Pharmacological activation of the p53 pathway by nutlin-3 exerts anti-tumoral effects in medulloblastomas

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Medulloblastomas account for 20% of pediatric brain tumors. With an overall survival of 40%–70%, their treatment is still a challenge. The majority of medulloblastomas lack p53 mutations, but even in cancers retaining wild-type p53, the tumor surveillance function of p53 is inhibited by the oncoprotein MDM2. Deregulation of the MDM2/p53 balance leads to malignant transformation. Here, we analyzed MDM2 mRNA and protein expression in primary medulloblastomas and normal cerebellum and assessed the mutational status of p53 and MDM2 expression in 6 medulloblastoma cell lines. MDM2 expression was elevated in medulloblastomas, compared with cerebellum. Four of 6 medulloblastoma cell lines expressed wild-type p53 and high levels of MDM2. The tumor-promoting p53-MDM2 interaction can be inhibited by the small molecule, nutlin-3, restoring p53 function. Targeting the p53-MDM2 axis using nutlin-3 significantly reduced cell viability and induced either cell cycle arrest or apoptosis and expression of the p53 target gene p21 in these 4 cell lines. In contrast, DAOY and UW-228 cells harboring TP53 mutations were almost unaffected by nutlin-3 treatment. MDM2 knockdown in medulloblastoma cells by siRNA mimicked nutlin-3 treatment, whereas expression of dominant negative p53 abrogated nutlin-3 effects. Oral nutlin-3 treatment of mice with established medulloblastoma xenografts inhibited tumor growth and significantly increased survival. Thus, nutlin-3 reduced medulloblastoma cell viability in vitro and in vivo by re-activating p53 function. We suggest that inhibition of the MDM2-p53 interaction with nutlin-3 is a promising therapeutic option for medulloblastomas with functional p53 that should be further evaluated in clinical trials.

Keywords: MDM2, medulloblastoma, nutlin-3, p21, wild-type p53.
potentially pre-neoplastic cells. Indeed, loss of p53 facilitates medulloblastoma development in mouse models, and up to 40% of medulloblastomas show p53 protein expression indicative of a dysfunctional p53 pathway. An important role for p53 inactivation in medulloblastoma pathogenesis is also suggested by the increased incidence of medulloblastomas in patients with Li Fraumeni syndrome, which is caused by a germ-line mutation in p53.

Dysfunction of p53 because of mutations in the TP53 tumor suppressor gene is rare in neuroectodermal embryonal tumors, including medulloblastomas. Fewer than 10% of sporadic medulloblastomas display TP53 mutations, which are associated with adverse outcome in pediatric patients. The mechanistic underlying the inactivation of the p53 pathway in the majority of medulloblastomas and other brain tumors has remained unclear for many years. It has been more recently observed in other cancers with wild-type p53 that p53 inactivation can be achieved by different alternative routes. One of these alternative routes of potential clinical significance for medulloblastoma is the rapid proteasomal degradation of p53 mediated by direct interaction of p53 with the E3 ubiquitin ligase, MDM2, which is promoted by the ubiquitination factor E4B. Amplification or overexpression of MDM2, leading to increased degradation of p53, is frequently observed in tumors with wild-type TP53. Although MDM2 gene amplification occurs in approximately 7% of human malignancies that lack TP53 mutations, it has not been detected in medulloblastomas. However, MDM2 protein overexpression has been observed at least in a subset of adult medulloblastomas. More significantly, the discovery that the loss of MDM2, leading to increased degradation of p53, is frequently observed in tumors with wild-type TP53. Although MDM2 gene amplification occurs in approximately 7% of human malignancies that lack TP53 mutations, it has not been detected in medulloblastomas. However, MDM2 protein overexpression has been observed at least in a subset of adult medulloblastomas. More significantly, the discovery that the loss of MDM2, leading to increased degradation of p53, is frequently observed in tumors with wild-type TP53. Although MDM2 gene amplification occurs in approximately 7% of human malignancies that lack TP53 mutations, it has not been detected in medulloblastomas. However, MDM2 protein overexpression has been observed at least in a subset of adult medulloblastomas. More significantly, the discovery that the loss of MDM2, leading to increased degradation of p53, is frequently observed in tumors with wild-type TP53. Although MDM2 gene amplification occurs in approximately 7% of human malignancies that lack TP53 mutations, it has not been detected in medulloblastomas. However, MDM2 protein overexpression has been observed at least in a subset of adult medulloblastomas. More significantly, the discovery that the loss of MDM2, leading to increased degradation of p53, is frequently observed in tumors with wild-type TP53. Although MDM2 gene amplification occurs in approximately 7% of human malignancies that lack TP53 mutations, it has not been detected in medulloblastomas. However, MDM2 protein overexpression

