Nutlin-3a Efficacy in Sarcoma Predicted by Transcriptomic and Epigenetic Profiling

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Abstract

Nutlin-3a is a small-molecule antagonist of p53/MDM2 that is being explored as a treatment for sarcoma. In this study, we examined the molecular mechanisms underlying the sensitivity of sarcomas to Nutlin-3a. In an ex vivo tissue explant system, we found that TP53 pathway alterations (TP53 status, MDM2/MDM4 genomic amplification/mRNA overexpression, MDM2 SNP309, and TP53 SNP72) did not confer apoptotic or cytostatic responses in sarcoma tissue biopsies (n = 24). Unexpectedly, MDM2 status did not predict Nutlin-3a sensitivity. RNA sequencing revealed that the global transcriptomic profiles of these sarcomas provided a more robust prediction of apoptotic responses to Nutlin-3a. Expression profiling revealed a subset of TP53 target genes that were transactivated specifically in sarcomas that were highly sensitive to Nutlin-3a. Of these target genes, the GADD45A promoter region was shown to be hypermethylated in 82% of wild-type TP53 sarcomas that did not respond to Nutlin-3a, thereby providing mechanistic insight into the innate ability of sarcomas to resist apoptotic death following Nutlin-3a treatment. Collectively, our findings argue that the existing benchmark biomarker for MDM2 antagonist efficacy (MDM2 amplification) should not be used to predict outcome but rather global gene expression profiles and epigenetic status of sarcomas dictate their sensitivity to p53/MDM2 antagonists. Cancer Res 74(3): 921–31. ©2013 AACR.

Introduction

Sarcomas constitute a heterogeneous group of bone and soft tissue malignancies that account for approximately 15% of all pediatric and 1% of all adult cancers (1). Many sarcoma subtypes are resistant to cytotoxic agents and even for those subtypes that are chemosensitive, long-term responders are rarely seen due to cumulative toxicity and a therapeutic ceiling that has now been reached. Overall 5-year survival rates for patients diagnosed with soft-tissue sarcomas remains at 60%, highlighting the pressing need for new targeted systemic therapies particularly for those patients with recurrent or advanced disease. In parallel with this, new approaches to better understand the biologic predictors of patient response to these agents is critical and remains the key enabler for us to be able to move away from empiricism to a more targeted approach. In essence, the implementation of genetic markers into treatment practices will allow us to match the correct drug with the right patient.

Detailed molecular analysis of the oncogenic pathways involved in sarcomagenesis has revealed that the integrity of the p53 tumor suppressor protein and its downstream pathways remain largely intact in the majority of sarcomas (2). A mechanism commonly used by these malignancies to subvert the antitumor activity of p53 during sarcomagenesis and disease progression is through amplification or overexpression of MDM2, the principal ubiquitin ligase for p53 (3, 4). Such characteristics suggest that sarcomas are ideal candidates for therapies that inhibit MDM2, thus reactivating the p53 pathway to subsequently drive apoptosis. To date, the most clinically advanced MDM2 antagonist is Nutlin-3a (RG7112, Hoffmann La Roche). Identified by Vassev and colleagues, this cis-imidazoline molecule blocks the hydrophobic p53-binding pocket of MDM2, resulting in rapid stabilization of the biologically active p53 protein (5).
The importance of MDM2 in sarcomagenesis has emanated from the observation that its gene amplification is present in more than 90% of well-/de-differentiated liposarcomas (6). Promising results from preclinical studies have shown that sarcoma cell lines with MDM2 amplification are more sensitive to Nutlin-3a than non--MDM2-amplified cell lines (7, 8). Thus, it has been much anticipated to evaluate the clinical efficacy of targeting MDM2 in MDM2-amplified sarcomas. One of the earliest clinical trials of Nutlin (RG7112) was recently conducted in 20 patients with sarcomas highly enriched for MDM2 amplification (well-/de-differentiated liposarcoma). Surprisingly, a poor clinical response rate was observed, with only one patient achieving a partial response (9). So, does this mean that MDM2 inhibition is not an attractive therapeutic approach, or alternatively have we overestimated the role that MDM2 amplification plays in the response to MDM2 antagonists? A clear answer to this question will be critical as new MDM2 inhibitors in addition to Nutlin (RG7112, RG7388), such as MI-773/SAR405838 (Sano) and FJN-2654165 (Johnson & Johnson) enter phase I clinical testing (10–12).

