Nutlin-3a Is a Potential Therapeutic for Ewing Sarcoma

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Abstract

Purpose: Although mutations in the TP53 gene occur in half of all cancers, approximately 90% of Ewing sarcomas retain a functional wild-type p53. The low frequency of TP53 alterations in Ewing sarcoma makes this tumor type an ideal candidate for p53-targeted therapies. In this study, we have examined the molecular and cellular responses of cultured Ewing sarcoma cell lines following exposure to Nutlin-3a, a recently developed MDM2 antagonist.

Experimental Design: The ability of Nutlin-3a to impart apoptosis or cell cycle arrest in a p53-dependent manner was determined in a comprehensive panel of Ewing sarcoma cell lines. The capacity of Nutlin-3a to augment the antitumor activity of MDM4 antagonists and cytotoxic agents currently used in the clinical treatment of Ewing sarcoma was also investigated.

Results: Apoptosis was the primary response of wild-type p53 expressing Ewing sarcoma cell lines. The cytotoxicity of Nutlin-3a was also synergistic with the chemotherapeutic agents, vincristine, actinomycin D, doxorubicin, and etoposide in a concentration-dependent manner. Significant MDM4 protein overexpression was observed in Ewing sarcoma cell lines of wild-type p53 status, providing a mechanism through which Ewing sarcomas can develop in the absence of TP53 alterations. This study provides the first evidence of synergism between targeted inhibition of MDM2 and MDM4.

Conclusion: Our findings suggest that p53-dependent apoptosis is the primary cellular response of Ewing sarcoma cell lines following exposure to Nutlin-3a. Furthermore, Nutlin-3a can synergize with the current Ewing sarcoma chemotherapy protocols, suggesting p53 activation as a novel systemic therapeutic approach for this disease. Clin Cancer Res; 17(3); 494–504. ©2010 AACR.

Introduction

The p53 tumor suppressor plays a pivotal role in the prevention of oncogenic transformation by inducing cell cycle arrest or apoptosis in response to various cellular stresses (1). TP53 is the most frequently altered gene in cancer, with approximately 50% of all human malignancies harboring dysfunctional, mutated p53 proteins (2). Most tumors that have retained a wild-type p53 still manage to evade the p53 response through alterations in p53 regulatory proteins or downstream pathways. In contrast, the Ewing sarcoma family of tumors (ESFTs) possesses unique properties related to the p53 signaling pathway. ESFTs retain wild-type p53 in approximately 90% of all cases, and the downstream DNA damage cell cycle checkpoints and p53 pathways remain functionally intact (3–5). The molecular alterations that abrogate the tumor surveillance role of p53 during the development of Ewing sarcoma are largely unknown, with infrequent reports of CDKN2A (p14ARF) deletion and of MDM2 amplification occurring in only 20% and 10% of all ESFT cases, respectively (5, 6). The importance of p53 pathway integrity in ESFT is clearly shown by observations that the minority of ESFTs with TP53 mutations or CDKN2A deletions are associated with reduced chemoresponse and markedly poorer outcomes (7–9).

The activity and protein levels of p53 are negatively regulated by the E3 ubiquitin ligase MDM2 (10). Association with MDM2 stimulates rapid degradation of p53 protein levels; a mechanism of p53 inactivation that is frequently exploited during tumorigenesis through MDM2 amplification (11). In addition, MDM2 also functions as a corepressor when recruited with p53 at the promoters of p53 target genes (12). Hence, a further consequence of MDM2 amplification in tumors is the repression of the ability of p53 to drive apoptosis and cell cycle arrest in
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Translational Relevance

Ewing sarcomas have the most unfavorable prognosis of all primary musculoskeletal tumors despite aggressive multimodality treatment involving intensive chemotherapy regimens. This strongly suggests the urgent need for systemic and targeted therapies to treat this aggressive pediatric malignancy. Although mutations in the TP53 gene occur in half of all cancers, approximately 90% of Ewing sarcomas retain a functional wild-type p53. This study has shown that Nutlin-3a, a recently discovered p53 activator, can induce apoptosis in wild-type p53 expressing Ewing sarcoma cell lines. Our findings also suggest that Nutlin-3a can synergize with the current Ewing sarcoma chemotherapy protocols, and we have defined the doses of these agents necessary in vitro to achieve the most potent synergistic effects. This study provides comprehensive evidence to support the rational use of p53 activators as a novel systemic therapeutic approach for wild-type p53 expressing Ewing sarcomas.