**Materials and Methods**

**Cell Lines and Nutlin-3 Treatment**

The human medulloblastoma cell lines, DAOY, HD-MB3, ONS-76, UW-228, and D-341, were grown in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics. Medium for cells used for xenografting into mice was also supplemented with 1% NEAA. D-283 cells were cultured in Eagle’s Minimum Essential Medium supplemented with 10% FCS and antibiotics. All cell lines were authenticated by STR DNA typing by the DSMZ (Braunschweig, Germany) prior to experiments. The HD-MB3 cell line was a gift from H. Deubzer, who generated this cell line in the DKFZ (Heidelberg, Germany). Nutlin-3 (Cayman Chemical Europe) was dissolved in ethanol and stored as a 10 mmol/L stock solution in small aliquots at −20°C. Cells were exposed to 0–32 μmol/L nutlin-3 for the period indicated, with the final ethanol concentration kept constant in each experiment. For rescue experiments, ONS-76 cells (p53 wt) were electroporated with a plasmid encoding dominant negative p53 (donated by Prof. M. Eilers) containing the Val135 p53 mutation or EGFP as a control. Selection of transfected cells was achieved by addition of puromycin (1 g/ml) to the medium.

**TP53 Mutation, Global Genomic, and Expression Analysis**

Mutation analysis of TP53 was done according to established protocols in the laboratory of H.-P. Seelig (Karlsruhe, Germany). For array comparative genome hybridization (array CGH), samples were profiled on 60K or 180K human whole-genome oligoarrays (Agilent Technologies). Data were uploaded to a publicly available database (http://medgen.ugent.be/arraycghbase) and analyzed as previously described. MDM2 and p21 gene expression was monitored using real-time polymerase chain reaction (PCR) using Assays on Demand (Applied Biosystems). Expression values were normalized to the geometric mean of GAPDH. For all these experiments, total RNA was isolated from cells with use of the RNeasyMini kit (Qiagen) and cDNA synthesis was performed using the SuperScript reverse transcription kit (Invitrogen).
Western Blot Analysis

Protein lysates were extracted from cells treated with 4 μmol/L nutlin-3 for 24, 48, or 72 h and blotted as described in Kahl et al.23 Transfer membranes were incubated for 1–12 h using the following antibodies and dilutions: p21 (polyclonal, Abcam), 1:500; p53 (clone 2B2.68, Santa Cruz), 1:500; MDM2 (clone IF2, Zymed Laboratories), 1:800; β-actin (Sigma-Aldrich), and 1:2000.

Cell Proliferation, Viability, and Cycle Analysis

Cells were seeded onto 96-well plates (2 × 103 per well) in triplicate, incubated for 6 h to permit surface adherence, and treated with 0–32 μmol/L nutlin-3 for 24, 48, or 72 h. Medium was replaced daily, and nutlin-3 and ethanol concentrations were constant throughout the experiment. Cell viability was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche), performed according to the manufacturer’s protocol. The IC50 was calculated using GraphPad Prism 5.0 (GraphPad Incorporation). For analysis of cell cycle distribution, cell lines were cultured in the presence of 4 μmol/L nutlin-3 or an equivalent amount of ethanol for 24, 48, or 72 h; trypsinized; washed with PBS; and incubated with propidium iodide for 15 min to stain DNA. Cellular DNA content was analyzed in an FC500 flow cytometer (Beckman Coulter). Apoptosis was assessed using the Cell Death enzyme-linked immunosorbent assay (ELISA; Roche), cell proliferation was assayed using the BrdU ELISA (Roche), and cell proliferation was assayed according to the manufacturer’s protocols. For both assays, cells were seeded onto 96-well plates (7 × 10³ per well) in duplicate, incubated for 6 h to permit surface adherence, and treated with 4 μmol/L nutlin-3 for 24, 48, or 72 h. Medium was replaced daily, and nutlin-3 concentration was constant throughout the experiment. To assess cellular survival in real time, HD-MB3 cells (7 × 10³ per well) were plated onto 96-well Xcelligence microelectronic cell sensor plates (Roche) in triplicate and cultured overnight in antibiotic-free complete media. Cells were transiently transfected with siRNAs directed against MDM2 (QIAGEN, Cat. No. SI02653392 and SI00300846) or control siRNA at a final concentration of 20 pmol, and adherence was continuously monitored for 144 h to assess cellular survival. All experiments were independently performed at least 3 times, if not otherwise indicated.