Using primary human sarcoma tissue biopsies from a broad range of sarcoma subtypes with varying MDM2 status; this study has re-evaluated the capacity for MDM2 amplification as a biomarker to identify those patients with sarcoma most likely to respond to Nutlin-3a. We conclusively show that MDM2 genomic amplification is a remarkably poor predictor of Nutlin-3a sensitivity and subsequently used transcriptomic approaches to identify more robust biomarkers for MDM2 antagonists.

Materials and Methods
Clinical material
Tumor specimens were collected from patients with sarcoma (n = 24) undergoing surgical resection/core biopsy at three clinical institutions: Royal Adelaide Hospital, Calvary Wakefield Hospital, and St. Andrew’s Hospital between 2010 and 2012. Written informed consent was obtained from each patient (RAH Protocol #100505). No patients received neoadjuvant chemotherapy or radiotherapy within 1 year before tissue collection. Patients 6, 11, and 14 were previously described in Pishas and colleagues (13).

Ex vivo tissue explant system
Tissue explant system was adapted from Singh and colleagues (14). Fresh tumor samples were collected immediately following surgical excision, dissected into 1-mm³ pieces, and explanted onto gelatin dental sponges (Spongostan, Johnson & Johnson) immersed in RPMI-1640 media (PAA Laboratories), supplemented with 5% FBS (Gibco), 2 mmol/L L-glutamine (Gibco), 0.11 µg/mL insulin (Gibco), 10 µg/mL hydrocortisone (Sigma-Aldrich), and 1 × antibiotic/antimycotic solution (100 units penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B; Sigma-Aldrich). Explants were incubated with Nutlin-3a (10 µmol/L) or vehicle control [dimethyl sulfoxide (DMSO)] in a humidified atmosphere at 37°C containing 5% CO₂ and collected 48 hours posttreatment. (→) Nutlin-3a was sourced from Cayman Biochemicals.

Immunohistochemistry
Formalin-fixed, paraffin-embedded (FFPE) sections were deparaffinized and quenched in 3% hydrogen peroxide (10 minutes) to eliminate endogenous peroxidase activity. After citrate buffer (0.001 mol/L, pH 6.0) antigen retrieval, sections were blocked with normal goat serum (20 minutes) and immunolabeled with the following antibodies: activated caspase-3 (ab4051, 1:100, Abcam), Ki67 (ab16667, 1:100, Abcam), and anti-human p53 (DO-7, 1:300, Dako) overnight at 4°C. Sections were subsequently incubated with biotinylated secondary antibody, anti-rabbit IgG (1:200, Vector Laboratories) or anti-mouse IgG (1:250, Vector Laboratories) for 30 minutes at room temperature, followed by incubation with avidin/biotin peroxidase complexes (Vector Laboratories) for an additional 30 minutes. Immunocomplexes were visualized using DAB (Invitrogen) and counterstained with hematoxylin. Digital images were acquired using a Nanozoomer Digital Pathology Scanner, magnification of ×40.

Total p53 protein expression was scored according to the intensity and percentage of positive cells from four fields of view. Staining intensity was scored: 1 = not detectable, 2 = weak, 3 = moderate, and 4 = strong. Percentage of positive cells was scored: 1 = 0%, 2 = 1%–20%, 3 = 21%–50%, and 4 = >51%. p53 histoscore was calculated by multiplying intensity scores with the extent of positivity scores.

TP53 and MDM2 genotype
Exons 2 to 11 of TP53 were sequenced using primers and parameters listed in Supplementary Table S1. Sequencing reactions were processed on an ABI Hitachi 3730 DNA analyzer. MDM2 SNP309 tumor genotype was determined as previously described (15).