cancer cells. As p53 and MDM2 play central roles in tumor development and progression, inhibition of the p53–MDM2 interaction has been explored as a promising mode of targeted therapy for malignancies that retain wild-type p53. The advent of p53-based biochemical and cell-based screens has facilitated the identification of small molecule activators of p53 that mediate their response through dissociation of the p53–MDM2 complex.

Nutlin-3a is a small cis-imidazole molecule antagonist of MDM2 and has been shown to effectively activate the p53 pathway in tumor cells both in vitro and in vivo (13). Nutlin-3a disrupts the p53–MDM2 interaction by blocking the p53-binding pocket of MDM2, resulting in rapid stabilization of biologically active p53 protein (13). Promising results from several preclinical studies have clearly showed the therapeutic potential of Nutlin-3a in a variety of tumor types with wild-type p53, including liposarcoma (14), rhabdomyosarcoma (15), osteosarcoma (13), synovial sarcoma (16), neuroblastoma (17), retinoblastoma (18), and leukemia (19–21). In an effort to accelerate clinical implementation of p53 activators, Lane and colleagues screened a library of clinically approved drugs and successfully identified actinomycin D (ActD) as a compound that mimics the action of Nutlin-3a when administered at nongenotoxic "low doses" (22). Given the frequent use of ActD in the clinic (albeit at higher doses) and its previously studied pharmacokinetics in humans (23, 24), low-dose ActD may be an attractive alternative to other p53 activators, which are currently being evaluated clinically and preclinically.

ESFT patients have the most unfavorable prognosis of all primary musculoskeletal tumors despite aggressive multimodality treatment involving the intensive VACDIE (vincristine, ActD, cyclophosphamide, doxorubicin, ifosfamide, and etoposide) chemotherapy regimen, radiotherapy, and surgery. Therefore, more effective systemic or targeted therapeutic modalities are required. Given the importance of p53 signaling during tumor responses to genotoxic agents, targeting p53 may augment current approaches to treatment of ESFTs. This study examines the ability of Nutlin-3a to activate the p53 pathway in cultured Ewing sarcoma cells, and shows its ability to augment the cytotoxic effects of the majority of chemotherapeutic agents used to treat Ewing sarcoma in the clinic. This preclinical study also reveals that MDM4 is specifically overexpressed in wild-type p53 Ewing sarcoma cells lines and evaluates the potential of a therapeutic approach involving a combination of MDM2 and MDM4 antagonists.

Materials and Methods

Cell culture and reagents

Nutlin-3a and Nutlin-3b enantiomers were purchased from Cayman Biochemicals. The MDM4 inhibitor SI-172550 was purchased from ChemBridge Corporation. Normal human osteoblasts (NHB) were derived from needle aspirates from the iliac crest of normal healthy donors and grown in oMEM (SAFC Biosciences) containing 10% fetal bovine serum and ascorbic acid 2-phosphate (NovoChem) as previously described (25). WE-68 and VH-64 were kindly supplied by F. van Valen (Department of Orthopaedic Surgery, Westfälische-Wilhelms-University, Germany). TC-252, TC-71, and SAL-2 cell lines were kindly supplied by G. Hamilton (Department of Surgery, University of Vienna, Austria), STA-ET1 was sourced from P. Ambros (Children’s Cancer Research Institute, St. Anna Children’s Hospital, Vienna, Austria), and CADO-ES-1 was kindly supplied by J. Sonnemann (Department of Pediatric Haematology and Oncology, University Children’s Hospital, Jena, Germany), SK-ES-1 and RD-ES were purchased from American Type Tissue Culture and cultured in DMEM media with 10% fetal calf serum. All other cell lines were cultured in RPMI 1640 media containing 10% fetal bovine serum, supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Cells were cultured in a humidified atmosphere at 37°C containing 5% CO2 on type-I collagen-coated plates (Iwaki Glass).