Immunohistochemistry

Tissue microarrays (TMA) were prepared from paraffin-embedded tissue specimens of 86 primary medulloblastomas and 10 cerebellum samples selected from archived files in the Department of Pediatric Oncology and Hematology, University Children’s Hospital, Zurich, Switzerland. Three different tissue cores in a single tumor were arrayed from formalin-fixed, paraffin-embedded tissue blocks using a manual device (Beecher Instruments). Two micrometer paraffin sections were cut from every tissue microarray and used for subsequent immunohistochemical analyses. In addition, xenografted tumors harvested from nu/nu mice treated either with nutlin-3 or with control were paraffin embedded, and 5 μm sections were cut from each block. For both TMA and xenograft tumors, immunohistochemical staining was conducted as previously described.23 In brief, formalin-fixed paraffin-embedded tissue sections were deparaffinized by routine techniques and placed in 200 mL of target retrieval solution (pH, 6.0; Envision Plus Detection Kit; Dako) for 20 min at 100°C. After cooling for 20 min, slides were quenched with 3% H2O2 for 5 min before incubating with primary antibody using a Dako Autostainer (Dako Cytomation). Primary antibodies used were: p21 (1:25, Cytomed), p53 (1:250, DAKO), MDM2 (1:100, Invitrogen), cleaved caspase 3 (1:200, Cell Signaling), and Mib-1 (1:25, DAKO). Slides were developed with EnVisionTM (Dako). Nuclear immunostaining for p53 and MDM2 was evaluated on the TMA with use of a semiquantitative scoring system by 2 independent researchers. In brief, the number and intensity of positive cells were counted and scored between 0 and 3 (0, no positive nuclei; 1, <20% nuclei display intense staining or more nuclei display weak staining; 2, 20%–80% intense staining or more nuclei display moderate staining; 3, 80%–100% nuclei display intense staining). Samples with a score of 2 or 3 were considered to be positive.

Growth of Xenograft Tumors in Nude Mice

HD-MB3 medulloblastoma cells were cultured to 80% confluency, harvested, and suspended in 200 μL Matrigel (BD Bioscience) for subcutaneous inoculation (1 × 10³ cells per mouse, n = 10 mice) into the right flank of 4-week-old female athymic NCR (nu/nu) mice. Mice were randomly assigned to either nutlin-3 or vehicle control groups (n = 5 mice per group) after tumors reached 100–300 mm³ in size. Nutlin-3 or vehicle control was administered by oral gavage twice daily at a dose of 200 mg per kg body weight in 2% Klucel and 0.2% Tween-80 (both from Fagron). Tumor growth was monitored using a caliper, and tumor volume was calculated using the formula (breadth × length × height)/2. Mice were euthanized by cervical dislocation when tumor size exceeded 1000 mm³. The tumor, liver, spleen, brain, and kidneys were removed, and half of each tissue was snap-frozen in liquid nitrogen then stored at −80°C, and the other half of tissue was formalin fixed and paraffin embedded. To examine the effects of nutlin-3 treatment on reactivation of the p53 pathway, nu/nu mice with established HD-MB3 xenografts were treated orally with 3 doses of nutlin-3 (200 mg per kg body weight) over a course of 24 h (doses were given at 0, 12, and 24 h; n = 2). Mice were euthanized by cervical dislocation 4 h after the last dose, and tumors were excised.
then snap frozen (half) and formalin fixed (half). All animal experiments were performed in accordance with the Council of Europe guidelines for accommodation and care of laboratory animals and protocols were approved by the Institutional Ethical Commission for Animal Experimentation.