Real-time PCR analysis
Tissue samples were homogenized using a Miccra RT homogenizer (ART-Labortechnik) with total RNA extracted using the RNeasy Mini Kit (Qiagen), using on-column RNase-free DNase digestion according to the manufacturer’s instructions. cDNA was synthesized by reverse transcribing the total RNA using random primers (Promega) and Moloney murine leukemia virus reverse transcriptase (H; Promega) as previously described (16). gDNA was extracted using DNeasy mini kit (Qiagen), with copy number variation (CNV) determined using a previously described method (7) and normalized using the α-albumin (AFM) housekeeping gene. Real-time PCR reactions were processed and normalized as previously described (17). Primer sequences are listed in Supplementary Table S1.

Expression microarray analysis
Expression profiling was conducted using Affymetrix Human Gene 1.0 ST array as per manufacturer’s protocol from RNA extracted from 6 sarcoma patient samples (patients 1, 2, 9, 11, 14, and 22) treated with Nutlin-3a or vehicle control. Data were processed by applying the RMA algorithm using Partex Genomics Suite 6.5. Genes were ranked in order of criteria 1: their magnitude of fold activation following Nutlin-3a treatment (vehicle control vs. Nutlin-3a treatment) averaged for all
cases and criteria 2: the correlation coefficients between increased gene activation by Nutlin-3a and percentage increase of apoptotic cells induced by Nutlin-3a. The cumulative total of these 2 independently ranked criteria provided the basis for the final ranking of these genes. Microarray data have been deposited in the Gene Expression Omnibus database (GEO GSE48296).

RNA deep sequencing
Illumina TruSeq LT protocol was used to generate cDNA and libraries. Nine individual samples were multiplexed and sequenced on 2 HiSeq2000 sequencers (2 × 100 bp). Tumor RNA from patients 1, 2, and 10 were sequenced at the Australian Genome Research Facility (AGRF, Melbourne, Australia) with the remaining patient samples (patients 3, 5, 12, 13, 17, and 23) sequenced at ACRF. This resulted in 41.5 to 80.1 million fragments per sample, using the Illumina 2 × 100 bp end protocol. Reads were mapped to the human genome (Version hg19) with Tophat2 (Version 2.0.4, using bowtie2, mate-inner-dist -20, mate-std-dev 180, UCSC splice junction gtf; ref. 18). Gene expression was quantified using HTSeq (Version 0.5.3p9; ref. 19) and log2-transformed after regularizing peaks containing no reads to one. Comparisons between samples using hierarchical clustering (single linkage) and heatmaps were carried out using Spearman rank correlation coefficient as the basis for the distance measure to avoid any normalization artifacts. However, after normalization of the samples using the median of the top quartile, clustering based on Pearson’s correlation coefficient yielded similar results. Variation between technical repeats of the transcriptome of patient 10, sequenced at 2 different facilities, was minimal (Supplementary Fig. S1).

GADD45A methylation
GADD45A CpG methylation approximately 700 bp upstream of the GADD45A transcriptional start site was analyzed as previously described (20). Represented here is the methylation of the first CpG residue out of a string of 4 CpG residues described in Perugini and colleagues (20). Consistent with Perugini, methylation of this individual CpG residue was representative of methylation across this site. gDNA extracted from untreated sarcoma specimens or sarcoma cell lines was submitted to AGRF for GADD45A promoter methylation analysis using Sequenom MassARRAY on bisulfate-converted gDNA.

GADD45A overexpression
TC252 GADD45A–overexpressing cells were generated using retroviral spin-infection as previously described (21).

Cell culture
Ewing sarcoma cell lines were maintained as previously described (17) and supplied by G. Hamilton (University of Vienna, Vienna, Austria; TC252), F. van Valen (Westfälische-Wilhelms-University, Germany; WE-68, VH-64), J. Sonnemann (University Children’s Hospital Jena, Germany; CADO-ES1), and P. Ambros (St. Anna Children’s Hospital, Austria; STA-ET1). Cell lines were not authenticated by our laboratory.

