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Selective inhibition of the p53–MDM2 interaction by nutlin drugs: a new therapeutic perspective for neuroblastoma

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Abstract

Neuroblastoma is one of the most common and most deadly childhood tumors. There is an unmet need to develop new therapeutic modalities for this malignancy that preferentially should be guided by our increasing knowledge of the biology of neuroblastoma. Proliferation and survival of neuroblastoma cells is critically dependent on suppression of the activity of the tumor suppressor protein p53, which is often mediated by increased activity of the MDM2 oncoprotein. Accordingly, small-molecule inhibitors of the interaction between MDM2 and p53 may provide a useful therapeutic option for the treatment of neuroblastoma by restoring the potent antitumor activity of wild-type p53. One of the most promising classes of selective inhibitors of the p53–MDM2 interaction are the nutlins, which have been extensively studied over the last years in several tumor types, including neuroblastoma. We discuss here preclinical data that support the notion that nutlin drugs may offer therapeutic benefit for children with neuroblastoma, on condition that wild-type p53 is present.

Keywords: Neuroblastoma, p53, MDM2, nutlin, targeted therapy

NEUROBLASTOMA

Although childhood cancer is relatively rare, it is the second most important cause of death in children after accidents. Neuroblastoma is the most common type of malignancy in infants and the second most prevalent tumor in the age group of one to four years (1). This neoplasm originates from primitive cells of the sympathoadrenal lineage of the neural crest. Accordingly, neuroblastoma tumors can be located in the adrenal medulla and anywhere in the sympathetic nervous system (e.g., in the abdomen, chest, neck, and pelvis). A remarkable feature of neuroblastoma is that a subset of tumors regress spontaneously, but approximately half of all neuroblastoma patients have an aggressive disease and a survival probability below 40% (2). Factors that are predictive of a poor prognosis include advanced tumor stage, older age at diagnosis, unfavorable histology, poor grade of tumor differentiation or undifferentiated morphology, amplification of the *MYCN* oncogene, loss of the long arm of chromosome 11, and near-diploid or hypodiploid tumor cell DNA content (3). There is a clear unmet need to develop new treatment options for patients who are classified as high-risk at diagnosis and for patients who experience a relapse of their disease or who have tumors that are primarily resistant to conventional therapy. Ideally, these new treatment modalities should be biologically driven and should target central aberrations in tumor cells, while sparing normal cells.

THE P53 PATHWAY

The biology of cancer is highly complex, but can in essence be reduced to a disruption of a small set of organizing principles (4). A limited number of core cellular pathways seem to control the proliferation and survival of normal cells and are therefore commonly altered in cancer cells. The most frequently inactivated protein in human cancer is probably p53 [encoded by the *TP53* (*p53*) gene], which is an evolutionary conserved coordinator of stress responses in metazoan species that has evolved to function as a safeguard against malignant transformation in higher organisms with a longer life span (5). The p53 protein is stabilized and activated by a variety of stress signals, such as DNA damage, and binds then to specific response elements in the DNA. This results in the induction or repression of expression of target genes that provide the appropriate answer to the stress signal. Examples include induction of the expression of *CDKN1A* ($p21^{WAF1/CIP1}$) and *GADD45A*, which encode proteins that mediate cell cycle arrest and DNA repair. If the stress stimulus persists or if the intensity of the stress is too severe, p53 will induce the expression of target genes that elicit apoptosis or inhibit survival signaling, such as *BAX*, *FAS*, *BBC3* (*PUMA*), *PMAIP1* (*NOXA*), and *PTEN*. The p53 transcription factor also induces gene expression alterations that result in cellular senescence, autophagy, antioxidant defense, regulation of metabolic pathways, and inhibition of angiogenesis. In addition, p53 has several transcription-independent

activities, like activation of mitochondrial apoptosis by binding to members of the BCL2 family of proteins. By means of these various mechanisms, p53 is capable of dealing with the cause of stress, repairing mild stress-induced damage, and eliminating severely damaged cells—thereby providing a major roadblock to tumor development (6).

The strong tumor suppressor activity of p53 implies that tumor cells need to find a way to circumvent this failsafe program against oncogenesis. Approximately 50% of all human tumors bypass this barrier by acquiring an inactivating mutation in the *p53* gene, whereas the other 50% of tumors suppress p53 activity by deregulating the normal function of other components in the p53 pathway. The E3 ubiquitin ligase MDM2 is a central molecule in the p53 pathway that directly interacts with p53 and that keeps p53 under control by targeting the p53 protein for proteasomal degradation and by blocking the transcriptional activity of p53 (7). The negative regulation of p53 by MDM2 is very important under physiological conditions to prevent unwanted and potentially lethal p53 activity, but a drawback of this tight control mechanism is that it also provides a tool to tumors to constitutively switch off the p53 protein. Indeed, tumors with wild-type p53 can have an amplification of the *MDM2* gene or increased expression or activity of the MDM2 protein to escape from p53-mediated growth control (8). In contrast to many adult cancers, neuroblastoma tumors rarely contain a *p53* mutation at diagnosis and at relapse (*p53* mutation prevalence of less than 2% and of 15%, respectively) (9, 10). Compelling evidence indicates that oncogenic activity of MDM2, e.g. resulting from MYCN-driven transactivation of *MDM2* expression, is the primary mechanism for inactivation of p53 in neuroblastoma (11).

