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Pharmacological inhibition of Mdm2 triggers growth arrest and promotes DNA breakage in mouse colon tumors and human colon cancer cells

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Abstract

The p53 tumor suppressor protein performs a number of cellular functions, ranging from the induction of cell cycle arrest and apoptosis to effects on DNA repair. Modulating p53 activity with Mdm2 inhibitors is a promising approach for treating cancer; however, it is presently unclear how the in vivo application of Mdm2 inhibitors impact the myriad processes orchestrated by p53. Since approximately half of all colon cancers (predominately cancers with microsatellite instability) are p53-normal, we assessed the anticancer activity of the Mdm2 inhibitor Nutlin-3 in the mouse azoxymethane (AOM) colon cancer model, in which p53 remains wild type. Using a cell line derived from an AOM-induced tumor, we found that four daily exposures to Nutlin-3 induced persistent p53 stabilization and cell cycle arrest without significant apoptosis. A four day dosing schedule in vivo generated a similar response in colon tumors; growth arrest without significantly increased apoptosis. In adjacent normal colon tissue, Nutlin-3 treatment reduced both cell proliferation and apoptosis. Surprisingly, Nutlin-3 induced a transient DNA damage response in tumors but not in adjacent normal tissue. Nutlin-3 likewise induced a transient DNA damage response in human colon cancer cells in a p53-dependent manner, and enhanced DNA strand breakage and cell death induced by doxorubicin. Our findings indicate that Mdm2 inhibitors not only trigger growth arrest, but may also stimulate p53's reported ability to slow homologous recombination repair. The potential impact of Nutlin-3 on DNA repair in tumors suggests that Mdm2 inhibitors may significantly accentuate the tumoricidal actions of certain therapeutic modalities.

Introduction

The p53 tumor suppressor protein is activated in response to DNA damage by phosphorylation of N-terminal serine residues, which prevents p53 from interacting with the Mdm2 ubiquitin ligase [1–3]. Activation of p53 arrests the cell cycle to facilitate accurate DNA repair or can trigger apoptosis [4]. p53 can also suppress tumor development after oncogene-induced activation of the p19 (mouse) or p14 (human) tumor suppressor proteins, which bind and neutralize Mdm2 [5–9]. Pharmacological inhibitors of Mdm2 have been developed that may enhance the anti-cancer activities of p53 [10,11]. The potential effectiveness of Mdm2 inhibitors is supported by the pre-clinical findings that genetic restoration of p53 activity in experimental mouse cancer models results in rapid and

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extensive tumor regression [12–14]. One potential advantage of the Mdm2 inhibitors is that unlike many current forms of chemotherapy, they activate p53 without first damaging DNA.

The Nutlin-3 and MI-63 Mdm2 inhibitors have been found to induce apoptosis of leukemic cells from acute myeloid leukemia (AML), B-cell chronic lymphocytic leukemia (CLL), and multiple myeloma patients, both on their own and in synergy with the chemotherapeutic agents doxorubicin, chlorambucil, and fludarabine [15–21]. A key finding from these studies is that while Mdm2 inhibition induces leukemia cell apoptosis normal cells are generally spared [17,19,21]. Other groups have also reported a degree of selectivity of Mdm2 inhibitors for the induction of apoptosis in cancer cells [22]. Although the sensitivity of cancer cells to Mdm2 inhibitors is dependent on the presence of p53, the basis of their increased apoptotic sensitivity relative to normal cells is not entirely clear but has in some cases been correlated with higher levels of Mdm2 expression in cancer cells [23].

In addition to serving as therapeutic agents, Mdm2 inhibitors may also be useful as “chemo-protective” agents [24]. Within this scenario, a patient with a p53-mutant cancer would be treated with an Mdm2 inhibitor prior to chemo- or radiation-therapy. The resulting cell proliferation arrest in normal tissues would increase the resistance of normal tissue to the therapy, whereas the p53-mutant cancer cells would continue to proliferate and maintain their high sensitivity. Additional data on how normal tissues respond to Mdm2 inhibition will however be necessary before the induced chemo-resistance application can be translated to clinical use.