Cell-cycle and viability assays
For decitabine/Nutlin-3a synergy experiments, cells were seeded in 96-well microtiter plates (2.5 × 10^4 cells per well) and left to adhere overnight. Cells were pretreated with 0 to 1,000 nmol/L of 5’aza-2’deoxycytidine (decitabine; Calbiochem) for 24 hours before the addition of Nutlin-3a (0–10 μmol/L) for an additional 24 hours. For apoptosis (7-amino-actinomycin-D) and cell-cycle analysis (propidium iodide), cells were harvested and processed (FACS Calibur flow cytometer, Becton Dickinson Immunocytometry Systems) as previously described (17).

Statistical analysis
P values were calculated using Student t test using GraphPad Prism version 6.

Results
Nutlin-3a induces robust apoptosis in a subset of sarcomas
To assess the biologic response of sarcoma tissue to Nutlin-3a, tumor tissue was collected immediately following surgical resection and treated ex vivo with Nutlin-3a (10 μmol/L) or vehicle control for 48 hours (Fig. 1A and B). This concentration of Nutlin-3a falls well within the range of saturating tissue concentrations that can be achieved in mouse tissue (22). The native tissue architecture, proliferative capacity, and cellular integrity of the tumor were maintained throughout the treatment period (Fig. 1C). Clinical characteristics and histopathology of the 24 patients with sarcoma are outlined in Table 1. Immunohistochemical analysis of explanted tissues for activated caspase-3, showed robust apoptotic responses (>25% increase in apoptotic cells) in 3 of 24 (12.5%) sarcomas following Nutlin-3a treatment (Fig. 1D and E). The biologic activity of Nutlin-3a was not restricted to specific sarcoma histotypes, as these major responses were observed in tissues from a myxoid liposarcoma (patient 1), Ewing sarcoma (patient 2), and a chondrosarcoma (patient 3). Tissues were additionally stained for Ki67 to assess the degree of cytostatic responses induced by Nutlin-3a. A marked decrease in proliferating cells (>50%) compared with vehicle control was exhibited from patients 2, 9, 15, and 22 (Supplementary Fig. S2A and S2B). No correlation between apoptotic and cytostatic responses following Nutlin-3a treatment were observed (Supplementary Fig. S2C).

MDM2 amplification does not confer Nutlin-3a sensitivity
In an effort to understand why sarcomas from patients 1, 2, and 3 showed strong apoptotic responses, we conducted a genetic study to assess the role of known TP53 pathway alterations on Nutlin-3a sensitivity. Currently, MDM2 amplification is the benchmark biomarker required for enhanced apoptotic response (7, 8, 23). Genomic amplification and mRNA overexpression of MDM2 (defined as >3-fold) was detected in 6 of 24 (25%) sarcomas (Table 1). A positive correlation between MDM2 genomic amplification and mRNA overexpression was observed (r^2 = 0.582; Supplementary Fig. S3A). Surprisingly, neither MDM2 amplification nor mRNA
expression levels showed significant correlation with cellular fate following Nutlin-3a treatment (Figs. 2A and Supplementary Fig. S3B). Even in the presence of MDM2 genomic amplification (sarcomas 4, 6, 9, 13, 21, and 22), weak or absent apoptotic responses following Nutlin-3a treatment were observed. Furthermore, MDM2 amplification was not observed in any of the 3 sarcoma cases that elicited the most significant apoptotic responses (sarcomas 1, 2, and 3). These results highlight the limited role that MDM2 amplification plays as a predictor of Nutlin-3a–induced apoptosis and counters the accepted view from preclinical studies that MDM2 amplification sensitizes cultured or xenografted cancer cell lines to Nutlin-3a.