TARGETED INHIBITION OF THE P53–MDM2 INTERACTION BY NUTLIN DRUGS

The general importance of the interplay between MDM2 and p53 in tumor biology has stimulated the development of small-molecule inhibitors of the interaction between both proteins as a means to restore p53 activity in tumors with wild-type p53. Targeting protein–protein interactions is, in general, challenging, as the interface of such interactions is typically large and flat and difficult to break by small molecules (12). However, in case of the p53–MDM2 interaction, drug development efforts have taken advantage of the existence of an interaction hot spot suitable for drug binding. Crystallographic studies have shown that three hydrophobic amino acids of p53 (Phe^{19}, Trp^{23}, and Leu^{26}) make the primary contact to MDM2 by binding to a deep hydrophobic cleft on the surface of this protein (13). Accordingly, a class of low-molecular-weight compounds with a *cis*-imidazoline backbone have been developed that have a similar spatial conformation as this triad of amino acids of p53 and that therefore can compete with p53 for binding to the hydrophobic pocket of MDM2 (Fig. 1) (14, 15). These small

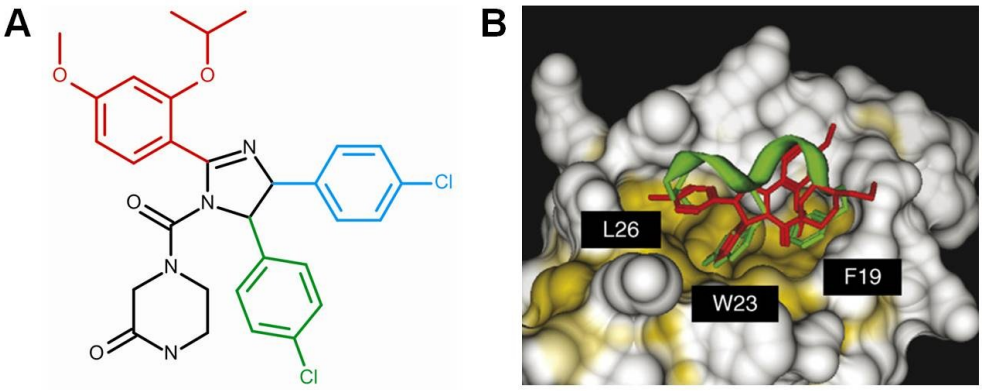

Figure 1. Structural aspects of nutlin binding. A, chemical structure of nutlin-3. The groups depicted in red, green, and blue have a similar spatial conformation as the Phe[19], Trp[23], and Leu[26] amino acid residues of the p53 protein, respectively. B, competition between nutlin-2 (red) and p53 (green; only the relevant portion of the p53 protein is shown) for binding to a hydrophobic pocket on the surface of the MDM2 protein (yellow). The nutlin drug binds to MDM2 at the same sites as the Phe[19], Trp[23], and Leu[26] amino acid residues of p53. This binding model is derived from superimposition of the coordinates from the crystal structure of the nutlin-2–MDM2 complex onto those of the p53–MDM2 complex [reproduced by permission of the publisher from (15)].

molecules, termed nutlins, were the first potent and selective inhibitors of the p53–MDM2 interaction at the time of their discovery and have now progressed to clinical development in adult cancer patients. However, there is ample evidence that several pediatric malignancies may be good candidates for nutlin treatment as well. We discuss here some of the preclinical studies with nutlin-3, which is the most widely used nutlin compound in a research setting, that support the potential use of nutlin drugs in the treatment of neuroblastoma.

