Small-Molecule MDM2 Antagonists as a New Therapy Concept for Neuroblastoma

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Abstract
Circumvention of the p53 tumor suppressor barrier in neuroblastoma is rarely caused by TP53 mutation but might arise from inappropriately increased activity of its principal negative regulator MDM2. We show here that targeted disruption of the p53-MDM2 interaction by the small-molecule MDM2 antagonist nutlin-3 stabilizes p53 and selectively activates the p53 pathway in neuroblastoma cells with wild-type p53, resulting in a pronounced antiproliferative and cytotoxic effect through induction of G1 cell cycle arrest and apoptosis. A nutlin-3 response was observed regardless of MYCN amplification status. Remarkably, surviving SK-N-SH cells adopted a senescence-like phenotype, whereas CLB-GA and NGP cells underwent neuronal differentiation. p53 dependence of these alternative outcomes of nutlin-3 treatment was evidenced by abrogation of the effects when p53 was knocked down by lentiviral-mediated short hairpin RNA interference. The diversity of cellular responses reveals pleiotropic mechanisms of nutlins to disable neuroblastoma cells and exemplifies the feasibility of exploiting, by a single targeted intervention, the multiplicity of anticancer activities exerted by a key tumor suppressor as p53. The observed treatment effects without the need of imposing a genotoxic burden suggest that selective MDM2 antagonists might be beneficial for treatment of neuroblastoma patients with and without MYCN amplification. (Cancer Res 2006; 66(19): 9646-55)

Introduction
As a key cellular gatekeeper, p53 is mutationally inactivated in ~50% of all human malignancies (1, 2). Tumors that retain the wild-type TP53 gene almost invariably harbor defects in other components of the p53 pathway, either impairing stabilization of p53 in response to stress signals or disrupting essential mediators of p53 transcriptional activity. One such common lesion involves overexpression of the E3 ubiquitin ligase MDM2. This nuclear phosphoprotein is a principal negative regulator of p53 activity and stability by binding to its transactivation domain, promoting its ubiquitination and degradation, favoring its nuclear export, and inhibiting its acetylation (3, 4). As MDM2 itself is a transcriptional target of p53, it is believed that this system constitutes a negative autoregulatory feedback loop that helps to switch off p53 at the end of a stress response (5, 6). Amplification of MDM2 or increased expression has been reported in many human neoplasms and has been shown to confer tumorigenic potential (3, 7).

Inhibition of the p53-MDM2 interaction provides an attractive strategy for activating wild-type p53 in tumors and has therefore been the focus of many efforts in anticancer drug discovery. Recently developed potent and selective small-molecule antagonists of MDM2 (8), termed nutlins, bind tightly into the p53 pocket of MDM2, release p53 from negative control, and activate the p53 pathway. This leads to cell cycle arrest and apoptosis in cancer cells with wild-type p53, whereas the response in normal cells, which do not carry the high apoptotic burden characteristic of tumor cells, is limited to a largely reversible growth arrest, except for some bone marrow cell types (8–11). Restoration of p53 function by nutlins may thus have profound therapeutic use for tumors that have retained wild-type p53, particularly if MDM2 activity is inappropriately increased.

Several lines of evidence indicate that nutlins could be effective in the treatment of the neural crest–derived childhood malignancy neuroblastoma. First, <2% of neuroblastoma tumors at diagnosis exhibit mutations in the TP53 gene, and many studies have revealed an intact p53 pathway in neuroblastoma cells (reviewed in ref. 12). Second, MDM2 is at least in part responsible for the characteristic phenotype of cytoplasmic p53 sequestration in neuroblastoma cells, which may, albeit not precluding p53 activity, limit chemotherapy-induced apoptosis (13). Third, deregulation of MDM2 expression or activity in neuroblastoma has been reported to occur by various means, including amplification of the MDM2 gene (14–17), loss of production of the MDM2 inhibitory protein p14ARF (17, 18), and transcriptional up-regulation in MYCN-amplified neuroblastomas that are associated with poor clinical outcome (19). Finally, inhibition of MDM2 expression or function in neuroblastoma cells results in accumulation of functional p53 in the nucleus (13, 20), further supporting the notion that targeting of MDM2 may offer therapeutic benefit.