Immunoprecipitations and Western blot analysis

Subconfluent cultures seeded in a 10 cm2 culture dish were treated as indicated: lysed in lysis buffer 1 (50 mmol/L Tris-HCl pH 7.5, 250 mmol/L NaCl, 1% Triton X-100, and 1× protease inhibitors), sonicated for 10 seconds at 25% amplitude, and clarified. Protein complexes were immunoprecipitated upon addition of an anti-MDM2 antibody (1 μg; 1 hour at 4°C) and protein A–sepharose fast flow beads (20 μL; 1 hour at 4°C). Beads were washed three times in SNNT buffer (5% sucrose, 1% NP-40, 0.5 mol/L NaCl, 50 mmol/L Tris-HCl pH 7.5, and 5 mmol/L EDTA), three times in radioimmunoprecipitation assay buffer.

www.aacrjournals.org Clin Cancer Res; 17(3) February 1, 2011 495

Published OnlineFirst November 23, 2010; DOI: 10.1158/1078-0432.CCR-10-1587

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(50 mmol/L Tris·HCl pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate), and protein complexes eluted in SDS loading buffer (5 minutes at 95°C), with subsequent Western blot analysis done as previously described (26). For direct Western blot analysis, cells were lysed in lysis buffer 1, sonicated for 10 seconds at 25% amplitude, and clarified. Protein concentration was assayed using the bicinchoninic acid kit (Pierce). Protein extracts were resolved using SDS PAGE electrophoresis on 8% polyacrylamide gels and electrotransferred to Hybond-C Extra nitrocellulose membranes (Amersham Bioscience). Membranes were probed with the following mouse monoclonal antibodies: p53 DO-1 (1:1,000; Santa Cruz Biotechnology); p21WAF1 and Ab-3 (1:500; Neomarkers); MDM2 and SMP14 (1:500; Santa Cruz) or rabbit polyclonal antibodies; MDM4 (1:500; Bethyl Laboratories); PARP (1:2,000; Roche Diagnostics); and β-tubulin (1:2,000; Abcam). Chemiluminescent detection of protein was done using appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham) and the enhanced chemiluminescence kit according to the manufacturer’s instructions (Amersham).

Real-time PCR analysis
Total RNA was extracted using RNeasy mini kit (Qiagen), using on-column RNase-free DNase digestion according to the manufacturer’s instructions. cDNAs were generated by reverse transcribing the total RNA using oligo(dT)24 and Moloney murine leukemia virus reverse transcriptase (H; Promega) as previously described (27). Genomic DNA was extracted using DNeasy mini kit (Qiagen), with copy number variation (CNV) determined using a previously described method (14) and normalized using the alpha-albumin (AFM) housekeeping gene. Primers used for real-time PCR-based detection of transcript levels or CNV for specified genes are listed in Supplementary Table 1. Real-time PCR reactions were processed using a Bio-Rad iCycler system (Bio-Rad) using IQ SYBR Green Supermix (BIORAD) with the following parameters: denaturation at 95°C for 3 minutes, annealing at 57°C for 15 seconds, and elongation at 72°C for 20 seconds, for 45 cycles. Relative target mRNA expression or genomic CNV was determined using the ACT method (28) from triplicate reactions, with the levels of gene expression normalized to the relative average Ct value of peptidylprolyl isomerase G (PPIG) or β-actin (ACTB) housekeeping genes.

Cell viability assays
Cells were seeded in 96-well microtiter plates at a density of 3×10^4 cells/well in the presence of Nutlin-3a and chemotherapy drugs as indicated. For SI-172550 synergy experiments, cells were pretreated with SI-172550 for 24 hours prior to the addition of Nutlin-3a. Cells were harvested for 48 hours after Nutlin-3a treatment, centrifuged at 1,300 × g, washed in phosphate buffered saline (PBS) and stained with 7-aminoactinomycin-D solution (2 μg/mL) (7AAD, Invitrogen) for 10 minutes at room temperature. Viable cells were determined with the use of a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems), and analyzed with the use of FLOWJO (Tree Star Inc.) and GraphPad Prism (GraphPad Software Inc.).