Several in vitro studies have shown that the ability of Nutlin-3a to activate the TP53 pathway is severely compromised in mutant TP53 tumor cells (24, 25). Sequencing revealed that 20
### Table 1. Clinical characteristics and histopathology of the sarcoma cohort

<table>
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<tr>
<th>Sarcoma patient</th>
<th>Gender</th>
<th>Age, y</th>
<th>Histologic subtype</th>
<th>Site</th>
<th>TP53 status</th>
<th>TP53 SNP72</th>
<th>MDM2 SNP309</th>
<th>MDM2 copy number</th>
<th>MDM2 mRNA level</th>
<th>MDM4 copy number</th>
<th>MDM4 mRNA level</th>
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<td>51</td>
<td>Myxoid liposarcoma</td>
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<td>0.85 ± 0.00</td>
<td>0.81 ± 0.03</td>
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<td>TT</td>
<td>12.84 ± 0.43</td>
<td>24.54 ± 3.06</td>
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<td>TT</td>
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<td>0.95 ± 0.02</td>
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<td>0.68 ± 0.03</td>
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<td>1.21 ± 0.02</td>
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</table>

NOTE: Patient cohort included 15 males and 9 females with a mean age at diagnosis of 56.5 years (range, 18–87 years). The different morphologic subtypes were represented by 6 liposarcomas (3 dedifferentiated, 1 well-differentiated, 1 pleomorphic, and 1 myxoid liposarcoma), 6 undifferentiated pleomorphic sarcomas, 3 myxofibrosarcomas, 2 PNET/Ewing sarcomas, 2 osteosarcomas, 2 leiomyosarcomas, and 1 chondrosarcoma, angiosarcoma, and radiation-induced sarcoma.

$^a$16-bp deletion resulting in a frameshift (exon 6).

$^b$Stop codon.
of 24 (83%) sarcomas were wild-type for TP53 (Table 1). Although those sarcomas that underwent robust apoptosis in response to Nutlin-3a were all wild-type TP53, no significant association between TP53 status and cellular response following Nutlin-3a treatment was observed (Figs. 2B and Supplementary Fig. S3C). This suggests that although wild-type TP53 may be necessary for Nutlin-3a to induce an apoptotic response, it is not useful as a stand-alone biomarker.

Finally, we assessed whether the sequence polymorphisms TP53 SNP72, MDM2 SNP309, and MDM4 genomic amplification/mRNA overexpression mediate Nutlin-3a sensitivity. MDM2 SNP309 tumor genotypes were as follows: TT (58.3%), TG (25.0%), and GG (16.7%). TP53 SNP72 tumor genotypes were 17 of 24 (71%) arginine and 7 of 24 (29%) proline. No significant statistical association was observed between any of these TP53 pathway alterations and cellular response following Nutlin-3a treatment (Table 1, Fig. 2C and D, Supplementary Fig. S3D and S3E).

The global transcriptomic profile of sarcomas predicts response to Nutlin-3a

Given that known genetic p53 pathway alterations were poor predictive markers of Nutlin-3a efficacy, RNA sequencing was used (in 9 sarcomas where sufficient RNA was available) to determine the basal gene expression profiles of sarcomas within our cohort. Hierarchical clustering analysis of these transcriptomes showed that those sarcomas that were highly sensitive to Nutlin-3a shared similar basal gene expression profiles (Fig 3). Given that these 3 sarcomas are of different histopathologic classifications, this suggests that molecular subtyping of sarcomas may be a useful approach to identify those patients with sarcoma most likely to respond to Nutlin-3a.

Activation of the canonical p53 pathway correlates with apoptotic responses

In an effort to define the downstream events induced by Nutlin-3a, induction of p53 protein levels in response to Nutlin-3a treatment was investigated through immunohistochemical analysis. A significant increase in p53 protein induction following Nutlin-3a treatment was observed in sarcomas undergoing apoptotic responses (P = 0.006; Fig. 4A and B). In contrast, p53 protein induction by Nutlin-3a was not an indicator of cytostatic responses (Fig. 4C), suggesting that activation of the canonical p53 pathway by Nutlin-3a in sarcomas is specifically linked to apoptotic but not cytostatic outcomes. Furthermore, moderate cytostatic responses (>20% reduction in proliferating cells) were observed in 2 sarcomas harboring TP53 mutations (patients 20 and 24), providing additional evidence that the cytostatic capabilities of Nutlin-3a may be mediated through a p53-independent pathway.