These considerations prompted us to investigate the therapeutic potential of disrupting the p53-MDM2 interaction in neuroblastoma. We show that the small-molecule MDM2 antagonist nutlin-3 activates the p53 pathway in neuroblastoma cells with wild-type p53 and elicits a dose- and time-dependent antiproliferative and cytotoxic effect through induction of both G1 cell cycle arrest and apoptosis regardless of MYCN amplification status. Surviving wild-type p53 cells engaged a senescent program in cell line SK-N-SH or a neuronal differentiation process in cell lines CLB-GA and NGP, which were prevented by lentiviral-mediated expression of p53 short hairpin RNA (shRNA). The pronounced effects of nutlin-3 on...
neuroblastoma cells and the identification of premature senescence and differentiation as drug-induced response programs, not yet described for selective MDM2 antagonists but of potential clinical importance, may provide a novel therapy concept for neuroblastoma patients with and without MYCN amplification.

Materials and Methods

Cell lines and nutlin-3 treatment. Nine human neuroblastoma cell lines were used for this study: CLB-GA, IMR-32, LA-N-5, LA-N-6, NBL-S, NGP, SK-N-BE(2c), SK-N-FL, and SK-N-SH. Cell lines were grown as monolayers in RPMI 1640 supplemented with antibiotics, 15% FCS, and 2 mm/L glutamine at 37°C and 5% CO2 in a humidified atmosphere. Nutlin-3 (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol and diluents 3 to 32 mm/L for 24 hours (all cell lines) and from 1 to 32 mm/L nutlin-3 for 1, 2, 3, and 7 days (NGP and CLB-GA cells). DNaT treatment, cDNA synthesis, and SYBR Green I reverse transcription-PCR (RT-PCR) were carried out as described previously (21). Two reference genes, TNRFSF17 (BCMA) and SDC4, were used for normalization. Primer sequences are available in the public database (1, 2) (TNRFSF17 (14), SDC4 (15), MYCN (11), and MDM2 (3498).

Real-time quantitative reverse transcription-PCR–based quantification of mRNA expression. Total RNA extraction from untreated cells treated with 0, 2, 4, 8, 16, and 32 mm/L nutlin-3 for 24 hours (all cell lines) and from cells treated with 0 and 16 mm/L nutlin-3 for 1, 2, 3, and 7 days (NGP and CLB-GA cells). DNaT treatment, cDNA synthesis, and SYBR Green I reverse transcription-PCR (RT-PCR) were carried out as described (23, 24). Primer sequences are available in the public database (22) (TNRFSF17 (BCMA) and SDC4, were used for normalization. Primer sequences are available in the public database (1, 2) (TNRFSF17 (14), SDC4 (15), MYCN (11), and MDM2 (3498).

TP53 mutation analysis. Total RNA extraction from untreated cells, DNaT treatment, and cDNA synthesis were done as detailed above. The entire coding region of TP53 was PCR amplified in two overlapping fragments using the following primer pairs: 5'-GTGACACGCTCCTCGTG-GATT-3' (forward) and 5'-GCACCCACACATATGTGCAA-3' (reverse) and 5'-GGCTCGGCAGCCAT-3' (forward) and 5'-GGACACAGGCTCAAGA-GACC-3' (reverse). After purification with eXonuclease I (New England Biolabs, Ipswich, MA) and anticyclic phosphatase (New England Biolabs), PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and the same primers as those used in the PCR. Sequencing reactions were cleaned up with the QIAquick spin columns (Qiagen, Hilden, Germany). DNA coding sequences were determined by using the in-house developed qBase analysis software using a 6-c relative quantification model with PCR efficiency correction and multiple reference gene normalization (GAPDH, UBC, and SDHA: ref 24).

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Cellular senescence assay. For senescence-associated β-galactosidase activity, 2.5 × 105 cells were seeded in 35-mm dishes, grown for 24 hours, and then exposed to 0 or 16 mm/L nutlin-3 or to an equivalent amount of ethanol for 1 and 7 days. Control cultures for 7 days were split every 3 days to avoid confluency. Fixation of cells and staining for SA-β-gal activity at pH 6.0 were done with the Senescent Cells Staining kit (Sigma). SA-β-gal activity was scored in 1,800 to 2,000 cells from three microscopy fields.

Lentiviral vector construction, virus production, and infection. The sequence of the human TP53 gene chosen to be shRNA targeted was 5'-GACTCCAAGTGTTAATCTAC-3'. As a negative control, we used a shRNA specific for the mouse form of p53 (5'-GATCCGCTGAGATCATTAGT-3') and its corresponding sequence in the human sequence. Additionally, control shRNA constructs for SK-N-SH cells were directed against a different region of the murine Trp53 gene (5'-GATCCGCTGAGATCATTAGT-3') and against a human sequence (5'-GATCCGCTGAGATCATTAGT-3'). Pairs of oligonucleotides containing these sequences were phosphorylated, annealed, and ligated into the pSIP-H1-Puro vectors (System Biosciences, Mountain View, CA).

