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) didn’t confer apoptotic or cytostatic responses in sarcoma tissue biopsies (n=24). Unexpectedly, MDM2 status didn’t 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 which 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 didn’t 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.
Introduction

Sarcomas constitute a heterogeneous group of bone and soft tissue malignancies that account for approximately 15% of all paediatric and 1% of all adult cancers (1). Many sarcoma subtypes are resistant to cytotoxic agents, and even for those subtypes that are chemo-sensitive, long term responders are rarely seen due to cumulative toxicity and a therapeutic ceiling that has now been reached. Overall five-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 biological 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 employed by these malignancies to subvert the anti-tumor 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 Vassilev et al., this cis-imidazoline molecule blocks the hydrophobic p53-binding pocket of MDM2 resulting in rapid stabilisation of biologically active p53 protein (5).
The importance of MDM2 in sarcomagenesis has emanated from the observation that its gene amplification is present in over 90% of well/de-differentiated liposarcomas (6). Promising results from preclinical studies have demonstrated 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 twenty 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 over-estimated 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 (Sanofi) and JNJ-26854165 (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 sarcoma patients most likely to respond to Nutlin-3a. We conclusively demonstrate 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 sarcoma patients (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 neo-adjuvant chemotherapy or radiotherapy within one year prior to tissue collection. Patients 6,11 and 14 were previously described in Pishas et al., 2013 (13)

Ex vivo tissue explant system

Tissue explant system was adapted from Singh et al., 2002 (14). Fresh tumor samples were collected immediately following surgical excision, dissected into 1mm³ pieces and explanted onto gelatine dental sponges (Spongostan, Johnson and Johnson) immersed in RPMI 1640 media (PAA Laboratories, Pasching, Austria), supplemented with 5% Fetal Bovine Serum (Gibco, Grand Island, NY), 2mM/L L-Glutamine (Gibco), 0.11µg/ml Insulin (Gibco), 10µg/ml Hydrcorticisone (Sigma-Aldrich, St Louis, MO) and 1X Antibiotic Antimycotic Solution (100units Penicillin, 100µg/ml Streptomycin, 0.25µg/ml Amphotericin B) (Sigma-Aldrich). Explants were incubated with Nutlin-3a (10µM) or vehicle control (DMSO) in a humidified atmosphere at 37°C containing 5% CO₂ and collected 48hrs post treatment. (-)- Nutlin-3a was sourced from Cayman Biochemicals (Ann Arbor, MI).

Immunohistochemistry

FFPE sections were deparaffinised and quenched in 3% hydrogen peroxide (10mins) to eliminate endogenous peroxidase activity. After citrate buffer (0.001M, pH 6.0) antigen retrieval, sections were blocked with normal goat serum (20mins) and immunolabelled with
the following antibodies: activated-caspase 3 (ab4051, 1:100, Abcam, Cambridge, UK), Ki67 (ab16667, 1:100, Abcam) and anti-human p53 (DO-7, 1:300, Dako, Carpinteria, CA) overnight at 4°C. Sections were subsequently incubated with biotinylated secondary antibody, anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) or anti-mouse IgG (1:250, Vector Laboratories) for 30mins at room temperature, followed by incubation with avidin-biotin peroxidase complexes (Vector Laboratories) for an additional 30mins. Immuno-complexes were visualised using DAB (Invitrogen, Grand Island, NY) and counterstained with haematoxylin. Digital images were acquired using a Nanozoomer Digital Pathology Scanner (Hamamatsu, Japan), magnification of x40.

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 2-11 of TP53 were sequenced using primers and parameters listed in Table S1. Sequencing reactions were processed on an ABI Hitachi 3730 DNA analyser. 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, Germany) with total RNA extracted using the RNeasy mini kit (Qiagen, Valencia, CA), 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, Madison, WI) and Moloney murine leukaemia virus reverse transcriptase (H; Promega) as previously described (16). Genomic DNA was extracted using DNeasy mini kit (Qiagen), with copy number variation (CNV) determined using a previously described method (7) and normalized using the alpha albumin (AFM) housekeeping gene. Real-time PCR reactions were processed and normalised as previously described (17). Primer sequences are listed Table S1.