Given that canonical p53 pathway activation was significantly associated with Nutlin-3a-induced apoptosis, gene expression microarray analysis was used to identify downstream TP53 target genes that were specifically activated only in those sarcomas that underwent significant apoptosis following Nutlin-3a treatment. The top 7 genes are listed in Table 2 and includes 4 established TP53 target genes GADD45A, BBC3, RPS27L, and pre-mir34A (26). GADD45A and BBC3 induction was validated through real-time PCR analysis and was correlated with Nutlin-3a–induced apoptosis (r² = 0.566 and 0.418, respectively; Supplementary Fig. S4).
**GADD45A methylation: a mechanism of Nutlin-3a resistance**

We next examined whether epigenetic silencing is a mechanism through which these p53 target genes were not activated in sarcomas that did not respond to Nutlin-3a. Aberrant proximal promoter methylation of *GADD45A*, approximately 700 bp upstream of the transcriptional start site, has been reported in solid tumors (27–29) and hematological malignancies (20). *GADD45A* CpG promoter methylation was highly prevalent in our cohort (0%–89% methylation; Fig. 5A). Hypermethylation was defined as methylation exceeding the highest level observed in 5 matching germline samples (>12%). Hypermethylation of *GADD45A* was absent from the 3 sarcomas in which the strongest apoptotic responses to Nutlin-3a were observed. In contrast, *GADD45A* hypermethylation was evident in 14/17 (82%) of wild-type TP53 sarcomas that did not show robust apoptosis to Nutlin-3a. Indeed, hypermethylation of *GADD45A* was significantly correlated with poor apoptotic response following Nutlin-3a treatment (*P* = 0.009), thus providing a possible mechanism through which several sarcomas suppress the downstream apoptotic pathways of p53.

On the basis of histologic subtype, hypermethylation of *GADD45A* was observed for all wild-type TP53 well-/de-differentiated liposarcomas, undifferentiated pleomorphic sarcomas, and myxofibrosarcomas (Supplementary Fig. S5A). Interestingly, none of the mutant TP53 tumors showed evidence of *GADD45A* promoter methylation, with methylation levels significantly lower than wild-type TP53 tumors (*P* = 0.042; Supplementary Fig. S5B). We next assessed whether hypermethylation of *GADD45A* was a reflection of global tumor hypermethylation. No correlation between *GADD45A* methylation and total global methylation (5-methylcytosine levels) was observed, suggesting that *GADD45A* methylation was a defined event (Supplementary Fig. S5C and S5D). Together, these results imply that epigenetic silencing of p53 target genes such as *GADD45A* is one mechanism through which sarcomas possess intrinsic resistance to Nutlin-3a.

**Overexpression of GADD45A induces sarcoma apoptosis**

We subsequently investigated whether reactivation of *GADD45A* using the clinically approved DNA methyltransferase inhibitor decitabine could enhance Nutlin-3a induced apoptosis. *GADD45A* CpG methylation was observed in a panel of 5 Ewing sarcoma cell lines but not in primary nonmalignant human osteoblasts (Supplementary Fig. S6). TC252 cells (displaying the highest level of *GADD45A* CpG methylation) were pretreated with decitabine for 24 hours before addition of Nutlin-3a for a further 24 hours. Increased apoptosis was observed when these 2 agents were combined (Fig. 5B) both in TC252 cells and an additional *GADD45A* hypermethylated cell line, STA-ET1. These findings provide a rationale for the use of epigenetic-based antitumor agents to enhance Nutlin-3a–induced apoptosis.