IN VITRO ANTITUMOR ACTIVITY OF NUTLIN-3 IN NEUROBLASTOMA

A first study has been performed to examine the antitumor effects of nutlin-3 as a single agent in a panel of 9 neuroblastoma cell lines with either wild-type or mutant p53 and with different genetic characteristics (16). Treatment with nutlin-3 was shown to selectively activate the p53 pathway in neuroblastoma cells with wild-type p53, as evidenced by accumulation of the p53 protein and by dose-dependent induction of expression of p53 target genes, like *p21*$^{WAF1/CIP1}$, *BAX*, *PUMA*, *TP53I3* (*PIG3*), and *MDM2*. This elicited a marked antiproliferative and cytotoxic effect through induction of both G_1 cell cycle arrest and apoptosis. These treatment effects were observed irrespective of the presence or absence of commonly found genomic aberrations in neuroblastoma, such as *MYCN* amplification, 1p deletion, 3p deletion, 11q deletion, and 17q gain, which suggests that nutlin-3 may have broad applications in the treatment of neuroblastoma on condition that wild-type p53 is present. Interestingly, the few neuroblastoma cells that survived nutlin-3 treatment acquired a senescence-like phenotype in cell line SK-N-SH or underwent neuronal differentiation in cell lines CLB-GA and NGP. The dependence

of these alternative nutlin-3 responses on p53 was demonstrated by the abrogation of these effects when *p53* was silenced using lentivirus-mediated short hairpin RNA interference, consistent with targeted inhibition of the p53–MDM2 interaction. The occurrence of premature senescence and neuronal differentiation as drug-induced response programs, in addition to G_1 cell cycle arrest and apoptosis, uncovered a unique combination of antitumor treatment effects induced by a single targeted intervention. These pleiotropic activities of nutlin-3 to eliminate neuroblastoma cells may increase therapeutic efficacy and may possibly help to avoid the emergence of treatment resistance. In addition, the observation of tumor cell differentiation after therapy with nutlin-3 makes selective MDM2 inhibitors an attractive approach for the treatment of tumors that are arrested in their maturation, such as neuroblastoma. Several other studies have confirmed the potent antitumor activity of nutlin-3 or nutlin-3a (i.e., the purified active enantiomer of nutlin-3) in neuroblastoma cell lines with wild-type p53 and have demonstrated strong synergistic effects when this targeted therapy was combined with conventional chemotherapeutic agents both in neuroblastoma cell lines with wild-type p53 and with mutant p53 (17-19).

IN VIVO ANTITUMOR ACTIVITY OF NUTLIN-3 IN NEUROBLASTOMA

In vivo trials of nutlin-3 have been conducted in a context of chemoresistance (20), since most cases of treatment failure and patient mortality are attributable to the emergence of neuroblastoma cells that are resistant to conventional chemo-therapeutics. New treatment options are therefore most urgently needed in the patient group with chemoresistant neuroblastoma tumors. It has been hypothesized that nutlin-3 may offer therapeutic benefit for the majority of patients with chemorefractory neuroblastoma, as most cases of chemoresistant neuroblastoma are not due to an inactivating *p53* mutation (10). For example, elevated expression of drug efflux pumps, high expression of detoxifying enzymes, disequilibrium between apoptotic and survival signals, and defects in the extrinsic cell death signaling pathway are some of the mechanisms that can cause chemoresistance in neuroblastoma. In principle, these different forms of chemoresistance remain amenable, with the exception of *p53* mutation, to therapeutic activity of nutlin-3. The antitumor effects of nutlin-3 have therefore been investigated in a well-established model system of acquired multidrug resistance that recapitulates several clinically relevant features of advanced-stage and chemoresistant neuroblastoma tumors (e.g., increased proliferation, higher survival capacity, enhanced invasiveness, elevated tumorigenicity, and marked resistance to several chemotherapeutic agents and to irradiation) and that allows to study treatment effects in relationship to p53 status (wild-type or mutant p53) (20). *In vitro* studies demonstrated that treatment with nutlin-3 induced activation of the p53

pathway, G_1 cell cycle arrest, and apoptosis in chemosensitive and chemorefractory neuroblastoma cells to a similar extent, on condition that wild-type p53 is present. Subsequently, mice with established subcutaneous chemorefractory neuroblastoma xenografts were treated with nutlin-3, which was administered orally at a dose of 200 mg/kg twice daily for three weeks or until euthanasia was required because of unacceptably large tumor size. This dosing schedule did not give rise to any overt sign of toxicity and reduced both the growth of the primary tumor and the extent of metastatic disease in liver and lungs when wild-type p53 was present. In contrast, and as expected, no treatment effects were observed in mice with p53-mutant chemorefractory neuroblastoma tumors. Investigation of mice carrying chemoresistant neuroblastoma xenografts with wild-type p53 demonstrated that oral administration of nutlin-3 for 36 hours induced activation of the p53 pathway and apoptosis in the xenograft tumors. Taken together, these data indicate that restoration of p53 function by administration of nutlin-3 may provide a new therapeutic option for the treatment of metastatic and chemorefractory neuroblastoma tumors with wild-type p53. Convincing *in vivo* data that support the clinical development of this therapeutic approach have also been obtained when treatment with nutlin-3a was combined with the VEGF-blocking monoclonal antibody bevacizumab (21). This combination treatment cooperatively suppressed tumor growth and angiogenesis in an orthotopic mouse model of neuroblastoma, suggesting that selective MDM2 inhibitors may have therapeutic utility as adjuncts to antiangiogenic strategies.