**Expression microarray analysis**

Expression profiling was performed using Affymetrix Human Gene 1.0 ST array as per manufacturer’s protocol from RNA extracted from six sarcoma patient samples (patients 1, 2, 9, 11, 14 and 22) treated with Nutlin-3a or vehicle control. Data was 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 versus 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 two independently ranked criteria provided the basis for the final ranking of these genes. Microarray data has been deposited in the Gene Expression Omnibus database (GEO GSE48296).

**RNA-deep sequencing**

Illumina TruSeq LT (San Diego, CA) protocol was used to generate cDNA and libraries. Nine individual samples were multiplexed and sequenced on two HiSeq2000 sequencers (2 x 100bp). 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 41.5-80.1 million fragments per sample, using the Illumina 2x100 base paired-end protocol. Reads
were mapped to the human genome (Version hg19) with Tophat2 (Version 2.0.4, using bowtie2, mate-innerdist -20, mate-std-dev 180, UCSC splice junction gtf) (18). Gene expression was quantified using HTSeq (Version 0.5.3p9) (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’s rank correlation coefficient as the basis for the distance measure in order to avoid any normalization artefacts. 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 two different facilities, was minimal (Fig S1).

**GADD45A Methylation**

GADD45A CpG methylation approximately 700bp upstream of the GADD45A transcriptional start site was analysed as previously described (20). Represented here is the methylation of the first CpG residue out of a string of four CpG residues described in Perugini et al., 2012 (20). Consistent with Perugini, methylation of this individual CpG residue was representative of methylation across this site. Genomic DNA extracted from untreated sarcoma specimens or sarcoma cell lines was submitted to AGRF for GADD45A promoter methylation analysis using Sequenom MassARRAY on bisulphite converted genomic DNA.

**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, 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 (2.5x10^4 cells/well) and left to adhere overnight. Cells were pre-treated with 0-1000nM of 5’Aza-2’Deoxycytidine (Decitabine) (Calbiochem, San Diego, CA) for 24hrs before the addition of Nutlin-3a (0-10µM) for an additional 24hrs. 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’s t-test using Graph Pad Prism version 6 (La Jolla, CA).
Results

Nutlin-3a induces robust apoptosis in a subset of sarcomas

To assess the biological response of sarcoma tissue to Nutlin-3a, tumor tissue was collected immediately following surgical resection and treated \textit{ex vivo} with Nutlin-3a (10µM) or vehicle control for 48hrs (Fig 1A/B). This concentration of Nutlin-3a falls well within the range of saturating tissue concentrations which can be achieved in mouse tissue (22). The native tissue architecture, proliferative capacity and cellular integrity of the tumor was maintained throughout the treatment period (Fig 1C). Clinical characteristics and histopathology of the 24 sarcoma patients are outlined in Table 1.

Immunohistochemical analysis of explanted tissues for activated-caspase 3, demonstrated robust apoptotic responses (>25% increase in apoptotic cells) in 3/24 (12.5%) of sarcomas following Nutlin-3a treatment (Fig 1D/E). The biological 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 to vehicle control was exhibited from patients 2, 9, 15 and 22 (Fig S2A/B). No correlation between apoptotic and cytostatic responses following Nutlin-3a treatment was observed (Fig S2C).

MDM2 amplification doesn’t confer Nutlin-3a sensitivity

In an effort to understand why sarcomas from patients 1, 2 and 3 showed strong apoptotic responses, we performed a genetic study to assess the role of known \textit{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 6/24 (25%) sarcomas (Table 1). A positive correlation between $MDM2$ genomic amplification and mRNA overexpression was observed ($r^2=0.582$) (Fig S3A). Surprisingly, neither $MDM2$ amplification nor mRNA expression levels showed significant correlation with cellular fate following Nutlin-3a treatment (Fig 2A and 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 wasn’t observed in any of the three 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 sensitises cultured or xenografted cancer cell lines to Nutlin-3a.

Several in vitro studies have demonstrated that the ability of Nutlin-3a to activate the $TP53$ pathway is severely compromised in mutant $TP53$ tumor cells (24, 25). Sequencing revealed 20/24 (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 (Fig 2B, 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 17/24 (71%) arginine, 7/24 (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/D Fig S3D/E).