Human embryonic kidney 293T cells were cultured at 37°C and 5% CO2 in DMEM supplemented with antibiotics, 10% FCS, and 2 mm/L glutamine. Transfections of 293T cells were done with the calcium phosphate precipitation technique. The pSIP-H1-HuRO vectors containing the shRNA sequences were cotransfected with two packaging plasmids: pFIV-3N and PVSV-G (System Biosciences). Viral supernatants were collected and...
filtered through a 0.45-μm filter. NGP and SK-N-SH cells were infected with lentiviruses carrying the shRNA constructs described above in the presence of 3 μg/mL polybrene (Sigma). Transduced cells were selected with 1 μg/mL puromycin (Sigma) for 48 hours.

Results

Genetic characterization of neuroblastoma cell lines and effect of nutlin-3 on cell viability. All nine cell lines used in this study were characterized with respect to the current TP53 mutational status and the gene copy number of MDM2 and MYCN (Table 1). cDNA sequence analysis of the entire coding region revealed wild-type TP53 in CLB-GA, IMR-32, LA-N-5, LA-N-6, NBL-S, NGP, and SK-N-SH cells. Cell line SK-N-NE(2c) carried a 404G>T (C135F) missense mutation in TP53 as previously reported (25). In SK-N-FI cells, we found a 737T>G (M246R) missense TP53 mutation, which has been described in other malignancies originating from the breast, colorectum, esophagus, liver, and skin (2). Gene copy number determination using a real-time quantitative PCR assay showed MDM2 amplification in one cell line, NGP, and MYCN amplification in cell lines IMR-32, LA-N-5, NGP, and SK-N-BE(2c).

To address if inhibition of the p33-MDM2 interaction might offer therapeutic potential for neuroblastoma, we examined the anti-proliferative and cytotoxic effect of nutlin-3 on this panel of cell lines (Fig. 1; Supplementary Fig. S1). In keeping with the normal TP53 status, a pronounced dose- and time-dependent decrease in cell viability on nutlin-3 treatment was recorded for cell lines LA-N-5, NBL-S, IMR-32, NGP, SK-N-SH, and CLB-GA (reduction in cell viability after 72 hours of incubation with 32 μmol/L nutlin-3: 100%, 98%, 98%, 96%, 95%, and 86%, respectively). LA-N-6 cells, which also carry wild-type TP53, displayed a more moderate decrease in cell viability throughout the whole range of tested nutlin-3 concentrations. The two cell lines with TP53 mutation, SK-N-BE(2c) and SK-N-FI, did not respond to low nutlin-3 concentrations but nonetheless exhibited a similar cell viability reduction as LA-N-6 cells at the highest tested concentration of 32 μmol/L.

We next looked at the possibility that amplification of MDM2 or MYCN, the latter being reported to exert its oncogenic activity partly through direct transcriptional up-regulation of MDM2 (19), influences the cell viability response to nutlin-3. No difference in nutlin-3-induced cell viability reduction was observed with respect to MDM2 or MYCN amplification status (P > 0.05, Mann-Whitney U test). Although for MDM2 this result should be interpreted with some caution because only one cell line displayed MDM2 amplification, the equal distribution of the four MYCN-amplified and the five MYCN-nonamplified cell lines in relation to cell viability response indicates that MYCN amplification does not enhance the antiproliferative and cytotoxic activity of nutlin-3.

Nutlin-3 induces expression of p53 target genes, cell cycle arrest, and apoptosis in neuroblastoma cells with wild-type p53. According to the proposed mechanism of action, the reduction in cell viability after nutlin-3 treatment should result from activation of the p53 pathway, leading to cell cycle arrest and apoptosis. In keeping with this assumption, exposure of cells with wild-type p53 to nutlin-3 for 24 hours induced an increase in mRNA levels of p53 target genes involved in autoregulation (MDM2), cell cycle arrest, and apoptosis in neuroblastoma cells with wild-type p53 (Fig. 2A). SK-N-BE(2c) cells with TP53 mutation exhibited a moderate accumulation of p53 protein on nutlin-3 treatment, compatible with the release from MDM2-mediated degradation, without induction of expression of p53 target genes (Fig. 2B). Similar results were observed at both mRNA and protein level for all other neuroblastoma cell lines in this study (Supplementary Figs. S2 and S3).