**Statistical Analysis**

Re-analysis of microarrays data was performed using R2 (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi). SPSS, version 18.0 (SPSS) was used for further statistical analysis. Student’s 2-sided t test was used for the comparison of all interval variables, and χ² test was used for the comparison for all categorical variables. Graph Pad Prism 5.0 was used to calculate IC50 concentrations and to perform Kaplan Meier survival analysis with log rank statistics for the mouse cohorts.

**Results**

**MDM2 mRNA and Protein Expression Is Elevated in Primary Medulloblastomas and Medulloblastoma Cell Lines**

Inactivation of p53 via mutation is known to occur in only a small percentage of primary medulloblastomas. MDM2 overexpression is an alternative mechanism to inactivate p53 function, which has not been investigated in pediatric medulloblastomas to date. We therefore analyzed MDM2 protein expression using immunohistochemistry on tissue arrays consisting of 86 primary medulloblastomas and 10 cerebellum samples, as a normal tissue control (Fig. 1A and B). MDM2 protein expression was detected in 25 of 86 primary medulloblastomas (29%), whereas no MDM2 protein expression was detected in normal cerebellum samples (P = .041. Kool, et al. previously published global mRNA expression profiles of 62 primary medulloblastomas and 9 samples of normal cerebellum performed on Affymetrix chips.26 We re-analyzed these data and detected significantly higher MDM2 mRNA expression levels in medulloblastomas, compared with cerebellum (P < .001) (Fig. 1C).

We then analyzed the mutational status of p53 in 6 established medulloblastoma cell lines and identified TP53 mutations in DAOY (C242F, previously reported27) and UW-228 (T155N) cells. HD-MB3 and ONS-76 medulloblastoma cells possess the R72P SNP in TP53, which affects neither p53 expression nor function. TP53 was not mutated in D-283 and D-341 medulloblastoma cells. Because mutant p53 is reported to accumulate at higher levels than wt-p53, we also analyzed p53 protein expression using immunohistochemistry on the tissue arrays described above (Supplementary material, Fig. S1). Expression of p53 protein was detectable in only 11 of 86 primary medulloblastomas (13%), whereas p53 protein expression was absent in all analyzed normal cerebellum samples. Of interest, MDM2 mRNA expression was higher in the 4 medulloblastoma cell lines (Fig. 1D), all of which expressed wild-type p53 but demonstrated p53 dysfunction indicated by low p21 expression. MDM2 protein expression was also higher in these 4 medulloblastoma cell lines without TP53 mutations, compared with the 2 with TP53 mutations (Fig. 1E). A frequent reason for elevated MDM2 mRNA expression in cancers is the amplification of the MDM2 gene.28 However, MDM2 amplification was not detected in any of the 6 medulloblastoma cell lines using array CGH (Fig. 1F and Supplementary material, Fig. S2). Taken together, we detected elevated MDM2 mRNA and protein expression in all medulloblastoma cell lines with wild-type p53 and in primary medulloblastomas. On the basis of these results and the published literature, we hypothesized that (1) elevated MDM2 expression is a potential mechanism for p53 inactivation in medulloblastoma cells with wild-type p53, which might ultimately lead to tumorigenesis, and (2) p53 tumor suppressor function could be restored in medulloblastoma cells by blocking the interaction between MDM2 and p53 with the small molecule, nutlin-3.