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 nine sarcomas where sufficient RNA was available) to determine the basal gene expression profiles of sarcomas within our cohort. Hierarchical clustering analysis of these transcriptomes demonstrated that those sarcomas that were highly sensitive to Nutlin-3a shared similar basal gene expression profiles (Fig 3). Given that these three sarcomas are of different histopathological classifications, this suggests that molecular subtyping of sarcomas may be a useful approach to identify those sarcoma patients 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 4 A/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 two 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 Nutlin-3a induced apoptosis, gene expression microarray analysis was employed to identify downstream TP53 target genes that were specifically activated only in those sarcomas that underwent significant apoptosis following Nutlin-3a treatment. The top seven genes are listed in Table 2 and includes four 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 ($r^2=0.566$ and 0.418 respectively) (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 didn’t respond to Nutlin-3a. Aberrant proximal promoter methylation of GADD45A, approximately 700bp upstream of the transcriptional start site, has been reported in solid tumors (27-29) and haematological 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 five matching germline samples (>12%). Hypermethylation of GADD45A was absent from the three 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 didn’t 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.

Based on histological subtype, hypermethylation of $GADD45A$ was observed for all wild-type $TP53$ well/de-differentiated liposarcoma, undifferentiated pleomorphic sarcoma and myxofibrosarcomas (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 ($p=0.042$) (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 (Fig S5C/D). 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 five Ewing sarcoma cell lines, but not in primary non-malignant human osteoblasts (Fig S6). TC252 cells (displaying the highest level of $GADD45A$ CpG methylation) were pre-treated with Decitabine for 24hrs prior to addition of Nutlin-3a for a further 24hrs. Increased apoptosis was observed when these two 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 anti-tumor agents to enhance Nutlin-3a induced apoptosis.
Given that \textit{GADD45A} transactivation by Nutlin-3a was associated with apoptotic outcomes (Table 2), we explored the biological effects of induced \textit{GADD45A} expression in sarcomas. Retroviral-mediated ectopic expression of \textit{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 \textit{GADD45A} (30-33). Colony formation assays comparing control and \textit{GADD45A} overexpressing TC252 cells plated at low density also showed an 82\% reduction in the number of discrete colonies formed, 10 days post seeding (p<0.0001) (Fig 5D).
Discussion

Substantial improvements in overall survival rates for most sarcoma patients haven’t 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 immortalised 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 don’t 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 employed 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.

Our results demonstrate 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 histological subtype. This is supported by several preclinical in vitro and mouse xenograft studies which demonstrated 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 demonstrated through cell culture and mouse xenograft studies that \textit{MDM2} amplification predisposes cells to undergo enhanced apoptotic responses following Nutlin-3a treatment (7, 8, 23). Based on these preclinical studies, it was presumed that \textit{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 \textit{MDM2} amplification nor overexpression was observed in any of the three sarcomas in which the most robust apoptotic responses were elicited (patient 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 \textit{MDM2} amplification (9). Taken together our observations imply that \textit{MDM2} amplification is not significantly associated with apoptotic outcomes induced by Nutlin-3a and questions the use of \textit{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 \textit{TP53} target genes that drive apoptosis. We identified that one of these target genes (\textit{GADD45A}) was hypermethylated in 82\% (14/17) of wild-type \textit{TP53} sarcomas that didn’t respond to Nutlin-3a. Indeed, absence of methylation was significantly correlated with apoptotic response following Nutlin-3a treatment ($p=0.0090$). Interestingly, methylation of \textit{GADD45A} was not evident in any mutant \textit{TP53} tumors supporting the hypothesis that inactivation of \textit{GADD45A} may be a central mechanism of innate resistance to p53 induced apoptosis and is therefore not selected for in \textit{TP53} mutant tumors. This is further supported by reports demonstrating that promoter methylation of \textit{GADD45A} is associated with poor overall
survival in acute myeloid leukemia, a tumor type in which \textit{TP53} mutations are infrequent (<10%) (20, 37, 38).