Apoptosis and cell cycle analysis
Cells were incubated with the indicated treatments and incubated for 16 hours (Annexin V and PARP-1 cleavage), 24 hours (cell cycle analysis), or 48 hours (sub-G₁ DNA content). Annexin V staining was done in accordance with the manufacturer’s protocol (BD Biosciences). Cell cycle profiles were determined as previously described (29). Briefly, cells were harvested, fixed in ice-cold ethanol (70%), and incubated overnight at 4°C. Cells were stained with propidium iodide solution (50 μg/mL; Sigma–Aldrich) and 100 μg/mL RNase A (Sigma–Aldrich) for 45 minutes at 37°C. DNA content was determined with the use of a FACS Calibur flow cytometer with cell cycle profiles analyzed using FLOWJO software.

Statistical analysis
A combination index (CI) (30) was calculated to determine the effects of Nutlin-3a on cell viability in the presence of cytotoxic agents. A CI value of less than 1 indicates synergism, whereas a CI greater than 1 suggests an antagonistic effect. A CI value equal to 1 represents an additive effect. P values were calculated using Student’s t-test.

Results

Nutlin-3a induces cell death in Ewing sarcoma cell lines in a p53-dependent manner
The ability of Nutlin-3a to induce p53-dependent cell death was determined in vitro using a comprehensive panel of 9 Ewing sarcoma cell lines with varying p53 status. Clinical and cytogenetic characteristics, including p53 status, are listed in Figure 1A. The viability of all Ewing sarcoma cell lines expressing wild-type p53 was markedly reduced following exposure to Nutlin-3a in a dose-dependent manner (Supplementary Fig. 1). The concentration of Nutlin-3a required to induce 50% cell death (IC₅₀) in wild-type p53 cell lines ranged from 3 to 5 μmol/L (Fig. 1A and B). At the maximal concentration tested (10 μmol/L), Nutlin-3a induced more than 70% cell death in all Ewing sarcoma cell lines expressing wild-type p53. The cytotoxic effects of Nutlin-3a were considered to be p53 dependent, as the viability of Ewing sarcoma cell lines expressing mutant p53 was unaffected at these concentrations. These dosages of Nutlin-3a are not expected to induce nonspecific cytotoxicity, as the viability of NHB-1 and NHB-2 was unaffected following exposure to Nutlin-3a (Fig. 1A).

Pharmacologic activation of p53 by Nutlin-3a leads to apoptosis in Ewing sarcoma cells
The major tumor suppressor function of p53 is its ability to impart either growth arrest or apoptosis following particular stimuli. Therefore, we investigated the cellular outcomes in Ewing sarcoma cells following p53 activation by Nutlin-3a. Activation of the p53 pathway by Nutlin-3a in
Ewing sarcoma cell lines expressing wild-type p53 (VH-64, WE-68, or CADO-ES1) induced a dose-dependent increase in the proportion of cells in sub-G₁ (Fig. 2A and Supplementary Fig. 2). This response was apoptotic, as both Annexin V-positive cells (Fig. 2B) and PARP cleavage (Fig. 2C) were detected in a dose-dependent manner following exposure of wild-type p53 cell lines to Nutlin-3a for 16 hours. The apoptotic response induced by Nutlin-3a was p53 dependent, as Ewing sarcoma cell lines expressing mutant p53 showed no changes in sub-G₁ content or PARP cleavage following treatment with Nutlin-3a (Fig. 2A and C). Exposure of Ewing sarcoma cells to Nutlin-3a was associated with a dose-dependent and time-dependent stabilization of p53 protein levels and accumulation of...

Figure 1. Nutlin-3a induces cell death in Ewing sarcoma cell lines with wild-type p53. A, Nutlin-3a sensitivities, genotypes, clinical, and biological characteristics of the ESFT cell lines used in this study. B, wild-type and mutant p53 Ewing sarcoma cell lines were treated with increasing concentrations of Nutlin-3a for 48 hours. Cell viability was determined by 7AAD staining and analyzed by flow cytometry. IC₅₀ Concentration of Nutlin-3a required to cause 50% cell death. A completed panel of dose–response curves is presented in Supplementary Figure 1.