**Nutlin-3 Reduces Viability of Medulloblastoma Cells with Wild-Type p53 In Vitro**

To test this hypothesis, we first assessed the effect of the small molecule, nutlin-3, in medulloblastoma cell lines with and without TP53 mutations. All 6 cell lines were treated with varying concentrations of nutlin-3 in vitro. Nutlin-3 treatment significantly reduced viability of the 4 medulloblastoma cell lines with wild-type p53 (D-283, D-341, HD-MB3, and ONS-76), whereas the DAOY and UW-228 cell lines harboring mutated p53 were remarkably less sensitive to nutlin-3 treatment, as indicated by the significantly higher IC50 values (Fig. 2A). We also observed that nutlin-3 treatment inhibited growth of HD-MB3 and ONS-76 cell lines with wild-type p53 but did not affect the growth of medulloblastoma cell lines with mutated p53 (Fig. 2B). This experiment was technically feasible only for the cell lines growing in monolayers, not for the D-283 and D-341 cell lines growing in suspension.

FACS analysis revealed that nutlin-3 treatment increased the fraction of apoptotic cells, as indicated by the higher percentage of sub-G1 cells in 3 of 4 medulloblastoma cell lines with wild-type p53, but not in the 2 cell lines with mutant p53 (Fig. 2C). These results were also confirmed using a cell death ELISA that detected mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates (Fig. 2D). Proliferation of HD-MB3 and ONS-76, measured by a BrdU ELISA, was dramatically reduced after 72 h of nutlin-3 treatment, whereas proliferation of the p53 mutant cell lines, DAOY and UW-228, remained unaffected (Fig. 2E). Of interest, siRNA-mediated knockdown of MDM2 performed representatively in HD-MB3 cells mimicked the effect of nutlin-3 treatment and inhibited cell growth, whereas transfection of scrambled control siRNA or mock transfection did not affect cell growth (Fig. 2F). These results
indicate an important role of MDM2 and its interaction with p53 for the viability and proliferation of medulloblastoma cells.

As further support for our hypothesis, we next investigated expression of p53 and its downstream target, p21, on both protein and mRNA levels to examine whether the p53 pathway is indeed dysfunctional or inhibited in medulloblastoma cells with wild-type p53 and whether it can be restored by nutlin-3 treatment. Baseline expression of p21 protein was very low in medulloblastoma cell lines with wild-type p53. Nutlin-3 treatment of these cells significantly elevated p21 and p53 protein expression in contrast to medulloblastoma cell lines without wild-type p53 (Fig. 3A and C). In real-time RT-PCR, mean p21 expression peaked at different times after the start of treatment (HD-MB3: 4.1-fold at 48 h, ONS-76: 9.2-fold at 24 h, D-283: 24.1-fold at 24 h, D-341: 59.4-fold for 72 h) but was significantly increased by nutlin-3 treatment in all cell lines expressing wild-type p53. Nutlin-3 treatment did not significantly affect p21 expression in DAOY or UW-288 cells with mutant p53. Nutlin-3 also significantly induced MDM2 expression on both mRNA and protein levels in medulloblastoma cells with functional p53 (Fig. 3A–C). Mean MDM2 mRNA expression peaked at different times after the start of treatment (HD-MB3: 5.9-fold at 48 h, ONS-76: 8.3-fold at 24 h, D-283: 9.9-fold at 72 h, D-341: 13.7-fold at 48 h). Nutlin-3 treatment had no significant effect on MDM2 expression in DAOY and UW-288 medulloblastoma cells with mutant p53. Taken together, these results indicate that nutlin-3 treatment leads to pharmacological...
activation of the p53 pathway, which induces cell cycle arrest and apoptosis in medulloblastoma cells with functional p53.

To show the critical role of functional p53 for nutlin-3 activity, we investigated whether expression of a dominant negative mutant of p53 (dn-p53) was able to abrogate nutlin-3 effects in medulloblastoma cell lines with wild-type p53. Stable, ectopic expression of dn-p53 in the ONS-76 cell line as a representative example increased basal levels of the p53 protein. However, nutlin-3 treatment of dn-p53 expressing ONS-76 cells did not significantly induce p21 protein expression, indicating that dn-p53 inactivated intrinsic p53. Induction of p21 was achieved in parental ONS-76 cells or ONS-76 cells expressing GFP from the same vector used to express the dn-p53 construct (Fig. 3D). Furthermore, the sensitivity to nutlin-3 was significantly reduced in ONS-76 cells expressing dn-p53 (IC50: ONS-76 dn-p53 = 58.2 μM vs, ONS-76 = 0.5 μM, ONS-76 EGFP = 2.3 μM, Fig. 3E). Expression of dominant negative mutant p53 blocked nutlin-3 action and further supports that the effect of nutlin-3 is based on reactivation of functional p53 via blocking the p53-MDM2 interaction.