The role of p53 in preventing colon cancer progression and improving patient response to therapy is well-documented [25–29]. The pharmacological enhancement of p53 activity in colon cancers maintaining a functional p53 gene may therefore be an effective and relatively safe therapeutic approach. The mouse AOM model is particularly well-suited for studying the efficacy of Mdm2 inhibitors on colorectal cancer because the tumors formed are p53 sequence-normal [30]. In addition, AOM-induced tumors form primarily within the distal colon, the anatomical location most frequently involved in human colon cancer [31]. Importantly, these tumors express elevated levels of Mdm2, and previous reports suggest that they may have a heightened sensitivity to Mdm2 inhibitors [30,32]. Here we test the in vivo efficacy of the Mdm2 inhibitor, Nutlin-3, in mice bearing AOM-induced colon tumors. Our findings demonstrate a growth-inhibitory effect of Nutlin-3. Analysis of adjacent normal tissue supports the potential of Mdm2 inhibitors to elicit chemo-resistance within normal tissue. We previously reported that Nutlin-3 can invoke a DNA damage response in some colon cancer cell lines [33]. This response to Nutlin-3 was found to occur during S phase and is possibly a consequence of p53’s ability to slow homologous recombination repair during DNA replication [33]. In this manuscript we extend these findings to the in vivo AOM model, and go on to show that Nutlin-3 may also slow DNA repair in human colon cancer cells following doxorubicin exposure. We discuss the implication of these findings with regard to the treatment of p53-normal colon cancers that do not respond well to 5-flourouracil-based therapy [34].

Material and Methods

Treatment of Animals

A/J Mice, purchased from Jackson Laboratory (Bar Harbor, ME), were housed in a ventilated, temperature controlled room with a 12-h light: 12-h dark cycle. Mice were allowed free access to laboratory rodent chow (Laboratory Rodent Diet 5001; PMI Nutrition International, Richmond IN) and water. At 6 weeks of age, mice were injected i.p. with 10mg/kg azoxymethane (AOM) weekly for 6 weeks. Tumors were then allowed to develop over an 18-week time period. Nutlin-3 (100 mg/mL stock in DMSO) was diluted in a 2%

(w/v) hydroxypropylcellulose (avg. M.W. 100,000) and 0.5% (v/v) Tween 80 vehicle (Sigma-Aldrich) and delivered via gastric lavage 18 weeks post AOM dosing. For the single dose experiment mice received 50mg/kg Nutlin-3, 200mg/kg Nutlin-3 or vehicle only. For the quadruple dose experiment mice received 200mg/kg Nutlin-3 or vehicle every morning for 4 days. For both experiments tissue was harvested five hours post final dose.

Cell Culture

The AJ02-NM₀ cell line was isolated from an AOM-induced tumor from the A/J mouse strain by the Rosenberg laboratory [35]. These cells were cultured in RPMI 1640 with Glutamax (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) fetal bovine serum (Lonza, Rockland, ME), 5% (v/v) heat-inactivated horse serum, 1% (v/v) insulin-transferrin-selenium (Gibco, Grand Island, NY), 100 mM non-essential amino acids (Invitrogen) and antibiotic-antimycotic (Invitrogen). HCT116 and HT29 cells were cultured in McCoy's 5A medium with 10% fetal bovine serum, non-essential amino acids and antibiotic/antimycotic. Nutlin-3 was purchased from Cayman Chemical (Ann Arbor, MI), and stored frozen as a 10 mM stock solution in DMSO. Nutlin-3 was employed at a final concentration of 20 μ M in the cell culture experiments (unless noted otherwise). For viable cell enumeration, cells were passed on to a 24-well tissue culture plate at a density of approximately 25%. Following the indicated time periods and treatments, cells were suspended using trypsin-EDTA (200 μ l) and mixed with trypan blue to a final concentration of 0.1%. Viable, non-staining cells were enumerated using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA).