Flow cytometric cell cycle profiling of CLB-GA, IMR-32, NGP, and SK-N-SH cells at 24 and 48 hours revealed that 16 μmol/L nutlin-3 confirmed stabilization of p53 protein and up-regulation of MDM2, p21\(^{WAF1/CIP1}\), and BAX expression in neuroblastoma cells with wild-type p53 (Fig. 2B). SK-N-BE(2c) cells with TP53 mutation exhibited a moderate accumulation of p53 protein on nutlin-3 treatment, compatible with the release from MDM2-mediated degradation, without induction of expression of p53 target genes (Fig. 2B). Similar results were observed at both mRNA and protein level for all other neuroblastoma cell lines in this study.

Table 1. TP53 mutational status, MDM2 copy number status, and MYCN copy number status of neuroblastoma cell lines studied

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TP53*</th>
<th>MDM2†</th>
<th>MYCN†</th>
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<tbody>
<tr>
<td>CLB-GA</td>
<td>wt</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>IMR-32</td>
<td>wt</td>
<td>N</td>
<td>amp</td>
</tr>
<tr>
<td>LA-N-5</td>
<td>wt</td>
<td>N</td>
<td>amp</td>
</tr>
<tr>
<td>LA-N-6</td>
<td>wt</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>NBL-S</td>
<td>wt</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>NGP</td>
<td>wt</td>
<td>amp</td>
<td>amp</td>
</tr>
<tr>
<td>SK-N-BE(2c)</td>
<td>404G&gt;T (C135F)</td>
<td>N</td>
<td>amp</td>
</tr>
<tr>
<td>SK-N-FI</td>
<td>737T&gt;G (M246R)</td>
<td>N</td>
<td>N</td>
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<tr>
<td>SK-N-SH</td>
<td>wt</td>
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Abbreviations: wt, wild-type; amp, amplified; N, nonamplified.

*From direct DNA sequencing of the entire coding region of TP53. Mutation is indicated by nucleotide change followed by amino acid change.

†Genomic amplification status.
whether targeted disruption of the p53-MDM2 interaction could trigger a premature senescence response in cells surviving nutlin-3 treatment.

Striking morphologic alterations characteristic of senescent cells were indeed observed in SK-N-SH cells on nutlin-3 administration, including a flattened and enlarged cell shape with increased cytoplasmic granularity (Fig. 4A). Gene expression of the cell cycle inhibitor CDKN1A was increased 14-fold after 24 hours of incubation with 16 μmol/L nutlin-3 (Fig. 2A), with ~70% of cells residing in G1 phase (Fig. 3A). SA-β-gal activity was examined to further define the phenotypic changes in SK-N-SH cells surviving nutlin-3 exposure. Approximately 2.5 × 10^5 SK-N-SH cells were seeded into 35-mm dishes, cultured for 24 hours, and then treated with 16 μmol/L nutlin-3 or vehicle control. Staining for SA-β-gal activity after 24 hours of treatment revealed that control cultures contained low numbers of SA-β-gal-expressing cells [45 per mm² cell culture area; 95% confidence interval (95% CI), 32-58; Fig. 4A]. Treatment with 16 μmol/L nutlin-3 significantly enhanced the number of SA-β-gal-expressing cells with rapid kinetics (411 per mm² after 24 hours; 95% CI, 371-451; P < 0.0001), suggesting that nutlin-3 does not just select for SA-β-gal-expressing cells but actually induces a senescence-like phenomenon in surviving SK-N-SH cells. After 7 days of exposure to 16 μmol/L nutlin-3, ~100% of SK-N-SH cells not subject to apoptotic cell death stained positive for SA-β-gal and had acquired a senescence-like morphology (Fig. 4A).

SA-β-gal analysis of all other neuroblastoma cell lines with a fraction of cells surviving nutlin-3 treatment [CLB-GA, LA-N-6, and NGP cells, all with wild-type p53, and SK-N-BE(2c) and SK-N-FI cells harboring a TP53 mutation] revealed that progressive SA-β-gal accumulation during nutlin-3 treatment also occurred in NGP and CLB-GA cells (data not shown). SA-β-gal staining intensity was however considerably weaker than in SK-N-SH cells, and morphologic signs of cellular senescence were absent in these two cell lines, suggesting that the increase in SA-β-gal activity did not concern a genuine senescence response.