Given that *GADD45A* transactivation by Nutlin-3a was associated with apoptotic outcomes (Table 2), we explored the biologic effects of induced *GADD45A* expression in sarcomas. Retroviral-mediated ectopic expression of *GADD45A* in TC252 cells resulted in a significant increase in apoptosis (Fig. 5C), which is consistent with previous studies showing a pro-apoptotic role for *GADD45A* (30–33). Colony formation assays comparing control and *GADD45A*-overexpressing TC252 cells plated at low density also showed an 82% reduction in the number of discrete colonies formed, 10 days after seeding (*P* < 0.0001; Fig. 5D).

**Discussion**

Substantial improvements in overall survival rates for most patients with sarcoma have not been achieved particularly for...
those with metastatic disease. Future progress will be dependent not on the discovery of new cytotoxic chemotherapeutics but rather from the development of non-genotoxic targeted therapies. The unique characteristic of sarcomas to retain functional wild-type TP53 status suggests that sarcomas are ideal candidates for targeted therapies that reactivate the TP53 pathway. The efficacy of such agents will be contingent on the identification of biomarkers that can prospectively identify patients most likely to respond to this novel therapeutic approach.

To date, the in vitro study of immortalized cancer cell lines has been the primary method used to examine the therapeutic potential of p53-MDM2 antagonists. Such culture systems have several limitations and do not accurately recapitulate the complex genetic characteristics and cellular heterogeneity of the original tumor from which the cell line was derived. To circumvent these limitations, we used an ex vivo methodology to directly assess the cellular response and clinical importance of genetic and epigenetic determinants underlying sarcoma apoptotic sensitivity to Nutlin-3a.

Table 2. Genes identified by microarray analysis involved in Nutlin-3a-induced apoptosis

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Cellular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_001071</td>
<td>TYMS</td>
<td>Thymidylate synthetase</td>
<td>Nucleotide biosynthesis</td>
</tr>
<tr>
<td>NR_002578</td>
<td>GASS</td>
<td>Growth arrest–specific 5</td>
<td>Noncoding RNA</td>
</tr>
<tr>
<td>NM_001924</td>
<td>GADD45A</td>
<td>Growth arrest and DNA damage-inducible, alpha</td>
<td>DNA damage repair and growth arrest</td>
</tr>
<tr>
<td>EF592573</td>
<td>Pre-mir34A</td>
<td>MicroRNA 34a</td>
<td>Noncoding RNA</td>
</tr>
<tr>
<td>NM_014578</td>
<td>RHOD</td>
<td>Ras homolog family member D</td>
<td>Muscle differentiation</td>
</tr>
<tr>
<td>NM_015920</td>
<td>RPS27L</td>
<td>RPS27L ribosomal protein S27-like</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>NM_001127240</td>
<td>BBC3</td>
<td>BCL2-binding component 3</td>
<td>Apoptosis</td>
</tr>
</tbody>
</table>
Our results show that restoration of the TP53 pathway through Nutlin-3a effectively induces apoptotic and cytostatic cellular fates in a subset of sarcoma biopsies that is not dependent on histologic subtype. This is supported by several preclinical \textit{in vitro} and mouse xenograft studies, which showed that Nutlin-3a can effectively activate the TP53 pathway, resulting in cell-cycle arrest or apoptosis in Ewing sarcoma (17, 34), liposarcoma (7), osteosarcoma (8), rhabdomyosarcoma (35), and synovial sarcoma (36).

The initial rationale for the development of MDM2 antagonists was for the targeted treatment of tumors with almost universal MDM2 amplification, such as well-/de-differentiated liposarcomas. Several groups have shown through cell culture and mouse xenograft studies that MDM2 amplification predisposes cells to undergo enhanced apoptotic responses following Nutlin-3a treatment (7, 8, 23). On the basis of these preclinical studies, it was presumed that MDM2 amplification is the key benchmark biomarker.
required for Nutlin-3a sensitivity. Although genomic amplification and/or mRNA overexpression of MDM2 was observed in 25% of sarcomas in this study, strong apoptotic responses were not elicited in any of these sarcomas following Nutlin-3a treatment. Furthermore, neither MDM2 amplification nor overexpression was observed in any of the 3 sarcomas in which the most robust apoptotic responses were elicited (patients 1, 2, and 3). These results are supported by a recent Nutlin (RG7112) early-phase clinical study conducted in patients with well-/de-differentiated liposarcomas. Only one partial response was observed in a patient harboring MDM2 amplification (9). Taken together our observations imply that MDM2 amplification is not significantly associated with apoptotic outcomes induced by Nutlin-3a and questions the use of MDM2 amplification as a biomarker to guide patient recruitment in clinical trials involving MDM2 antagonists.