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ES, Ewing’s sarcoma; NHB, normal human osteoblast; pPNET, peripheral primitive neuroectodermal tumour; PE, pleural effusion.

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Figure 2. Nutlin-3a induces cell death in Ewing sarcoma cell lines with wild-type p53. A, Nutlin-3a sensitivities, genotypes, clinical, and biological characteristics of the ESFT cell lines used in this study. B, wild-type and mutant p53 Ewing sarcoma cell lines were treated with increasing concentrations of Nutlin-3a for 48 hours. Cell viability was determined by 7AAD staining and analyzed by flow cytometry. IC₅₀ Concentration of Nutlin-3a required to cause 50% cell death. A completed panel of dose–response curves is presented in Supplementary Figure 1.
established p53 target genes p21, MDM2, and BAX both at the protein (Fig. 2C) and mRNA level (Supplementary Fig. 3). Neither stabilization of p53 protein levels nor transactivation of p53 target genes was observed following Nutlin-3a treatment of Ewing sarcoma cell lines (SK-ES1 and RD-ES) expressing mutant p53 (Fig. 2C and Supplementary Fig. 3).

The apoptotic effects of Nutlin-3a required the loss of the p53–MDM2 interaction, as p53–MDM2 complex dissociation was observed within 2 hours of exposure to Nutlin-3a (Supplementary Fig. 4). Furthermore, treatment with equivalent concentrations of Nutlin-3b (an inactive enantiomer) did not influence the viability (Supplementary Fig. 5A) or apoptotic response (Supplementary Fig. 5B) of Ewing sarcoma cell lines. The growth arrest capabilities of activated p53 in Ewing sarcoma cells were also investigated. Given the robust apoptotic response of Ewing sarcoma cell lines following p53 activation, it was only possible to determine the growth arrest response using reduced dosages of Nutlin-3a (up to 5 μmol/L). Nutlin-3a did not dramatically influence S phase progression in the VH-64, CADO-ES1, or TC71 cell lines; however, a mild G1 arrest was observed in WE-68 cells (Supplementary Fig. 6).

Figure 2. Nutlin-3a induces apoptosis through activation of the p53 pathway in Ewing sarcoma cell lines with wild-type p53. A, induction of apoptosis by Nutlin-3a. Wild-type (VH-64, WE-68, and CADO-ES1) and mutant p53 (TC71) Ewing sarcoma cell lines were treated with the indicated concentrations of Nutlin-3a for 48 hours. Cell cycle distribution was analyzed by flow cytometry of propidium iodide-stained cells. Percentages of cells in sub-G1 from a typical experiment are shown. A complete panel of cell cycle profiles for VH-64, WE-68, CADO-ES1, and TC71 cell lines is presented in Supplementary Fig. 2. B, representative flow cytometric results showing the effect of increasing concentrations of Nutlin-3a on the detection of Annexin V. WE-68 cells were treated for 16 hours with the indicated concentrations of Nutlin-3a, with the proportion of Annexin V-positive cells determined by flow cytometry. C, Western blot analyses of p53, p21, and MDM2 expression levels in Ewing sarcoma cell lines with wild-type (WE-68 and VH-64) and mutant p53 (SK-ES1 and RD-ES) after treatment with 0, 2.5, and 10 μmol/L of Nutlin-3a for the indicated times. Full length (113 kDa) and cleaved PARP (89 kDa) fragments are also shown. Total β-tubulin levels were used as a protein loading control.