Nutlin-3 has Antitumoral Activity Against Human Medulloblastoma Cells Grown as Xenografts in Mice

To add further evidence for a therapeutic benefit of nutlin-3 for medulloblastoma, we treated mice with established HD-MB3 xenografts orally with nutlin-3 to assess its in vivo efficacy against medulloblastoma cells with functional p53. The treatment regimen was well tolerated and did not alter the physical status or behavior of the mice, induce weight loss, or produce any other obvious signs of toxicity. The growth of
HD-MB3 xenografts was significantly suppressed in mice treated with nutlin-3 (n = 5), compared with mice treated with vehicle control (n = 5) (Fig. 4A). After 8 days of treatment, the mean volumes of HD-MB3 xenografts were 388 mm³ for nutlin-3-treated mice and 1018 mm³ for vehicle-treated mice (difference in mean tumor volume between treatment groups = 630 mm³, 95% CI = 195–212 mm³, P = .004). With a primary end point defined as a tumor volume > 1000 mm³, the survival of the mice treated with nutlin-3 was significantly prolonged when compared to mice treated with vehicle control (Fig. 4B, Supplementary material, Table S1). Nutlin-3 treatment of the mice activated the p53 pathway in the xenograft tumor tissue, as we showed for medulloblastoma cell lines grown in vitro. Both p53 and p21 proteins were elevated in comparison with the xenograft tumors from nontreated mice (Fig. 4C and D). Also consistent with our in vitro data, immunohistochemical analysis of the xenograft tumors indicated increased apoptosis and decreased proliferation after nutlin-3 treatment by an increase of cleaved caspase 3 and reduced KI-67, respectively. Nutlin-3 treatment also induced MDM2 expression in the xenograft tumors through a feed-back loop. Thus, nutlin-3 activated the p53 pathway in vivo, decreased xenograft tumor growth, and increased mouse host survival.

**Discussion**

We report here that nutlin-3 shows anti-tumoral activity against medulloblastoma cells with wild-type p53 grown both in vitro and as mouse xenograft tumors in vivo. Nutlin-3 exerted its effect through re-activation of the p53 pathway, an effect that was mimicked by MDM2 knockdown. Our data confirmed elevated expression of MDM2 mRNA and protein in primary medulloblastomas in comparison with cerebellum. MDM2 mRNA expression was also elevated in medulloblastoma cell lines with wild-type p53, but we detected no amplification of the MDM2 gene in medulloblastoma cell lines as a possible explanation for elevated expression. Our data confirm 2 smaller previous studies analyzing MDM2 expression in primary medulloblastomas, in which also no amplification of the MDM2 gene was detected, whereas overexpression of MDM2 protein in a subset of adult medulloblastomas could be shown. Our data indicate that MDM2 is mainly upregulated at the transcriptional level in medulloblastoma cells and that high levels of MDM2 were at least partly responsible for functional inactivation of p53 in medulloblastoma cell lines with wild-type p53.

Mechanisms that lead to upregulation of MDM2 in the absence of gene amplification might include changes in the expression levels of upstream regulators of MDM2 transcription but might also include epigenetic changes at the MDM2 locus or downregulation of miRNAs that target MDM2. Further research to elucidate the mechanisms that lead to increased MDM2 expression is warranted, because targeting these regulatory pathways could be alternative or even synergistic to targeting MDM2 with nutlin, as analyzed in this study.

A series of genetic studies in mouse models have shown that loss of p53 induces tumor formation and that restoration of p53 leads to a rapid regression of established tumors. 

