Small Molecule Inhibition of HDM2 Leads to p53-Mediated Cell Death in Retinoblastoma Cells

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**Purpose:** To determine the efficacy of inducing p53-mediated cell death in retinoblastoma cells by Nutlin 3A, a small molecule HDM2 inhibitor.

**Methods:** Retinoblastoma cell lines WERI-RB-1 and Y79 were treated with Nutlin 3A. Cell viability assays, Western blot analyses, confocal microscopy, and flow cytometry were performed to measure cell survival, p53 protein levels, activation of downstream targets, and apoptosis. To determine whether the effects of Nutlin 3A were p53-dependent, cell viability assays were performed on Y79 cells expressing short interfering RNA (siRNA) against p53.

**Results:** Nutlin 3A induced cell death in Y79 and WERI-RB-1 in the 5- to 10-µM dose range. Treated cells demonstrated increased protein levels of p53 and the p53 targets p21 and HDM2. Phosphorylation of p53-serine-15, a marker for activation of p53 via genotoxic mechanisms, was absent. Y79 cells expressing siRNA against p53 demonstrated resistance to Nutlin 3A.

**Conclusions:** Nutlin 3A induced p53-mediated apoptosis in a dose-dependent, nongenotoxic fashion in 2 retinoblastoma cell lines.

**Clinical Relevance:** Nutlin 3A is effective against retinoblastoma cells in a nongenotoxic manner. Because the mutagenic effects of radiation and chemotherapy may increase risks of secondary tumor formation, targeted p53 activation may be a safer alternative treatment for retinoblastoma in the future.

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the interaction between p53 and HDM2. Nutlin 3A treatment led to activation of the p53 pathway, apoptosis in various p53 wild-type tumor cell lines, as well as oral efficacy with minimal systemic adverse effects in an osteosarcoma model. Furthermore, Nutlin 3A was not associated with phosphorylation of p53 on serine residues that are phosphorylated as part of the cellular stress response, suggesting that the drug is not genotoxic. In this article, we examine the efficacy of Nutlin 3A to induce p53-mediated cell death in retinoblastoma cells.

### METHODS

#### CELLS AND DRUG TREATMENT

Y79, WERI-RB-1, and MDA-MB-435 cells were grown in the recommended media supplemented with 10% fetal bovine serum at 37°C in a humidified environment with 5% carbon dioxide. Nutlin 3A and 3B, gifts from Dr Lubomir Vassilev (Hoffman-La Roche, Nutley, NJ), were dissolved in dimethyl sulfoxide (DMSO) and kept at −20°C as 10-mM stocks. Doxorubicin (Sigma, St Louis, Mo) was dissolved in DMSO to 10 mM and stored at 4°C. For cell viability assays, cells were grown in 12 well plates (at initial concentrations of 10^5 cells per mL for WERI-RB-1, 2×10^5 cells per mL for Y79, and 5×10^4 cells per mL for MDA-MB-435) and counted using the trypan blue exclusion assay.

#### IMMUNOCYTOCHEMISTRY

WERI-RB-1 and Y79 cells were incubated with the drug or with a DMSO vehicle for 8 hours. Cells were centrifuged and resuspended in 50 µL of Accutase (Innovative Cell Technology, Inc, San Diego, Calif). They were centrifuged again, resuspended in fresh media, and incubated for 30 minutes at 37°C on poly-L-lysine slides to attach. Cells were washed with phosphate-buffered saline, incubated for 10 minutes in 500 µL 4% paraformaldehyde/phosphate-buffered saline, and rewashed. Slides were incubated with p53 antibody 1:100 (SC263; Santa Cruz Biotechnology, Santa Cruz, Calif) for 24 hours, followed by the nuclear stain 4′,6-diamidino-2-phenylindole 1:10 000 (catalog #D1036; Molecular Probes, Invitrogen, Carlsbad, Calif) and the fluorescent secondary antibody Alexa 488 1:500 (A11029; Invitrogen). Slides were then examined with the Leica TCS SP confocal microscope (Leica Microsystems, Bannockburn, Ill).

#### WESTERN BLOTTING

Y79 cells were lysed in RIPA (radioimmunoprecipitation assay) lysis buffer containing 150 mM sodium chloride; 50 mM Tris at pH 7.5; 1 mM EDTA; 1% nonidet-P-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate; and protease inhibitors aprotinin (10 µg/mL), leupeptin (2.5 µg/mL), and 1 mM phenylmethylsulfonyl fluoride. Aliquots of lysates containing 10 µg/mL of protein were separated on 12% Tris-glycine polyacrylamide gels (Invitrogen). Proteins were detected using enhanced chemiluminescence reagents (Amersham, Piscataway, NJ) using antibodies specific for human p53 (SC263; Santa Cruz Biotechnology), Phospho-p53 (PC386; Calbiochem, San Diego), p21 (OP64; Oncogene Research Products, San Diego), MDM2 (SC695; Santa Cruz Biotechnology), and β-actin (Sigma).