Nutlin-3a augments the cytotoxic effects of chemotherapeutic agents in Ewing sarcoma cells

Given that administration of multiagent chemotherapy (VACD-IE) is an integral part of the standard of care for pediatric ESFT patients, we investigated if activation of the p53 pathway by Nutlin-3a could enhance the antitumor effects of these cytotoxic agents. The viability of Ewing sarcoma cell lines following 48 hours of treatment with doxorubicin (DOX), ActD, etoposide (ETO), or vincristine (VIN); both in the presence and absence of Nutlin-3a were evaluated. Cell death induced by Nutlin-3a in VH-64 cells was significantly enhanced in combination with DOX, ActD, or ETO at the indicated concentrations (Fig. 3A and Supplementary Fig. 7A). Synergy of all chemotherapeutic agents with Nutlin-3a was also observed in WE-68 and STA-ET1 cell lines (Fig. 3B and Supplementary Figs. 7B and C). Surprisingly, synergy with Nutlin-3a was only observed at particular doses of the cytotoxic drugs. The observed synergism required p53 pathway activation, as Nutlin-3a did not influence the cytotoxic activity of these drugs in the mutant p53 expressing cell line TC-71 (Supplementary Fig. 8) or in NHB (Supplementary Fig. 9). Furthermore, Nutlin-3b (the inactive enantiomer) did not alter the cytotoxic activity of these drugs in Ewing sarcoma cell lines.
sarcoma cell lines expressing either wild-type (VH-64) or mutant p53 (TC-71), providing further evidence that activation of the p53 pathway is a critical requirement for the synergistic effects of Nutlin-3a with chemotherapeutic agents (Supplementary Figs. 10A and B).

**Nutlin-3a protects normal osteoblasts from the cytotoxic effects of chemotherapeutic agents**

Pharmacologic activation of p53 by Nutlin-3a has been shown to cause a growth arrest in normal dividing cells, thereby protecting them from the cytotoxic effects of chemotherapeutic agents that target rapidly dividing cells—a concept termed “cyclotherapy” (31, 32). We examined the cyclotherapeutic potential of Nutlin-3a on primary osteoblasts in vitro. Indeed, Nutlin-3a treatment of NHB-1 and NHB-2 was associated with a significant reduction in chemotherapy-related cytotoxicity (Fig. 4). This dose-dependent effect was observed with all of the drugs tested.

**MDM4 is overexpressed in Ewing sarcoma cell lines**

The ability of ESFTs to evade the tumor surveillance capacity of p53 during transformation cannot be accounted for by infrequent TP53 and CDKN1A mutations or MDM2 amplifications. Thus, further investigations into the key genetic and molecular events that allow these sarcomas to develop in the presence of a wild-type p53 were required.
We determined the protein levels of MDM4 in the panel of Ewing sarcoma cell lines. Western blot analysis revealed that MDM4 is overexpressed in the majority of wild-type p53 Ewing sarcoma cell lines, as compared with MDM4 expression in NHB (Fig. 5A). Overexpression of MDM4 in these cell lines was comparable to that observed in MCF-7, a breast cancer cell line that has been previously shown to exhibit MDM4 protein overexpression and gene amplification (33). A strong correlation was observed between MDM4 protein expression and wild-type p53 status (Fig. 5A), supporting the notion that MDM4 overexpression is the key mechanism responsible for ablation of p53 activity in ESFTs.

To gain further insight into the mechanisms responsible for elevated MDM4 protein expression in Ewing sarcoma cell lines, we examined the levels of MDM4 copy number and mRNA expression in these cell lines. Compared with NHB, MDM4 gene copy number and mRNA expression were similarly increased (≥1.5-fold) in 6 of 9 (67%) and 8 of 9 (89%) of Ewing sarcoma cell lines, respectively (Fig. 5B). However, several wild-type p53 Ewing sarcoma cell lines did not show either MDM4 gene amplification (WE-68, VH-64, and SAL-2) or mRNA overexpression (WE-68), suggesting that further posttranslational mechanisms are responsible for the elevated MDM4 protein levels observed in these cell lines.