**Fig. 3.** Treatment of medulloblastoma cells with nutlin-3 activated the p53 pathway in wild-type p53 cells. (A–C) Activation of the p53 pathway was shown by induction of p21 and MDM2 protein and mRNA in a medulloblastoma cell line that harbors wt TP53 (ONS-76) in comparison to a cell line with p53 mutated (DAOY). (D and E) Induction of p21 by nutlin-3 in p53-wt ONS-76 cells was abrogated by transfection with dominant negative p53 (dn-p53), but not by a control-transfected plasmid encoding GFP, as shown by Western blotting. Cell viability assays (MTT) confirmed that dn-p53 rescues ONS-76 from nutlin-3 induced cell death.
tumors, providing strong evidence for designing antican-
cer drugs that restore p53 function.\textsuperscript{31–33} Several different therapeutic approaches have been attempted with this
goal.\textsuperscript{21,34–36} Among these, targeting the MDM2-p53
interaction by small molecules for the reactivation of
p53 has emerged as a promising approach for the

Fig. 4. Nutlin-3 has antitumoral activity against medulloblastoma tumor xenografts in mice. (A) Tumor growth was significantly delayed and
(B) survival was significantly prolonged in mice treated with nutlin-3 ($n = 5$), compared with controls ($n = 5$). (C) Induction of MDM2, p53,
and p21 protein was detectable in tumors from mice treated with nutlin-3 ($n = 2$, tumor C + D) but not in controls ($n = 2$, tumor A + B). (D)
Immunohistochemical analysis also showed induction of MDM2, p53, and p21 protein, in addition to increased apoptosis (cleaved caspase 3)
and decreased proliferation (Ki-67) in tumors from mice treated with nutlin-3 (tumor C + D) but not in controls (tumor A + B).
treatment of cancers that retain wild-type p53. The atomic-level understanding of the MDM2-p53 interaction through X-ray crystallography provided the solid foundation for structure-based design of small-molecule antagonists of this interaction. A breakthrough in the design of potent small-molecule MDM2 inhibitors was obtained through the discovery of small molecules, termed nutlins by Vassilev et al. The cis-imidazoline, nutlin-3, has a high binding affinity and specificity to MDM2 and a highly desirable pharmacokinetic profile. It has previously been shown to reduce the viability of several tumor cell lines expressing wild-type p53 in vitro. The cell lines assessed were derived from the neuroectodermal tumor, neuroblastoma, and from Ewing sarcoma, osteosarcoma, melanoma, and several carcinomas. Here, we show, for the first time to our knowledge, that nutlin-3 was effective at reducing the viability of medulloblastoma cell lines with wild-type p53 in vitro and in vivo. Nutlin-3 induced either cell cycle arrest characterized by depletion of S-phase cells and accumulation at G1/S phase boundaries of the cell cycle or p33-dependent apoptosis in medulloblastoma cell lines with functional p53. For the cell line in which nutlin-3 did not induce apoptosis, ONS-76, proliferation was suppressed, suggesting that nutlin-3 induced senescence in ONS-76 cells. In addition to upregulation of MDM2 expression in medulloblastoma cells with wild-type p53, activation of p53 by nutlin-3 also caused upregulation of the p53 target gene, the p21Waf1/Cip1 cyclin-dependent kinase inhibitor, which is an essential mediator of p53-induced cell cycle arrest. These results are consistent with previously published results that nutlin-1 treatment of tumor cell lines with wild-type p53 lead to accumulation of p53 and subsequent elevation of MDM2 and p21 expression, consistent with activation of the p53 pathway. Of interest, the levels of p53 or MDM2 do not increase steadily over the time points analyzed. Because nutlin-3 activates p53 immediately after treatment, the observed levels of apoptotic cells can be interpreted by an early p53 reactivation, causing a peak of apoptosis at 48 h after nutlin-3 treatment. As a compensatory mechanism to activation of p53, MDM2 levels may increase with a different kinetic than activation of pathways downstream of p53, and these effects can be cell line specific. Knockdown of MDM2 expression using siRNA phenocopied the effect of nutlin-3 on cell viability in medulloblastoma cells, further emphasizing the central role of MDM2 in the tumorigenic effect of the MDM2/p53 axis and in the targeting mechanism of nutlins. In contrast, enforced expression of dominant-negative mutant p53 abrogated the nutlin-3 effect, indicating that an intact p53 pathway is needed for sensitivity of medulloblastoma cells to MDM2 inhibitors. Conclusive evidence of the dependence of the activity of MDM2 inhibitors on p53 status has previously been provided by a recent study with 100 B-CLL patients treated with nutlin-3, in which the p53 status was identified as the major determinant of response. We showed in the first part of our study that treatment with the MDM2 inhibitor, nutlin-3, is effective against medulloblastoma cell lines with wild-type p53 and that this effect occurred through re-activation of the p53 pathway.