#### ANNEXIN V STAINING

Y79 cells were incubated with 10 µM of Nutlin 3A for 36 hours or the equivalent volume of DMSO. Cells were washed twice with phosphate-buffered saline and stained with propidium iodide and Annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit I; BD Pharamingen, San Diego) according...
to the manufacturer’s instructions. This was followed by flow cytometry.

PRODUCTION OF CELLS EXPRESSING P53 SHORT INTERFERING RNA

pRETRO-SUPER and pRETRO-SUPER-p53 virus, gifts from Dr Reuven Agami, were produced in GP2 cells (Clontech, Mountain View, Calif). Supernatant was filtered and then added to Y79 cells in the presence of 4 mg/mL Polybrene (hexadimethrine bromide; Sigma) followed by selection for 7 days with 1 µg/mL puromycin.

RESULTS

NUTLIN 3A UP-REGULATES LEVELS OF P53, HDM2, AND P21

We first examined the effects of Nutlin 3A on p53 protein levels. Under normal growth conditions, WERI-RB-1 cells and Y79 cells expressed minimal p53 protein. Treatment with 5 µM of Nutlin 3A resulted in both cell lines accumulating nuclear and cytoplasmic p53 at 8 hours based on confocal microscopy (Figure 1A). Incubation with Nutlin 3B, the less potent enantiomer, had no discernable effect. We performed Western blot analysis on Y79 cells to determine whether Nutlin 3A affected levels of p53 and 2 of its transcriptional targets, p21 and HDM2. Nutlin 3A increased p53, p21, and HDM2 protein levels in a dose-dependent fashion at 8 hours. Incubation with Nutlin 3B appeared to have a minimal effect (Figure 1B).

NUTLIN 3A INDUCES CELL DEATH BY APOPTOSIS

Cell viability assays were performed on exponentially growing WERI-RB-1 and Y79 cells exposed to DMSO or Nutlin 3A at varying concentrations. Nutlin 3A-treated cells exhibited a dose-dependent reduction in cell viability with cell death in the 5- to 10-µM range (Figure 2). To determine whether cell death occurred via apoptosis, Annexin V staining was performed on WERI-RB-1 cells exposed to either DMSO or 10 µM of Nutlin 3A for 36 hours. Cells treated with Nutlin 3A demonstrated a significant increase in Annexin V but not propidium iodide staining, indicating early cell death by apoptosis (Figure 3).

NUTLIN 3A ACTIVITY IS P53-DEPENDENT

Cell viability assays were performed on MDA-MB-435 cells, which contain mutated p53, in parallel with WERI-RB-1 and Y79 cells (Figure 4). MDA-MB-435 cells were resistant to Nutlin 3A. To determine specifically whether retinoblastoma cells required p53 for Nutlin 3A sensitivity, we developed a Y79 cell line that stably expressed short interfering RNA (siRNA) directed against p53 and a control line infected with a control siRNA vector. Multiple p53 bands were detected on the Western blot, which may represent previously described p53 splice variants. The retinoblastoma cell line containing p53 siRNA had an approximately 70% reduction in the amount of p53 protein detected by Western blotting.

After incubation with Nutlin 3A, cells expressing siRNA to p53 exhibited significant resistance to the drug as compared with vector control (Figure 5B and C). This resistance was not complete but correlated with the approximately 70% reduction in the amount of p53 protein detected by Western blotting.

NUTLIN 3A DOES NOT INDUCE PHOSPHORYLATION OF P53-SERINE-15

We next evaluated whether activation of p53 could occur via a mechanism independent of Nutlin 3A’s effects on HDM2. Known genotoxic drugs such as doxorubicin lead to stabilization of p53 via phosphorylation of specific serine residues on the protein, most commonly serine 15. We performed Western blot experiments on cells exposed to DMSO, Nutlin 3A, or doxorubicin and assessed levels of p53-serine-15 phosphorylation using an antibody specific to this modified residue (Figure 6). Neither Nutlin 3A nor DMSO induced phosphorylation whereas doxorubicin induced phosphorylation of p53-serine-15.