Tissue RNA Extraction

RNA was extracted from tumors using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA) according to the manufacturer's protocol following homogenization of the tissue in extraction buffer with a motorized pestle. RNA was extracted from liver tissue with TRIzol reagent (Invitrogen, Carlsbad, CA). Tissue was homogenized with a motorized pestle in 10 μ l TRIzol/mg tissue. The homogenate was centrifuged at 12,000 $\times$ g for 15 min at 4°C and the aqueous phase was collected. Approximately 300 μ l isopropanol was added and incubated at room temperature for 10 min followed by centrifugation at 12,000 $\times$ g for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed with approximately 150 μ l 75% ethanol followed by air-drying. The pellet was re-suspended in 50 μ l nuclease free water.

Reverse Transcription and Real-Time Quantitative PCR

Reverse transcription was performed using the ABI High-Capacity cDNA reverse transcription kit (Applied Biosystems Inc., Foster City, CA). A total of 2 μ g RNA was reverse transcribed according to the manufacturer's protocol. Real-time quantitative PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR system and software. Taqman gene expression assays for Bax, Mdm2, p21, and β -Actin were purchased from Applied Biosystems (Carlsbad, CA).

Cell Fractionation

Approximately 25 mg of tissue was homogenized in lysis buffer A [10 mM Hepes, pH 7.6, 15 mM KCl and 2 mM MgCl₂ plus 0.1% (v/v) Nonidet P-40] supplemented with proteinase/phosphatase inhibitor cocktails (Sigma) and 1 mM DTT, and incubated on ice for 8 minutes. The tissue homogenate was centrifuged for 10 min at 4°C (14,000 rpm). The supernatant was the cytoplasmic extract and the resulting nuclear pellets were rinsed with the above buffer A without NP-40. Nuclear extracts were prepared by resuspending nuclear pellets with a high-salt buffer C [20 mM Hepes, pH 7.6, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM

EDTA, 1 mM DTT, 5% (v/v) glycerol and proteinase/phosphatase inhibitor cocktails (Sigma)], incubating on ice for 40 min and then centrifuging for 10 min at 4°C. Total protein in the extracts was quantified using the Bio-Rad protein assay (BioRad, Hercules, CA).

Senescence Associate β -galactosidase (SABG) Assay

This assay was performed according to the chromogenic SABG assay procedure published by Debacq-Chainiaux et. al. [36].

Cell Cycle Analysis

Cells were harvested by trypsinization and fixed with 4% (w/v) PFA at room temperature for 10 min followed by permeabilization with PBS plus 0.5% (v/v) Triton-X100 for 10 min. Cells were stained with 30 μ g/ml propidium iodide with 0.3 mg/ml RNase A. At least 10,000 cells that fell within an analysis gate excluding clumped cells and cells with greater than 4N DNA content were evaluated using a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA). Cell cycle analysis was performed with FlowJo flow cytometry analysis software (Tree Star, Ashland, OR) using the Watson-pragmatic algorithm.

Caspase 3 activity assay

HCT116 cells were cultured and treated on 24 well plates as described. Cells were then scraped from the plate (in growth medium), washed with cold PBS and lysed in 100 μ l of buffer containing 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA and 0.01% Triton X-100 using a single freeze-thaw cycle. 50 μ l of this extract was combined with 50 μ l 2X Reaction buffer [containing 20 mM PIPES, pH 7.4, 4 mM EDTA, 0.2% CHAPS and 0.2 mM of caspase 3 substrate Z-DEVD-AMC (Enzo Life Sciences International, Plymouth Meeting, PA)]. The increase in fluorescence was determined over the course of thirty minutes. The change in fluorescence was then normalized to the protein content of the extract.