NUTLIN-3 AS A TOOL FOR PROBING THE P53 PATHWAY IN NEUROBLASTOMA CELLS

Understanding the mechanisms used by tumor cells to circumvent the p53-driven antitumor barrier is important to select the right patient subsets for nutlin therapy. From the studies above and the targeted mechanism of action of nutlin-3, it is clear that wild-type p53 is a first prerequisite to obtain potent antitumor activity of nutlin-3. To gain more insight into additional determinants of the response to nutlin-3 in neuroblastoma cells, a detailed analysis has been performed in a set of 34 human neuroblastoma cell lines which were also characterized by sequencing of the *p53* gene and gene copy number analysis of *MDM2*, *CDKN2A* ($p16^{INK4a}/p14^{ARF}$), and *MYCN* (22). As expected, a highly significant difference in the sensitivity to nutlin-3 was observed between the neuroblastoma cell lines with wild-type p53 and the neuroblastoma cell lines with mutant p53. At 72 hours of treatment with nutlin-3, the median IC_{50} value (i.e., the concentration of nutlin-3 that caused 50% growth inhibition) was 4.6 μM for the 25 neuroblastoma cell lines with wild-type p53 and >32 μM (the highest nutlin-3 concentration tested) for the 9 lines with mutant p53. Marked reductions in cell viability were noted after nutlin-3

treatment in 23 out of the 25 neuroblastoma cell lines with wild-type p53, indicating that downstream defects in the p53 pathway are uncommon in neuroblastoma. Neither amplification of the *MDM2* gene nor *MYCN* amplification was predictive of the response to nutlin-3. However, deletion of the $p16^{INK4a}/p14^{ARF}$ gene correlated with a reduced sensitivity to nutlin-3. Knockdown and overexpression experiments demonstrated that the expression levels of $p14^{ARF}$, but not of $p16^{INK4a}$, sensitize neuroblastoma cells to nutlin-induced apoptosis. This suggests that $p14^{ARF}$ is not just an upstream regulator of the p53 protein, but also actively provides a coactivating signal for driving the p53 response towards apoptosis. Collectively, this study yields several insights into the spectrum of p53 pathway lesions in neuroblastoma cells that could be useful for the clinical translation of therapeutic strategies aimed at restoration of p53 function. It remains to be determined in clinical trials whether treatment with nutlin drugs will result in the acquisition of *p53* mutations and $p14^{ARF}$ loss by tumor cells, but these data already provide sufficient evidence to stimulate the preclinical development of complementary therapeutic approaches that reactivate mutant p53 and counteract $p14^{ARF}$ defects. Interestingly, an independent study has also identified the expression levels of *MYCN* as a factor that determines the response of neuroblastoma cells to nutlin-3 and to another inhibitor of the p53–MDM2 interaction, MI-63 (23). The authors of this study therefore speculated that selective MDM2 inhibitors may be particularly effective in the treatment of high-risk *MYCN*-amplified neuroblastoma. The occurrence of a strong apoptotic response in *MYCN*-amplified neuroblastoma cells treated with nutlin-3 has been attributed to a tumor type–dependent induction of HIPK2, which is a p53-activating proapoptotic kinase (24), and to repression of the β-galactoside–binding protein galectin-3 (25). However, it is not yet clear whether *MYCN* amplification or high *MYCN* expression will be a clinically useful biomarker for predicting treatment outcome, as several authors have failed to detect a significant better response to nutlin-3 upon MYCN induction or in *MYCN*-amplified neuroblastoma cells compared to *MYCN*-nonamplified neuroblastoma cells (16, 17, 22, 26). The reasons for these contrasting findings are unclear and may, for instance, relate to the use of different model systems. Further study is clearly needed to clarify this issue.

CONCLUSIONS

The existing preclinical data lend strong support to the view that nutlin drugs may be a viable therapeutic option for patients with neuroblastoma. However, several issues still need to be addressed prior to introduction of these drugs in the clinic. For instance, the *in vivo* activity of nutlin-3 against neuroblastoma has at present only been investigated using tumor xenograft modeling in nude mice and not yet in immunocompetent transgenic mouse models of neuroblastoma. There is also a clear need to define the best combinatorial

treatment regimen for clinical implementation and to identify pharmacodynamic biomarkers for noninvasive monitoring of treatment response. Another important requirement is that nutlin drugs are found to be safe and tolerable in phase I clinical trials in adult cancer patients, which are currently ongoing. If these outstanding questions can be answered in a satisfactory way, we hope that the concept of reactivation of wild-type p53 may mature to a new therapeutic avenue for the treatment of children with neuroblastoma.

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