COMMENT

p53 is a transcription factor that plays a critical role in the defense against tumorgenesis. It prevents uncontrolled cell division through 2 mechanisms. The first is by inducing a
cell cycle arrest through expression of p21, a protein that can prevent entry into the S phase. p21 regulates cell division indirectly by maintaining the Rb protein in an active hypophosphorylated state. The second mechanism is to initiate apoptosis, resulting in a caspase-mediated cell death. Either action can be triggered by oncogenic stimuli or DNA damage.22-24 Because of its powerful effects, many cancers acquire the ability to inhibit the p53 pathway with almost 50% of human cancers having mutations within the p53 gene.25 Many other cancers will evolve alternative mechanisms to circumvent p53 such as overexpression of HDM2. Prior studies have demonstrated that human retinoblastoma tumors are the exception to this pattern and rarely contain p53 mutations.26,27 One reason may be that the tumor can circumvent a cell cycle arrest since it does not have functional Rb. This in turn would allow unrestricted entry into cell division. However, it is not clear whether the tumor still needs to inactivate or attenuate the p53 pathway to prevent the other option of cell death. Some animal models of retinoblastoma suggest that this is required for tumor formation in the mouse retina. In the initial retinoblastoma mouse models, loss of both the Rb and p53 pathways were required for tumorgenesis and inactivation of Rb alone resulted in photoreceptor cell death.28,29 More recent mouse models, however, have shown that inactivation of p53 is not required if both Rb and its relative p107 or p130 are inactivated together during retinal development.30-32

The role of p53 in the development of human retinoblastoma remains unclear. There is empirical evidence that suggests p53 is active based on expression patterns in poorly differentiated primary human retinoblastoma tumors.27,33 In 2 studies, the highest expression of nuclear p53 occurred in tumor cells that were the furthest away from a central blood vessel. This also correlated with the region with the highest rate of apoptosis. Interestingly, in poorly differentiated tumors, regions with p53 expression had the lowest p21 expression, implying that the p53 pathway was driving the cell toward apoptosis as opposed to differentiation.

There is some functional evidence to suggest that the pathway is intact based on the observation that retinoblastoma cell death resulting from either radiation or chemotherapy is associated with increased levels of p53.34,35 Nork et al33 were the first to show that specific up-regulation of p53 induces retinoblastoma cell death. They transiently transfected retinoblastoma cells with a p53 expression vector and observed elevated p53 levels and cell death. Harbour et al36 provide the best evidence to date that it is possible
to activate p53 and achieve retinoblastoma cell death without overexpressing the gene. They developed transducible peptides to interfere with HDM2, which allowed p53 to accumulate in a more physiologic range. They observed effective killing of retinoblastoma cells in the 200-µM dose range in association with an up-regulation of the p53 target Bax. Their data was the first to suggest that inhibition of the HDM2-p53 interaction could be a potential therapeutic option in retinoblastoma.

We sought to build on this work and investigate whether Nutlin 3A, a small-molecule inhibitor of the p53-HDM2 interaction, could induce accumulation of p53 protein and consequent apoptosis in retinoblastoma cells. We first examined Nutlin 3A's effects on p53 levels by immunocytochemistry and confocal microscopy. Previous studies have suggested that p53's presence in the cytoplasm may not be sufficient for activation of the apoptotic pathway, presumably because the protein is unable to act in its capacity as a transcriptional activator. Nuclear exclusion has been proposed as a means of p53 inactivation in retinoblastoma as well as in other tumors, possibly via an HDM2-mediated mechanism. Our findings are consistent with prior observations and demonstrated low levels of predominantly cytoplasmic p53 protein in untreated Y79 and WERI-RB-1 cells. After Nutlin 3A treatment, there was a significant amount of both nuclear and cytoplasmic p53 protein in both cell lines.

Nutlin 3A treatment also led to decreased viability in both cell lines in the 5- to 10-µM dose range, similar to the dose range needed for up-regulation of p53 transcriptional targets. It was unclear, however, whether this was a function of forced differentiation, necrosis, or apoptosis. Normally, a p21-related forced differentiation occurs via Rb-mediated cell cycle arrest. That scenario seems unlikely in this system given the loss of Rb function, but it has been proposed that related proteins such as p107 or p130 may be able to compensate for Rb. To address this issue, we examined phosphatidyserine, which is translocated to the outer leaflet of the cell membrane in cells undergoing apoptosis. The external presence of this phospholipid can be detected by the phospholipid-binding protein Annexin V. WERI-RB-1 cells exposed to Nutlin 3A for 36 hours demonstrated an increase in Annexin V staining as compared with those treated with the vehicle DMSO. This is direct evidence that Nutlin 3A treatment is associated with apoptosis in these retinoblastoma cells.