**Induction of sympathetic neuronal differentiation in the surviving fraction of NGP and CLB-GA cells by nutlin-3.** The morphologic changes induced by nutlin-3 in surviving NGP and CLB-GA cells were markedly different from those in SK-N-SH cells. As shown in Fig. 4B, addition of nutlin-3 to NGP and CLB-GA cultures provoked hallmarks of neuronal differentiation in cells surviving the treatment, such as the acquisition of a bipolar or multipolar cell shape, the development and extensive outgrowth of neurites with varicosities and growth cone-like endings, and the formation of netlike arrangements of neuronal processes.

Phenotypic characterization of the differentiation process was done by assessing mRNA expression levels of 23 neuronal or neuroendocrine differentiation marker genes at 1, 2, 3, and 7 days of treatment with 16 μmol/L nutlin-3. Selected results for NGP cells are shown in Fig. 4C (all results available in Supplementary Fig. S4). The neurite outgrowth in nutlin-3-treated NGP cells was confirmed by increased expression of genes encoding neurofilament constituents (NEFL, NEF3, and NEFH), a gene encoding an axonal protein with an established role in growth cone and synapse formation (GAP43), and a gene reported to promote neurite outgrowth and adhesion (HNT). Expression of several marker genes typically associated with neuroendocrine chromaffin differentiation in the developing sympathetic nervous system (27, 28) did not change (CHGB and TH), decreased (CHGA), or increased (IGF2) on nutlin-3 treatment of NGP cells, the latter possibly related to a role of IGF2 as an autocrine and paracrine survival factor in a negative autoregulatory feedback fashion (29). As for TH, no significant increase in mRNA levels of other genes involved in catecholamine biosynthesis (DDC, DBH, and Pnmt) was detected, but expression of the neuropeptide NPY, which in the developing sympathetic nervous system is confined to the neuronal lineage (27), was significantly up-regulated. The most dramatic gene expression change in nutlin-3-treated NGP cells consisted of an ~500-fold increase in expression of the gene responsible for acetylcholine biosynthesis (CHAT), indicative of differentiation along a cholinergic neuronal lineage. Interestingly, nutlin-3 treatment of NGP cells also induced a shift in the neurotrophin receptor expression profile toward up-regulation of NTRK1 (TRKA) and suppression of NTRK2 (TRKB) expression, consistent with the acquisition of a less aggressive phenotype (reviewed in ref. 30).

Neuronal/neuroendocrine marker expression analysis of CLB-GA cells after nutlin-3 treatment also confirmed the process of neuronal differentiation as evidenced by elevated expression of several genes involved in neurite outgrowth (NEFL, PRPH, and...
For this cell line, however, we observed a marked down-regulation of \(\text{CHAT}\) expression along with a transient up-regulation of expression of several catecholaminergic genes (\(\text{TH}\) and \(\text{DBH}\)), sympathetic neuroendocrine markers (\(\text{CHGA}, \text{CHGB}, \text{IGF2}\), and \(\text{TH}\)), and the sympathetic neuronal marker \(\text{NPY}\).

**Induction of premature senescence in SK-N-SH cells and neuronal differentiation in NGP cells by nutlin-3 is \(p53\) dependent.** To evaluate whether the premature senescence response in SK-N-SH cells and the neuronal differentiation in NGP cells are a direct consequence of nutlin-3-induced activation of wild-type \(p53\), we infected SK-N-SH and NGP cells with a lentiviral vector encoding a shRNA directed specifically against human \(p53\) (LV-h-\(p53\)) or murine \(p53\) (LV-m-\(p53\)). Knockdown of human \(p53\) severely attenuated the cell viability reduction after nutlin-3 treatment in both SK-N-SH and NGP cells (Fig. 5A). In contrast, control infection with LV-m-\(p53\) did not affect the nutlin-3 response in NGP cells, but unexpectedly, we observed some attenuation of response in SK-N-SH cells infected with LV-m-\(p53\) (Fig. 5A). Control infection of SK-N-SH cells was therefore repeated using the same LV-m-\(p53\) vector, a lentiviral vector encoding a shRNA against a different target site in the murine \(\text{Trp}53\) gene (\(\text{LV-m2-}p53\)), and a lentiviral vector carrying a shRNA construct specific for firefly luciferase (LV-luc). This yielded in all cases a distinct dose- and time-dependent cell viability response to nutlin-3, similar to the example shown in Fig. 5A and indicative of \(p53\) functionality.

Western blot analysis of \(p53\) and \(\text{p21WAF1/CIP1}\) expression showed induction of a \(p53\) response after treatment with 16 \(\mu\text{mol/L}\) nutlin-3 for 24 hours in all three types of control-infected SK-N-SH cells but not in LV-h-\(p53\)-infected SK-N-SH cells (Fig. 5B), validating selective impairment of \(p53\) function in the latter cells.