Immunoblotting and Immunofluorescence

For immunoblotting studies, 10 μ g of protein was denatured under reducing conditions, separated on 10% (v/v) SDS-polyacrylamide gels and transferred to nitrocellulose by voltage gradient transfer. The resulting blots were blocked with 5% (w/v) non-fat dry milk in PBS + 0.1% (v/v) Tween 20. Specific proteins were detected with appropriate antibodies using enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology, Santa Cruz, CA) as recommended by the manufacturer. Immunoblotting antibodies were p53 (CM5) (Novocastra, Bannockburn, IL), Mdm2 (Ab-2) (Calbiochem, Gibbstown, NJ), PCNA (PC10) (Santa Cruz Biotechnology, Santa Cruz, CA) and β -actin (I-19) (Santa Cruz Biotechnology, Santa Cruz, CA). For immunofluorescence, distal colon tissue was fixed in 10% (w/v) neutral buffered formalin for 4 hours at room temperature (~23–25°C), embedded in paraffin and cut in 5 μ m sections. Tissue sections were heated in an oven at 60°C for 20 min followed by deparaffinization, re-hydration, and antigen retrieval in 10 mM citrate buffer pH 6.0 with 0.05% (v/v) Tween 20 at 90°C. Tissue sections were then blocked with Background Buster (Innovex Biosciences, Richmond, CA) for 30 min at room temperature. Incubation of primary antibodies was performed overnight at 4°C with 1:100 dilutions of all primary antibodies in 5% (v/v) goat serum in PBS, followed by incubation with 1:100 dilution of cy3 or FITC labeled secondary antibodies for 1 hour at room temperature (Jackson ImmunoResearch Labs Inc, West Grove, PA). Tissue sections were counterstained with DAPI (1mg/ml) for 10 min at room temperature. Primary antibodies used for immunofluorescence were p-Histone H2A.X (Ser-139) (Santa Cruz Biotechnology, Santa Cruz, CA) and PCNA (PC10) (Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescent

imaging was performed on a Zeiss epifluorescent microscope using a 40X objective. Images were acquired using a CCD camera.

DNA analysis

Field inversion gel electrophoresis (FIGE) was used to resolve high molecular weight fragments resulting from DNA cleavage in whole cells. Agarose plugs containing $\sim 8 \times 10^5$ cells were casted in end-cut tuberculin syringes and treated for 24 h at 56°C in buffer A (0.5M EDTA, 1% sarkosyl, 1mg/ml Proteinase K). The plugs were washed six times in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at 4°C. Plugs sections ($\sim 2.5 \times 10^5$ cells) were loaded onto a 14 cm long gel casted with 1% (w/v) SeaKem^R Gold Agarose (Lonza, Rockland, ME) in 0.5x TBE (45 mM Tris, 45 mM borate, 1 mM EDTA) and electrophoresis was carried out at 22°C using the FIGE Mapper electrophoresis system with a buffer circulation pump (BioRad, CA, USA). The total run time was 10 hours with forward and reverse voltages of 100V and 60V, respectively. The forward switch time was increased linearly to 21 sec, and the reverse switch time was increased linearly to 8 sec over the 10 hr run. Two sets of standards were used; the Yeast Chromosome PFG Marker (225 – 1,900 kb) and the MidRange PFG Marker I (15 – 300 kb) [New England Biolabs, MA]. The gel was stained in ethidium bromide for visualization and photography.

Fluorescent TUNEL staining

Briefly, 5 μ m sections were deparaffinized, rehydrated and treated with 20 μ g/ml proteinase K (Roche Applied Science, Indianapolis, IN) for 30 min at room temperature. Tissue sections were then incubated with terminal deoxynucleotidyl transferase and labeling solution according to the manufacturers protocol for 1 hour at 37°C. Tissue sections were counterstained with DAPI (1mg/ml) for 10 min at room temperature.

Image analysis

Quantification of γ H2AX and TUNEL staining was performed using ImageJ image analysis software (<http://rsb.info.nih.gov/ij/>). Background subtraction from 8-bit images was accomplished by setting the minimum displayed brightness/contrast value to the value of a region of known background. Images were then converted to binary using the convert to mask function. To remove any false γ H2AX or TUNEL signal, a binary image of the colocalized points was generated using the colocalization plugin of Bourdoncle (<http://rsbweb.nih.gov/ij/plugins/colocalization.html>). The integrated densities of the total area of the colocalized binary image and the corresponding DAPI image were then measured. To generate an index of staining, the integrated density of the colocalized image was divided by the integrated density of the corresponding DAPI image and multiplied by 100. Because PCNA staining generated relatively high levels of stromal non-nuclear staining, PCNA was quantified by manual counting with the individual blinded to the image source.