In addition to co-ordinating DNA excision repair and cell cycle regulation, growing evidence has implicated that GADD45 proteins possess pro-apoptotic functions through activation of the JNK/p38 signalling pathway (39, 40). Recently, it has been reported that \textit{MDM2} functions as an E3 ubiquitin ligase for \textit{GADD45A}. Enhanced association between MDM2 and RPS7 following arsenite treatment, attenuated MDM2-mediated GADD45A ubiquitination resulting in GADD45A protein stabilisation and apoptosis (41). Interestingly our microarray analysis identified RPS27L, a direct \textit{TP53} target that modulates cell fate in response to genotoxic stress (42), to be up-regulated 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 co-localizes with MDM2 in response to p53 inducing signals (43). Like RPL7, RPS27L is a physiological 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 pro-apoptotic 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 \textit{GADD45A} promoter methylation with innate resistance to MDM2 antagonists. Indeed, treatment of \textit{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 sarcoma patients in future clinical trials, and provides a rationale for combining MDM2 antagonists with demethylating agents in selected patients, an combinational approach that has not been investigated in any disease type prior to this study.

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


Table Legend

Table I: Clinical characteristics and histopathology of the sarcoma cohort

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

Figure Legends

Fig 1. Nutlin-3a induces apoptotic responses in sarcoma patient tissue *ex vivo*

(A) Sarcoma tissue was collected following surgical resection, and explanted onto gelatine sponges immersed in culture media containing either Nutlin-3a (10µM) or vehicle control for 48hrs. (B) Representative image of sarcoma tumor pieces cultured on a gelatine sponge. (C) H&E and Ki67 immunostaining of sarcoma tissue biopsies collected following surgical resection (untreated) and 48hrs *ex vivo* incubation with vehicle control. Images depict sarcoma tissue from patients 8, 18 and 20 (myxofibrosarcoma, leiomyosarcoma and undifferentiated pleomorphic sarcoma respectively). Scale bar = 300µM. Number of Ki67 positive cells was averaged from four fields of view (mean ± SE shown). (D) Percentage increase in apoptosis compared to vehicle control following Nutlin-3a treatment. Data represents average percentage of apoptotic cells determined from immunohistochemical analysis (activated-caspase 3), counted from four fields of view. (D) Representative images of activated-caspase 3 immunohistochemistry. Scale bar = 100µm.

Fig 2. *MDM2* genomic amplification doesn’t confer Nutlin-3a induced apoptosis

Correlation between (A) MDM2 copy number/mRNA expression levels, (B) *TP53* status, (C) *MDM2* SNP309, (D) *TP53* SNP72 and percentage increase in apoptotic cells following
Nutlin-3a treatment (10µM). Apoptotic cells determined through immunohistochemical analysis of activated-caspase 3. MDM2 amplification or overexpression defined as >3 fold. Horizontal bars indicate the mean of data. Open symbols denote sarcomas 1, 2 and 3 (strongest apoptotic response).

**Fig 3. Global transcriptomic profile of sarcomas predicts response to Nutlin-3a**

(A) Hierarchical clustering of transcriptomic response to Nutlin-3a. Strongest apoptotic responders highlighted in bold. (B) Heatmap of Spearman’s rank correlation coefficients, range: 0.68 to 0.92, average within-group correlation being 0.87 vs. an average across-group correlation of 0.75.

**Fig 4. p53 protein levels following Nutlin-3a treatment predict apoptotic response**

(A) Representative images of p53 immunohistochemistry from sarcoma biopsies (patient 1, 2 and 16) treated with Nutlin-3a (10µM) and vehicle control. Scale bar= 300µm. Correlation between induction of p53 protein levels (compared to vehicle control) and (B) percentage increase in apoptotic cells, and (C) percentage decrease in proliferating cells following Nutlin-3a treatment. Horizontal bars indicate the mean of data. Asterisk denotes statistical significance (**p<0.01**). Open symbols denote sarcomas 1, 2 and 3 (strongest apoptotic response).

**Fig 5. GADD45A is hypermethylated in sarcoma patient samples**

(A) Sarcoma GADD45A promoter CpG methylation levels. Asterisk denotes statistical significant difference in apoptosis induced by Nutlin-3a. (B) TC252 and STA-ET1 cells were
pre-treated with the Decitabine for 24hrs (0-1000nM) before the addition of Nutlin-3a (0-10µM) for a further 24hrs. Cell viability was determined through 7AAD staining and analyzed by flow cytometry. The percentage of cell death ± STDEV from duplicate reactions is shown. (C) TC252 cells were transduced with control or GADD45A virus. Percentage of apoptotic cells (SubG1) was determined through propidium iodide staining. Data represents mean percentage of cells in SubG1 ± STDEV from duplicate reactions. (D) TC252 control and GADD45A overexpressing cells were seeded at low density (4x10³ cell per well), fixed and stained with Giemsa (10 days post seeding). Data represents mean number of colonies ± STDEV from duplicate wells. Asterisk denotes statistical significance (**p<0.01, ***p<0.001).
Figure 1

(A) Schematic diagram illustrating the experimental setup for the study. Tissue explant, sponge, and culture media are shown with the addition of Vehicle control or Nutlin-3a 10 µM after 48 hrs.