Transduction of SK-N-SH cells with LV-h-\(p53\) prevented nutlin-3-induced premature senescence as evidenced by cell morphology evaluation and staining for SA-\(\beta\)-gal activity (Fig. 5C). Incubation of LV-h-\(p53\)-infected SK-N-SH cells with 16 \(\mu\text{mol/L}\) nutlin-3 for 24 hours did not induce phenotypic alterations and yielded a similar low density of SA-\(\beta\)-gal-expressing cells (53 per mm\(^2\) cell culture area; 95% CI, 39-67) as treatment of a parallel culture, plated at the same initial cell density, with vehicle control for 24 hours (58 per mm\(^2\); 95% CI, 43-73; \(P > 0.05\)). Induction of neuronal differentiation by nutlin-3 in NGP cells was also completely abolished when human \(p53\) was silenced. Cultures of NGP cells

**Figure 2.** Nutlin-3 treatment stabilizes \(p53\) and selectively induces expression of \(p53\) target genes in neuroblastoma cells with wild-type \(p53\). A. CLB-GA, IMR-32, NGP, and SK-N-SH cells with wild-type \(p53\) and SK-N-BE(2c) cells with \(TP53\) mutation were treated with 0 to 32 \(\mu\text{mol/L}\) nutlin-3 for 24 hours, and expression of \(p53\) target genes with a role in autoregulation (MDM2), cell cycle arrest (CDKN1A (\(\text{p21WAF1/CIP1}\)), and apoptosis (BAX, BBC3 (PUMA), and TP53I3 (PIG3)) was analyzed by real-time quantitative RT-PCR. Results are fold induction of mRNA expression compared with vehicle-treated cells. The lack of up-regulation of \(TP53I3\) expression in NGP cells was unexpected and suggests that PIG3 is nonfunctional in this cell line. Columns, mean of two different RT-PCR measurements; bars, SE. B. Western blot analysis of \(p53\), MDM2, \(\text{p21WAF1/CIP1}\), and BAX expression in neuroblastoma cells with wild-type \(p53\) (IMR-32, SK-N-SH, CLB-GA, and NGP) or with \(TP53\) mutation (SK-N-BE(2c)) after treatment with 16 \(\mu\text{mol/L}\) nutlin-3. A or an equivalent amount of solvent (--) for 24 hours. Expression of \(\gamma\)-tubulin for each lysate as a loading control. Results of nutlin-3-induced changes in mRNA and protein expression levels of \(p53\) target genes for all cell lines in this study are available as Supplementary Data, Figs. S2 and S3.
infected with LV-h-p53 did not exhibit morphologic changes when treated with 16 μmol/L nutlin-3 for 7 days (Fig. 5C), which was confirmed by the absence of consistent changes in the mRNA expression pattern of neuronal/neuroendocrine marker genes after 7 days of treatment with 16 μmol/L nutlin-3 compared with vehicle control (Fig. 5D). These results indicate that nutlin-3 induces premature senescence in SK-N-SH cells and neuronal differentiation in NGP cells in a p53-dependent manner, consistent with targeted inhibition of the p53-MDM2 interaction.