Results

Nutlin-3 induces transcriptional activation of target genes in vivo

Activation of p53 can lead to the increased expression of pro-apoptotic and cell cycle arrest genes. p53 also activates its own inhibitor, Mdm2, as part of a negative feedback loop. In order to determine the appropriate Nutlin-3 dosing level, A/J mice bearing AOM-induced colon tumors received a single dose of Nutlin-3 via gavage at 0, 50, or 200 mg/kg. RNA was isolated from colon tumors and quantitative PCR (qPCR) analysis was performed. Three genes activated by Nutlin-3 in cell culture models [32], Mdm2, p21, and Bax, were increased in a dose-dependent manner in the AOM-induced tumors. The highest dose of

Nutlin-3 (200 mg/kg) also caused a significant increase in Mdm2 transcription (Fig 1A). Similarly, qPCR analysis of these genes in the liver also showed a similar response profile, indicating a systemic effect of Nutlin-3 (Fig 1B).

Nutlin-3 induces proliferation arrest following multiple daily doses in the AJ02-NM₀ cell line

Although a single dose of Nutlin-3 is sufficient to activate p53 in colon tumors, it is unlikely that a single dose will lead to significant tumor stasis or regression. To determine an appropriate dosing strategy, we examined the effect of multiple daily doses of Nutlin-3 on a cell line derived from AOM-induced tumors, the AJ02-NM₀ cell line [35]. Although pharmacokinetic data on a 200 mg/kg dose of Nutlin-3 in the colon is not available, analysis of plasma, liver and other organ sites have shown that Nutlin-3 levels peak rapidly at 2 hours, and then maintain active levels (of over 20 μ M) for several additional hours [37]. To conservatively simulate in vivo dosing kinetics, AJ02-NM₀ cells were treated with Nutlin-3 for 2–3 hours followed by removal of the medium and replacement with fresh media once daily for up to 4 days (Fig 2A)[37]. At 4 hours post-treatment each day, viable cells were counted and prepared for cell cycle analysis by flow cytometry. After two days of treatment, the cells began to exhibit growth arrest, with significantly lower cell numbers counted at days 3 and 4 (Fig 2B). Cell cycle analysis revealed increases in the G1 and G2/M phase populations and a decrease in the S-phase populations at days 2 through 4 (Fig 2C), consistent with a growth arrest response at these time points. Few apoptotic cells were observed during with this treatment regimen (data not shown).

Growth arrest of the AJ02-NM₀ cells following three daily Nutlin-3 pulses was accompanied by the stabilization of p53 and increased expression of Mdm2 (Fig 2D). As reported for other cell lines, the increased level of Mdm2 expression is likely to result from the activation of Mdm2 transcription by p53 [38,39]. p53 accumulated within the cells by day 3 and was not further increased by drug exposure on day 4. Analysis of Mdm2 levels showed that two Mdm2 isoforms were expressed in AJ02-NM₀ cells, the 90 and 70 kD form. The primary 90 kD product is the full-length form that binds and ubiquitinylates p53, and the 70 kD form is an alternative splice product that lacks the N-terminus and can inhibit p53 ubiquitinylation by the long form [40]. Accumulation of the shorter product may therefore contribute to p53 stabilization through the daily pulsing protocol.

Further investigation of the Nutlin-3-induced growth arrest response of AJ02-NM₀ cells indicated that growth arrest was accompanied by an increased number of senescent cells in the culture. Assay for senescence associated β -galactosidase revealed a small but significant increase in senescence in cells treated once daily for four days with Nutlin-3. The number of AJ02-NM₀ cells exhibiting a large flat morphology with blue staining indicative of senescence increased from 5 to 15 per microscopic field (Fig 2E). In general however, the fraction of cells in which senescence was induced by Nutlin-3 was relatively small and accounted for approximately 5% of cells.