(B) Photograph showing the tissue explant after 48 hrs under Vehicle control condition.

(C) H&E and IHC: Ki67 images for different sarcoma patient specimens under Untreated and Vehicle control conditions.

(D) Bar graph showing the % increase in apoptosis for different sarcoma patients under Vehicle control and Nutlin-3a 10 µM conditions. The colors represent different types of sarcomas: Angiosarcoma, Chondrosarcoma, Leiomyosarcoma, Myxofibrosarcoma, Myxoid Liposarcoma, Pleomorphic Liposarcoma, Osteosarcoma, PNET/Ewing sarcoma, Radiation induced sarcoma, Undifferentiated Pleomorphic sarcoma, Well/De-Differentiated Liposarcoma.

(E) IHC: activated-caspase 3 images for Patient 1 (myxoid liposarcoma) and Patient 2 (Ewing sarcoma) under Vehicle control and Nutlin-3a 10 µM conditions.
**A**

MDM2 copy number/mRNA overexpression

% Increase in apoptosis

- 3 Fold
- > 3 Fold

\[ p = 0.551 \]

**B**

TP53 status

% Increase in apoptosis

- Wild-type
- Mutant

\[ p = 0.285 \]

**C**

MDM2 SNP309

% Increase in apoptosis

- TT
- TG
- GG

\[ p = 0.926 \]

**D**

TP53 SNP72

% Increase in apoptosis

- Proline
- Arginine

\[ p = 0.926 \]
Figure 3

A

- Patient 13 (Well-differentiated Liposarcoma)
- Patient 23 (Leiomyosarcoma)
- Patient 17 (Myxofibrosarcoma)
- Patient 12 (Undifferentiated Pleomorphic sarcoma)
- Patient 5 (Undifferentiated Pleomorphic sarcoma)
- Patient 10 (Ewing sarcoma)
- Patient 2 (PNET/Ewing sarcoma)
- Patient 1 (Myxoid Liposarcoma)
- Patient 3 (Chondrosarcoma)

B

[Heatmap diagram showing gene expression levels for different patients]
Figure 4

A

Vehicle control

Nutlin-3a 10µM

Strong apoptotic response to Nutlin-3a

patient 1

patient 2

patient 16

IHC: p53

B

C

Percentage increase in apoptosis

p = 0.006

**

Δ

ΔΔ

% Increase in TP53 histoscore

% Decrease in proliferation

p = 0.235

< 4

> 5

< 4

> 5

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Figure 5

A

Wid-type TP53

Mutant TP53

Germline

% GADD45A CpG methylation

Strongest apoptotic response to Nutlin-3a

1 3 2 10 14 21 9 6 13 11 17 7 8 16 22 12 4 18 15 19 23 20 5 24 12 20 5 17 23

B

TC252

STA-ET1

% Cell death

Nutlin (0.37µM) – + – – – + – +
Nutlin (1.1µM) – + – + + + + +
Decitabine (250nM) – + + + + + + +
Decitabine (1000nM) – + + + + + + +

C

TC252

% Cells in SubG1

Control

GADD45A overexpression

D

Control

GADD45A overexpression

Number of colonies

TC252

***

Decitabine (250nM)

Nutlin (1.1µM) – + – – – + – +
Nutlin (0.37µM) – + – + + + + +
Decitabine (250nM) – + + + + + + +
Decitabine (1000nM) – + + + + + + +

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Table I: Clinical characteristics and histopathology of the sarcoma cohort