In an effort to identify biomarkers that would better predict Nutlin-3a sensitivity, gene expression array analysis was undertaken to identify alterations in key downstream TP53 target genes that drive apoptosis. We identified that one of these target genes (GADD45A) was hypermethylated in 82% (14 of 17) of wild-type TP53 sarcomas that did not respond to Nutlin-3a. Indeed, absence of methylation was significantly correlated with apoptotic response following Nutlin-3a treatment (P = 0.0090). Interestingly, methylation of GADD45A was not evident in any mutant TP53 tumors supporting the hypothesis that inactivation of GADD45A may be a central mechanism of innate resistance to p53-induced apoptosis and is therefore not selected for in TP53-mutant tumors. This is further supported by reports showing that promoter methylation of GADD45A is associated with poor overall survival in acute myeloid leukemia, a tumor type in which TP53 mutations are infrequent (<10%; refs. 20, 37, 38).

In addition to coordinating DNA excision repair and cell-cycle regulation, growing evidence has implicated that GADD45 proteins possess proapoptotic functions through activation of the JNK/p38 signalling pathway (39, 40). Recently, it has been reported that MDM2 functions as an E3 ubiquitin ligase for GADD45A. Enhanced association between MDM2 and RPS7 following arsenite treatment attenuated MDM2-mediated GADD45A ubiquitination, resulting in GADD45A protein stabilization and apoptosis (41). Interestingly, our microarray analysis identified RPS27L, a direct TP53 target, that modulates cell fate in response to genotoxic stress (42), to be upregulated only in those sarcomas in which robust apoptotic responses were observed. RPS27L has been shown to be translocated from the cytoplasm to the nucleoplasm where it colocalizes with MDM2 in response to p53-inducing signals (43). Like RPL7, RPS27L is a physiologic substrate of MDM2 and binds MDM2 via its central acidic domain (43, 44). Therefore, it may be plausible for sarcomas in which enhanced apoptotic response was observed that Nutlin-3a exposure induced the proapoptotic activity of GADD45A by blocking its constitutive ubiquitination and degradation by enhancing the interaction between ribosomal proteins such as RPS27L and MDM2. However, it is clear that further investigations are required to elucidate the role of GADD45A in Nutlin-induced apoptosis.

Epigenetic alterations (hypermethylation of 5′ regulatory CpG genomic regions) have been implicated in the repression and inactivation of cellular pathways that contribute to tumorigenesis such as those governing apoptosis. This is the first study to link GADD45A promoter methylation with innate resistance to MDM2 antagonists. Indeed, treatment of GADD45A hypermethylated sarcoma cell lines with decitabine augmented the apoptotic potential of Nutlin-3a. Collectively, our findings suggest that promoter methylation of GADD45A represents a novel marker that could potentially be used to stratify patients with sarcoma in future clinical trials and provides a rationale for combining MDM2 antagonists with demethylating agents in selected patients, a combinational approach that has not been investigated in any disease type before this study.

In summary, our findings provide a clearer insight into the current lack of understanding of predictive markers that mediate Nutlin-3a sensitivity and offer a rationale to redefine the inclusion criteria for patient recruitment in clinical trials involving MDM2 antagonists.

Disclosure of Potential Conflicts of Interest
M.P. Brown has commercial research grant from and is a consultant/advisory board member of Roche Products Pty Ltd Australia. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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