Translating a promising MDM2 inhibitor with excellent in vitro properties into an anticancer drug requires that the inhibitor have excellent pharmacological properties and be nontoxic for normal tissues. Previously published pharmacokinetic studies have shown that oral administration of nutlin-3 induced robust accumulation and activation of p53 in osteosarcoma and prostate cancer xenografts, as was also demonstrated in the tumor tissue of our medulloblastoma mouse model. Of most importance, the nutlin-3 effect appears to be selective for cancer over normal cells, as revealed by the lack of toxicity to peripheral blood mononuclear cells or bone marrow-derived hematopoietic progenitors and bone marrow stromal epithelial cells in patients with leukemia. In addition, nutlin-3 has been shown to protect normal cells against S-phase and M-phase-specific chemotherapeutic drugs without altering their activity against cancer cells. However, the latter data from in vitro studies await confirmation in vivo. We performed an initial evaluation of the efficacy of nutlin-3 treatment in vivo in a medulloblastoma xenograft mouse model, using a dosage regimen that has previously been shown to be safe for control mice and effective against osteosarcoma tumor xenografts. Nutlin-3 potently inhibited growth of medulloblastoma tumor xenografts expressing wild-type p53 and significantly prolonged mouse survival. These findings were consistent with previous studies of the drug in several xenograft models of human cancer with wild-type p53. Radioactive tissues, such as the thymus and crypts of the small intestine, are extremely susceptible to p53-induced apoptosis. Restoration of p53 by a genetic approach in the absence of MDM2 resulted in severe pathological damage to radiosensitive mouse tissues and death of all animals within 5 days, raising concern that p53 activation by MDM2 inhibitors could be toxic to healthy tissues. However, in our mouse model, nutlin-3 achieved its antitumor activity against medulloblastoma xenografts without causing visible signs of toxicity in the animals, as assessed by necropsy studies and assessment of body weight. Nutlin-3 had previously also shown little toxicity to animals at therapeutically efficacious dose schedules against osteosarcoma xenografts. In this study by Vassilev et al., nutlin-3 was well tolerated at doses of 200 mg/kg administered twice daily orally for 20 days.

Here, we provide first evidence that the p53 pathway can be re-activated by nutlin-3 treatment in medulloblastoma xenograft tumors and that targeting the MDM2/p53 axis using nutlins represents a potentially attractive therapeutic strategy for medulloblastomas retaining wild-type p53, because nutlin-3 has promising anti-tumoral activity against medulloblastoma cells with wild-type p53 both in vitro and in vivo. The effects of nutlin-3 against medulloblastoma cells occur through re-activation of the p53 pathway by prevention of p53 degradation through disruption.
of the p53-MDM2 interaction and lead either to cell death or to cell cycle arrest. Intensive research efforts in the past decade have yielded nutlin-3 as a potent and specific inhibitor of the MDM2-p53 interaction with desirable pharmacological properties. Nutlin-3, meanwhile, has progressed to advanced preclinical development or early-phase clinical trials for several tumor entities. In light of the fact that about 30% of patients with medulloblastoma experience relapses and that relapse tumors are often resistant to chemotherapeutic agents, it may be interesting to see whether extended treatment of human medulloblastoma xenografts for several months will create resistance to nutlin-3 or provoke changes associated with drug resistance in medulloblastoma cells. Testing nutlin-3 in an orthotopic xenograft model could also provide additional information about whether nutlin-3 is able to cross the blood-brain barrier, easing treatment in patients with medulloblastoma. Ultimately, clinical testing of nutlin-3 against medulloblastoma is necessary to provide the ultimate proof of the usefulness of this therapeutic strategy for the treatment of this highly aggressive pediatric malignancy.

Supplementary Material

Supplementary material is available at Neuro-Oncology Journal online (http://neuro-oncology.oxfordjournals.org/).

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