<table>
<thead>
<tr>
<th>Sarcoma Patient</th>
<th>Gender</th>
<th>Age (Years)</th>
<th>Histological 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>1</td>
<td>Male</td>
<td>51</td>
<td>Myxoid liposarcoma</td>
<td>Leg</td>
<td>Wildtype</td>
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<td>TG</td>
<td>0.81 ± 0.02</td>
<td>0.85 ± 0.00</td>
<td>0.81 ± 0.03</td>
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<td>PNET/Ewing sarcoma</td>
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<td>Wildtype</td>
<td>TG</td>
<td>0.80 ± 0.02</td>
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<td>Thigh</td>
<td>Wildtype</td>
<td>Arg</td>
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<td>0.57 ± 0.05</td>
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<td>0.54 ± 0.08</td>
<td>0.11 ± 0.07</td>
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<td>4</td>
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<td>Dedifferentiated Liposarcoma</td>
<td>Retroperitoneum</td>
<td>Wildtype</td>
<td>Arg</td>
<td>TT</td>
<td>12.84 ± 0.43</td>
<td>24.54 ± 3.06</td>
<td>0.91 ± 0.03</td>
<td>0.12 ± 0.12</td>
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<td>Undifferentiated Pleomorphic sarcoma</td>
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<td>0.96 ± 0.03</td>
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<td>0.39 ± 0.12</td>
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<td>Humerus</td>
<td>Wildtype</td>
<td>Arg</td>
<td>TT</td>
<td>8.26 ± 0.23</td>
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<td>Pro</td>
<td>TT</td>
<td>1.22 ± 0.04</td>
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<td>0.95 ± 0.02</td>
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<td>Myxofibrosarcoma</td>
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<td>TT</td>
<td>80.46 ± 3.28</td>
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<td>TT</td>
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<td>1.68 ± 0.16</td>
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<td>0.66 ± 0.07</td>
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<td>Well-differentiated Liposarcoma</td>
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<td>36.73 ± 2.58</td>
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<td>Radiation Induced sarcoma</td>
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<td>Arg</td>
<td>TT</td>
<td>0.43 ± 0.04</td>
<td>0.50 ± 0.01</td>
<td>0.64 ± 0.05</td>
<td>0.13 ± 0.02</td>
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<td>Angiosarcoma</td>
<td>Thigh</td>
<td>Wildtype</td>
<td>Pro</td>
<td>GG</td>
<td>0.72 ± 0.05</td>
<td>1.16 ± 0.11</td>
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<td>0.24 ± 0.16</td>
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<td>0.23 ± 0.12</td>
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<td>18</td>
<td>Osteosarcoma</td>
<td>Femur</td>
<td>Wildtype</td>
<td>Arg</td>
<td>TT</td>
<td>0.69 ± 0.03</td>
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<td>0.08 ± 0.06</td>
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<td>Fibula</td>
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<td>GG</td>
<td>12.10 ± 0.90</td>
<td>49.84 ± 3.55</td>
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<td>0.39 ± 0.02</td>
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<td>Leiomyosarcoma</td>
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<td>H168R</td>
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<td>0.48 ± 0.13</td>
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<td>R196*</td>
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<td>1.21 ± 0.02</td>
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<td>0.40 ± 0.02</td>
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</table>

Patient cohort included fifteen males and nine females with a mean age at diagnosis of 56.5 years (range, 18-87 years). The different morphological subtypes were represented by six liposarcomas (three de-differentiated, one well-differentiated, one pleomorphic and one myxoid liposarcoma), six undifferentiated pleomorphic sarcomas, three myxofibrosarcomas, two PNET/Ewing sarcomas, two osteosarcomas, two leiomyosarcomas, and one chondrosarcoma, angiosarcoma and radiation-induced sarcoma.

# 16bp deletion resulting in a frameshift (Exon 6); * Stop codon
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>
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<tr>
<td>NM_001071</td>
<td>TYMS</td>
<td>Thymidylate synthetase</td>
<td>Nucleotide biosynthesis</td>
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<tr>
<td>NR_002578</td>
<td>GAS5</td>
<td>Growth arrest-specific 5</td>
<td>Noncoding RNA</td>
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<tr>
<td>NM_001924</td>
<td>GADD45A</td>
<td>Growth arrest and DNA-damage-inducible,</td>
<td>DNA damage repair and growth arrest</td>
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<td>EF592573</td>
<td>Pre-mir34A</td>
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<td>BCL2 binding component 3</td>
<td>Apoptosis</td>
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Nutlin-3a efficacy in sarcoma predicted by transcriptomic and epigenetic profiling


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Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/12/13/0008-5472.CAN-13-2424.DC1

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