**Discussion**

Neuroblastoma accounts for ~15% of all childhood cancer deaths (31) and often confronts survivors later in life with severe genotoxic side effects of treatment. Direct and specific activation of the p53 pathway without inducing collateral DNA damage offers a tantalizing answer to the shortcomings of current therapeutic regimens and seems a reasonable approach for neuroblastoma in view of the infrequent occurrence of TP53 mutations. Cumulative evidence of inappropriately increased MDM2 activity in neuroblastoma incited us to examine the effects of targeted inhibition of the p53-MDM2 interaction by nutlin-3 in neuroblastoma cells. We found that treatment with nutlin-3 stabilizes p53 and selectively induces expression of p53 target genes in neuroblastoma cells with wild-type p53, leading to G1 cell cycle arrest and apoptosis. Of particular interest is the observation of alternative nutlin-3 response programs in surviving cells (i.e., premature cellular senescence in SK-N-SH cells and neuronal differentiation in NGP...
Figure 4. Induction of premature cellular senescence in surviving SK-N-SH cells and sympathetic neuronal differentiation in surviving NGP and CLB-GA cells by nutlin-3. A, cellular morphology and SA-β-gal staining of SK-N-SH cells treated with vehicle control (−) or 16 μmol/L nutlin-3 (+) for 1 day (left) or 7 days (right). Cells surviving incubation with nutlin-3 entered a state of premature cellular senescence as evidenced by their increased volume, flattened morphology, enhanced cytoplasmic granularity, and augmented SA-β-gal activity. Bar, 50 μm. B, phase-contrast images of NGP (left) and CLB-GA (right) cells under control conditions (−) and after treatment with 16 μmol/L nutlin-3 (+) for 7 days. Both NGP and CLB-GA cells surviving exposure to nutlin-3 displayed signs of neuronal differentiation, including a polar morphology and long neuritic processes, which formed a netlike arrangement. Bar, 50 μm. C, fold change of mRNA expression in NGP cells treated with 16 μmol/L nutlin-3 for 7 days relative to vehicle-treated cells for the neuronal/neuroendocrine differentiation marker genes NEFL, NEF3, NEF4, GAP43, HNT, CHGA, CHGB, IGF2, TH, DDC, DBH, PNMT, NPY, CHAT, NTRK1, and NTRK2. Asterisk, NTRK2 expression no longer detectable after 16 μmol/L nutlin-3 for 7 days due to profound down-regulation; minimal fold change of NTRK2 expression is displayed. Columns, mean of two different RT-PCR analyses; bars, SE. D, relative mRNA expression levels of NEFL, HNT, and PRPH and mRNA expression ratio of NTRK1 to NTRK2 in CLB-GA cells after exposure to 0 or 16 μmol/L nutlin-3 for 1, 2, 3, or 7 days. Asterisk, HNT expression not detectable in vehicle-treated CLB-GA cells. Columns, mean of two different RT-PCR measurements; bars, SE. Relative mRNA expression levels of the complete set of neuronal/neuroendocrine differentiation marker genes studied are available as Supplementary Data, Figs. S4 (NGP) and S5 (CLB-GA).
and CLB-GA cells), which depend on functional p53, as shown by abrogation of these responses when p53 is silenced using a lentiviral vector expressing shRNA. These findings reveal pleiotropic activities of nutlin-3 to incapacitate neuroblastoma cells and make this class of compounds particularly attractive for treatment of tumors that are arrested in their differentiation as is the case in neuroblastoma.

Functional outcome of nutlin-3 treatment was primarily dependent on the mutational status of TP53. Nutlin-3 activity was observed in all neuroblastoma cell lines with wild-type p53 regardless of MDM2 and MYCN amplification status. The absence of correlation between MYCN amplification status and responsiveness to nutlin-3 might suggest that MDM2 deregulation is not the critical oncogenic switch by which MYCN-amplified neuroblastomas acquire a more aggressive behavior than MYCN single copy tumors, in contrast to what has been recently proposed (32), and indicates that both neuroblastoma patients with and without MYCN amplification could benefit from treatment with MDM2 antagonists. Several additional genomic aberrations are commonly found in neuroblastoma, including loss of 1p, 3p, and 11q and gain of 17q, enabling classification of neuroblastoma patients into different clinicogenetic subgroups (33). Genomic copy number alterations for the panel of neuroblastoma cell lines in this study will be published elsewhere.7 For none of the frequently altered

Figure 5. Induction of premature senescence in SK-N-SH cells and neuronal differentiation in NGP cells by nutlin-3 is p53 dependent. A, effect of nutlin-3 on cell viability of uninfected cells (top), cells infected with a lentiviral vector carrying a shRNA construct against human p53 (LV-h-p53; middle), and cells infected with a control lentiviral vector encoding a shRNA specific for murine p53 (LV-m-p53; bottom) for cell lines NGP and SK-N-SH. Results are percentage cell viability with respect to vehicle-control-treated cells. Points, average of three different experiments, each done in duplicate; bars, SD. B, Western blot analysis of p53 and p21WAF1/CIP1 expression after treatment with 16 μmol/L nutlin-3 for 24 hours in SK-N-SH cells infected with LV-h-p53, LV-h-p53, and two additional control shRNA lentiviral vectors targeting a different region of the murine Trp53 gene (LV-m2-p53) and firefly luciferase (LV-luc), showing selective impairment of p53 function in LV-h-p53-infected cells. Expression of γ-tubulin as a loading control. C, SA-β-gal staining of SK-N-SH cells infected with LV-h-p53 after treatment with 0 μmol/L (−) or 16 μmol/L nutlin-3 (+) for 1 day (top) and cellular morphology of NGP cells infected with LV-h-p53 after exposure to 0 μmol/L (−) or 16 μmol/L nutlin-3 (+) for 7 days (bottom). No differences were observed between the 0 and 16 μmol/L treated cells. Bar, 50 μm. D, fold change of mRNA expression in NGP cells infected with LV-h-p53 after treatment with 16 μmol/L nutlin-3 for 7 days relative to vehicle-treated cells for 7 days for the same panel of neuronal/neuroendocrine differentiation marker genes as reported in Fig. 4C (please mind the different scale). Columns, mean of two different RT-PCR analyses; bars, SE.