**Nutlin-3a synergizes with an MDM4 antagonist**

Cell lines with MDM4 overexpression have been previously reported to exhibit less sensitivity to Nutlin-3a (33). Therefore, we examined whether targeted inhibition of MDM4 could enhance the antitumor effects of Nutlin-3a. MDM4 inhibition was achieved using a recently identified specific MDM4 antagonist, SJ-172550 (34). Exposure of wild-type p53 expressing Ewing sarcoma cell lines to SJ-172550–induced dose-dependent apoptosis, as showed through an increased proportion of cells in sub-G1 (Supplementary Fig. 11). The viability of WE-68 cells in response to Nutlin-3a treatment was synergistically enhanced in the presence of SJ-172550 (Fig. 6A), illustrating the potential of a dual targeted therapeutic approach. Surprisingly, combinations of Nutlin-3a and SJ-172550 was only able to reduce the viability of VH-64 and STA-ET1 cell lines in an additive manner (Fig. 6A), suggesting that synergy between these MDM2 and MDM4 antagonists is cell line specific. Interestingly, we observed that the synergistic effects of MDM2 and MDM4 inhibitors in the WE-68 cell line were associated with a rapid degradation of MDM4 following exposure to Nutlin-3a (Fig. 6B). However, Nutlin-3a exposure did not stimulate the degradation of MDM4 in VH-64 cells, suggesting a plausible mechanism for the lack of synergy with SJ-172550 in this cell line.

**Discussion**

This study shows that pharmacologic activation of p53 in Ewing sarcoma results in a proapoptotic response. These findings are also in agreement with published studies of other cancer cell lines with functional apoptotic pathways (35). This suggests that apoptotic pathways are intact in all 6 wild-type p53 Ewing sarcoma cell lines used in this study.
consistent with previous studies (4). In Ewing sarcoma cell lines tested, the capacity of Nutlin-3a to block cell cycle progression was limited (Supplementary Fig. 6). From a clinical therapeutic perspective, this is an ideal response as cells that evade the apoptotic response of Nutlin-3a will be dividing and therefore will remain sensitive to conventional cytotoxic drugs.

Multiagent chemotherapy protocols are essential components of the current standard of care for Ewing sarcoma. Since their introduction, the survival rates of Ewing sarcoma patients have increased from 10% to 60% (36). Nevertheless, Ewing sarcoma still has the worst prognosis of all musculoskeletal tumors. Further improvements in these survival rates may result if p53 activators such as Nutlin-3a could be incorporated in the current VACD-IE treatment protocol. The antitumor activity of DOX, ActD, ETO, and VIN was enhanced in the presence of Nutlin-3a across multiple cell lines expressing wild-type p53 (Fig. 3) and was dependent upon activation of the p53 pathway. Thus, these findings support clinical testing of systematically administered Nutlin-3a alone and in combination with VACD-IE chemotherapy. However, because the synergistic effects of Nutlin-3a in combination with conventional cytotoxic agents were only observed in vitro using limited dose ranges of each of these agents, detailed preclinical pharmacokinetic studies of these combinations will be required. Nutlin-3a has been shown to synergize with these drugs in other sarcoma types, with previously reported CIs similar to those reported in this study at optimal dosages (15, 16). Unfortunately, these previous studies were limited to investigations of synergy at a single dose of these agents. Our findings have identified the specific doses required to achieve the most potent synergy between p53 activators and clinically relevant drugs (Fig. 3).

Previous studies have shown that MDM4 is amplified in 65% of human retinoblastomas (18) and overexpressed in 19% of breast and colon carcinomas (37), and this study presents evidence that MDM4 is overexpressed in a significant proportion of wild-type p53 Ewing sarcoma cell lines. These findings are consistent with a recent study that identified MDM4 amplification in more than half of ESFT cases (D.M. Thomas, personal communication). Collectively, these findings suggest that MDM4 amplification or protein overexpression is the key mechanism that facilitates Ewing sarcomagenesis in the presence of wild-type p53. Thus, targeted inhibition of both MDM2 and MDM4 may result in a more robust activation of the p53 pathway in ESFTs. However, findings from this study suggest that the synergistic effects of combined MDM2 and MDM4 antagonists were cell line dependent and may rely on the ability of free MDM2 to turn over MDM4. It has been previously established that Nutlin-3a treatment results in an indirect degradation of MDM4 levels through dissociation of the p53–MDM2 complex, which redirects the ubiquitin ligase activity of MDM2 toward MDM4 (33). Forced expression of MDM4 conferred resistance to Nutlin-3a (33), suggesting that the lack of synergy between MDM2 and MDM4 antagonists observed in VH-64 cells is a result of the poor kinetics of MDM4 degradation by MDM2 (Fig. 6B).

