hoffmann-La Roche	Binding of the p53-binding site of MDM2	Phase 1	AML/CML	Recruiting	NCT01677780
				Haematologic neoplasm	Completed	NCT00623870
				Solid tumours	Completed	NCT00559533
			PK study (Phase 1)	Solid tumours	Active, not recruiting	NCT01164033
			Exploratory Biomarker	Liposarcoma	Completed (published)	ref 80 NCT01143740
			Phase 1B-combination with Doxorubicin	Soft Tissue Sarcomas	Recruiting	NCT01605526
			Phase 1B – combination with Cytarabine	AML	Recruiting	NCT01635296
RO5503781 (RG7388)	Hoffmann-La Roche	Binding of the p53-binding site of MDM2	Phase 1	Solid tumours	Recruiting	NCT01462175
			Phase 1/1B – alone vs combination with cytarabine	AML	Recruiting	NCT01773408
Kevetrin	Cellceutix Pharmaceuticals	Phosphorylation of p53 at Ser15	PK and PD	Solid tumours	Recruiting	NCT01664000

Another new class of drugs that acts by restoring the activity of mutant p53 is emerging and could be used in combination with MDM2-p53 interaction inhibitors to overcome resistance [106].

CONCLUSIONS

The MDM2-p53 interaction represents a new target for both adult and paediatric malignancies. Children with neuroblastoma and sarcomas could particularly benefit from this therapy provided the tumour is p53 wild-type. p53 wild-type retinoblastoma has also shown response in a pre-clinical setting through a topical approach. Fortunately, these tumours rarely harbour mutations or deletions of the p53 gene or of p53 downstream pathway effectors. As MDM2 is a principal effector of the p53 upstream regulator pathway, inhibition of the p53 and MDM2 interaction would overcome most of the mechanisms by which paediatric solid tumours escape from p53-mediated apoptosis, cell cycle arrest, differentiation, senescence and anti-angiogenesis.

Collectively, these preclinical results provide strong support for the further development and implementation of this new class of drugs in the treatment strategy for paediatric solid tumours, where there is an unmet need for novel treatment approaches.

CONFLICT OF INTEREST

JSM, LM, AJP and TVM participated to advisory board for clinical development of MDM2-p53 interaction inhibitors of Genentech, Roche.

Genentech (Roche) has supplied DAT, JMS, LC and TVM with RG7112 for preclinical testing in neuroblastoma.

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