genomic regions in neuroblastoma could a significant association with nutlin-3 responsiveness be shown, although it is possible that additional genetic defects may exist that attenuate or modify the response to nutlin-3. Of note is that SK-N-BE(2c) and SK-N-FI cells with TP53 consistently displayed some degree of cell viability reduction when exposed to high nutlin-3 concentrations. This might theoretically reflect general cytotoxicity of nutlin compounds, residual activity of p53 missense mutants, or p53-independent activities of nutlin-3 and is thus an issue that needs to be addressed more closely to uncover whether this might curtail or favor therapeutic usefulness. One possible explanation concerns the p53 homologues p63 and p73, which are also able to suppress cell growth and induce apoptosis. Physical interaction between p73 and MDM2 resulting in inhibition of p73 transactivating activity has been clearly established (34, 35), whereas for p63 conflicting results on the relationship with MDM2 exist (36–40). Interestingly, the ability of p53 to bind MDM2 is primarily dependent on a triad of hydrophobic amino acids (F19-W23-L26), which insert deeply into a hydrophobic cleft on the surface of MDM2 (41), and this FWL motif is evolutionary conserved among p53, p63, and p73 despite a high degree of degeneration of the surrounding region. Activity of nutlin compounds relies on their structural mimicry to the conformation of the FWL motif (8). Thus, the conserved FWL motif in p63 and p73 might act as a binding site for MDM2, and nutlins might in turn disrupt the interaction between MDM2 and p53 family members, which could result in the observed increase in caspase-3 and caspase-7 activity in SK-N-BE(2c) cells after treatment with 32 μmol/L nutlin-3 for 12 and 24 hours.

From a clinical perspective, the observation of premature senescence and neuronal differentiation as treatment effects parallel to G1 arrest and apoptosis is of substantial interest. Contribution of cellular senescence and differentiation to treatment outcome in vivo has been shown for conventional chemotherapy (26, 42). Our results suggest that a similar situation may be applicable to targeted therapeutics and indicate the feasibility of exploiting, by a single targeted intervention, the multitude of anticancer activities carried out by major tumor suppressors as p53. In addition, addressing multiple cellular programs could provide important failsafe mechanisms countering attempts of cancer cells to escape from therapy.

The differentiation process elicited by nutlin-3 in NGP and CLB-GA cells was characterized by expression analysis of well-established sympathetic neuronal or neuroendocrine marker genes. Neuroblastoma cells are arrested at immature stages of sympathetic differentiation, impairing the normal development into neuronal/ganglionic and neuroendocrine chromaffin cell lineages. The extensive neurite outgrowth, the expression of NEFH, NEF3, NEFH, HNT, and GAP43, the decrease in CHGA expression, and the neurotransmitter profile (NPY and CHAT) strongly suggest that the surviving fraction of nutlin-3-treated NGP cells matures along a sympathetic cholinergic neuronal lineage, a phenomenon also reported on retinoic acid treatment of cultured neuroblastoma cells (43, 44). In CLB-GA cells, nutlin-3 promoted a noncholinergic sympathetic neuronal differentiation, with an initial transient coexpression of sympathetic neuronal and neuroendocrine marker genes reminiscent of the mixed neuronal/neuroendocrine phenotype in some maturing neuroblastoma tumors (27). Furthermore, both nutlin-3-treated NGP and CLB-GA cells acquired a >100-fold shift in the neurotrophin receptor expression profile toward a higher NTRK1 to NTRK2 ratio, indicative of a less aggressive phenotype.

In summary, our data show that targeted disruption of the p53-MDM2 interaction unchains the powerful antitumor capacities of p53 in neuroblastoma cells by inducing a unique combination of G1 arrest, apoptosis, premature senescence, and neuronal differentiation. If confirmed in vivo, selective MDM2 antagonists could offer a novel therapy concept for treatment of neuroblastoma patients with and without MYCN amplification by inducing tumor growth inhibition, regression, and maturation.

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