In addition to infrequent TP53 alterations, ESFTs are associated with a genetic rearrangement involving the EWS
gene and a member of the ETS transcription factor gene family. This translocation oncoprotein is present in 95% of ESFTs (38) and has been shown to repress the transcriptional activity of p53 through both direct (39) and indirect (40) mechanisms. The cross-talk between p53 and the EWS–FLI1 oncoprotein is also evident in vivo, as specific expression of this oncoprotein in the mesenchymal progenitor cells of transgenic mice influenced sarcoma onset and development only in the presence of p53 deletion (41). However, EWS–FLI1 expression alone is not sufficient to transform fibroblasts, as expression of EWS–FLI1 engages oncogene-induced p53 response and initiates cell cycle arrest (42). Such studies suggest the requirement of a "second event" for EWS–FLI1 to drive Ewing sarcomagenesis without stimulating the p53 pathway. Our findings strongly support the notion that overexpression of MDM4 is the "second event" required to silence p53 activity during the expression of the EWS–FLI1 oncoprotein.

Interestingly, ActD at low doses (1 to 10 nmol/L) was reported to mimic Nutlin-3a in its highly specific activation of the p53 pathway (26). ActD was an extremely potent antitumor agent for wild-type p53 expressing Ewing sarcoma cell lines (average IC_{50} of 2.5 nmol/L), whilst cell lines harboring TP53 mutations were only sensitive to ActD at higher genotoxic concentrations (average IC_{50} of 18 nmol/L; Supplementary Fig. 12). These findings suggest that Ewing sarcoma cell lines are sensitive to the p53 dependent antitumor activity of "low-dose" Act D, further supporting the notion of p53 activation as an attractive approach for the treatment of Ewing sarcoma.

Altogether, these results have showed the potent antitumor activity of pharmacologic p53 activation in Ewing sarcoma. Our findings have defined in vitro concentrations required to achieve maximal antitumor effects of Nutlin-3a either as a single agent or in combination with drugs currently employed in protocols used for treatment of Ewing sarcoma.

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**Figure 5.** MDM4 is overexpressed in Ewing sarcoma cell lines with wild-type p53. A, Western blot analyses of MDM4 protein expression levels in Ewing sarcoma cell lines with wild-type and mutant p53, as compared with normal human osteoblasts. The MCF-7 cell line is used as a positive control for MDM4 protein overexpression. Total β-tubulin levels were used as a protein loading control. B, MDM4 copy number and transcript levels were determined in Ewing sarcoma cell lines using real-time PCR and relativised against normal human osteoblasts. MCF-7 cells are used as a positive control for MDM4 amplification and mRNA overexpression.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This study was supported in part by the ASSG Sarcoma Research Award funded through the Rainbows for Kate Foundation in memory of Tom Wood. We wish to acknowledge San Remo for their financial support and Prof Yukihide Iwamoto and Associate Prof Yoshihiro Matsumoto for assistance with cell culture. This study is dedicated to Brad Neilsen.

Grant Support

ASSG Sarcoma Research Award, San Remo Early Career Fellowship, and National Health and Medical Research Council Project Grant ID511303.

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Received June 14, 2010; revised September 29, 2010; accepted October 21, 2010; published OnlineFirst November 23, 2010.

Figure 6. MDM2 and MDM4 antagonists are synergistic. A, VH-64, WE-68, and STA-ET1 cells were pretreated for 24 hours with 0, 12.5, 25, 50, and 100 μmol/L of SJ-172550 before the addition of increasing concentrations of Nutlin-3a for 48 hours. Cell viability was determined by 7AAD staining and analyzed by flow cytometry. The percentage of cell death for each treatment is shown. Combination index (CI) values for these cell lines are listed. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. IC50 values for SJ-172550 obtained for each cell line are also shown. B, the kinetics of MDM4 degradation following exposure to Nutlin-3a. Western blot analyses of MDM4 expression levels in WE-68 and VH-64 Ewing sarcoma cell lines after treatment with 0, 2.5, and 10 μmol/L of Nutlin-3a for the indicated times. Total β-tubulin levels were used as a protein loading control.

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