The data thus far demonstrate that Nutlin 3A treatment leads to up-regulation of p53 and its downstream targets as well as apoptosis in retinoblastoma cell lines. Nutlin 3A's specific fit within HDM2's binding site for p53 suggests that these actions should be p53-mediated. We next evaluated whether Nutlin 3A associated cell death could occur independently of p53, potentially via nonspecific toxicity or by alternative effects on HDM2. We first examined a p53 mutant breast cancer cell line, MDA-MB-435, and found it to be resistant to Nutlin 3A, suggesting that the drug's effects on cell viability are p53-dependent. To further evaluate this specifically in retinoblastoma, we developed a Y79 cell line expressing siRNA to p53, which exhibited an approximately 70% reduction in p53 levels. As expected, these cells were more resistant to Nutlin 3A treatment. This suggests that p53 activation by Nutlin 3A does not involve DNA damage.

**Figure 5.** Y79 with short interfering RNA (siRNA) to p53 is resistant to Nutlin 3A. A, Western blots demonstrating decreased p53 expression in siRNA infected cells vs vector control and Y79 cells. B, Y79 cells with vector alone were plated and incubated with 0 (dimethyl sulfoxide vehicle), 1, 5, or 10 µM of Nutlin 3A for 5 days. Viable cells were counted using the trypan blue exclusion assay. C, Y79 cells with siRNA to p53 were incubated with Nutlin 3A as described and found to have an attenuated response to the small molecule. HPF indicates high-power field.

**Figure 6.** Absence of phosphorylation at serine-15 p53. Western blot analysis on protein lysate from WERI-RB-1 and Y79 cells treated with dimethyl sulfoxide (DMSO), 5 µM of Nutlin 3A, or 1 µM of doxorubicin (Dox) for a period of 24 hours. Nutlin 3A showed no evidence of inducing phosphorylation of Serine-15 p53 in contrast to doxorubicin, implying that p53 activation by Nutlin 3A does not involve DNA damage.

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cells exhibited an attenuated response to the drug, but only after an initial lag time during which the growth curves appeared to be blunted. The initial flattening of the growth curve most likely represents an equilibrium between the death of the minority p53-containing cells vs the growth of those with the siRNA knockdown of p53.

The most important aspect of this study is that Nutlin 3A activates p53 in a manner that bypasses the genotoxic effects of radiation and chemotherapy. DNA damage induces p53 via phosphorylation of specific serine residues, most frequently the serine in the 15 position. Retinoblastoma cells treated with Nutlin 3A had no increase in p53-serine-15 phosphorylation. In contrast, cells treated with doxorubicin, a known genotoxic compound, exhibited a significant increase in serine-15 phosphorylation. Thus, as suggested by Nutlin 3A’s mechanism of action, the drug appears to selectively activate the p53 pathway without causing DNA damage.

The predisposition of retinoblastoma patients to the development of secondary tumors is important when considering treatment options because the mutagenic effects of traditional therapies such as chemotherapy and radiation may increase a child’s risk of developing secondary nonocular tumors. Although the cure rate for retinoblastoma is 98% in the United States, patients with germline disease have a 50% chance of developing a secondary nonocular tumor later in life, and half of these tumors are fatal.

Nutlin 3A shows great promise as a treatment for retinoblastoma, but it may be susceptible to inactivation by resistance acquired by tumor cells. Because Nutlin 3A’s mechanism of action is dependent on activation of the p53 pathway, mutation of p53 could lead to resistance to the drug. Although p53 mutation in retinoblastoma is considered to be extremely rare, it has been reported in a patient with a tumor metastatic to the lung. Acquired p53 mutations have been documented in nonocular tumors such as neuroblastoma and lymphoma as a consequence of treatment with DNA-damaging agents. If the development of these mutations were a result of the mutagenic effects of the treatments, it is possible that their occurrence may be less likely after treatment with a nongenotoxic drug like Nutlin 3A.

This study demonstrates that targeted inactivation of HDM2 by a small molecule can achieve significant death of retinoblastoma cells while bypassing the genotoxic effects associated with traditional therapies. Nutlin 3A may provide a novel treatment option whose specific mechanism of action theoretically portends a narrow adverse effect profile in terms of risk of secondary tumors, bone marrow suppression, and deformity as compared with ionizing radiation and chemotherapy. Its small size could be an advantage in the setting of periocular administration for the achievement of effective intravitreal doses. Further studies are needed to address the efficacy of in vivo dosing as well as retinal and systemic adverse effects.

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