Nutlin-3 induces p53 stabilization and expression of Mdm2 isoforms in vivo

To determine how daily Nutlin-3 treatments influence p53 and Mdm2 expression levels in vivo, tumor-bearing mice were gavaged daily for 4 days with 200 mg/kg Nutlin-3, and protein extracts were prepared from colon tumors and liver. As found in cell culture, immunoblot analysis of colon tumor and liver protein extract showed p53 accumulation following four days of Nutlin-3 treatment (Fig 3). The 70 kD Mdm2-short form was preferentially activated in the liver by Nutlin-3, suggesting a role for this isoform in p53 stabilization (Fig 3A). Mdm2 expression in the colon tumors was highly variable, with no consistent changes in expression observed following Nutlin-3 treatment. It is not clear why

Mdm2 protein levels were not increased following Nutlin-3 administration, since Mdm2 mRNA levels were responsive to the drug (Figure 1). Mdm2 protein turnover rates in the tumors may be high enough to suppress Mdm2 accumulation.

Nutlin-3 treatment reduces tumor cell proliferation but does not induce apoptosis

Activation of p53 can result in cell cycle arrest or apoptosis. To determine whether Nutlin-3 affected cell proliferation in AOM-induced tumors and adjacent normal tissue, PCNA staining was performed. Both tumor and normal tissue from mice that received four doses of Nutlin-3 showed a significant reduction in PCNA staining (Fig 4A). This tumor response was additionally supported by a significant reduction in tumor PCNA protein levels as determined by immunoblotting (Fig 4B). The quantification of apoptosis via TUNEL assay revealed a small, but non-significant trend towards increased apoptosis in tumor tissue (Fig 5A and 5B). A similar trend to increased caspase 3 activity was observed in extracts prepared from Nutlin-3 treated mice, but this increase also failed to reach statistical significance (Figure 5C). Interestingly, Nutlin-3 significantly decreased apoptosis in normal tissue following four doses with Nutlin-3, consistent with a potential protective effect of Nutlin-3 on normal tissue.

Nutlin-3 induces a DNA damage response in the colon tumors that is attenuated following multiple doses

There are a number of reports indicating that p53 can accentuate or stabilize DNA strand breaks by slowing homologous recombination repair [41–44], and this activity of p53 may be stimulated by Nutlin-3 [45]. Specifically, Nutlin-3 activated p53 has been reported to increase DNA damage in cells during S phase, potentially by slowing their ability to repair strand breaks that occur spontaneously during DNA replication [45]. To determine if this Nutlin-3 activity was manifested in vivo, tumor tissue from mice that received a single dose of Nutlin-3 (200mg/kg) was analyzed for expression of the DNA damage response marker γ H2AX (Figure 6). The DNA damage response in tumors was increased 24 hours after Nutlin-3 administration, whereas adjacent normal tissue was not significantly affected. Interestingly, the DNA damage response in tumor tissue was attenuated following four daily doses of Nutlin-3 (Figure 6B). Although it is not entirely clear why the DNA damage response subsides in tumors after 24 hours, it may arise from the fact that Nutlin-3 decreases the number of cells in S phase, the cell cycle phase in which Nutlin-3 has its maximal effect on DNA damage (Figure 4)[33].

Effect of Nutlin-3 on the DNA damage response in human colon cancer cells

To determine whether Nutlin-3 influenced the DNA damage response in human colon cancer cells, the p53-normal HCT116 cell line was treated with Nutlin-3 in the presence and absence of the topoisomerase II inhibitor doxorubicin (Figure 7A). As expected, doxorubicin increased the appearance of γ H2AX at 24 and 48 hours after treatment. However, combining doxorubicin and Nutlin-3 generated the highest level of γ H2AX, consistent with Nutlin-3 stabilizing double strand breaks induced by doxorubicin. Nutlin-3 alone induced a transient increase γ H2AX, which may result from slowing DNA repair required during the replication process, which is ultimately halted by the induction of growth arrest by Nutlin-3. DNA strand breakage was also determined by field inversion gel electrophoresis, and results from this analysis support the strand-breakage activities of the Nutlin-3/doxorubicin combination (Figure 7B). p53 was required for the increased γ H2AX expression following treatment with the Nutlin-3/doxorubicin combination, since p53-null HCT116 cells did not show a heightened sensitivity to the combination treatment (Figure 7C).

We then determined the growth-inhibitory effects of the Nutlin-3/doxorubicin combination. As shown in Figure 8A, the combination treatment reduced cell viability by over an order of

magnitude after a 48-hour exposure; approximately 5-fold more than the individual treatments. Growth inhibition by the combination treatment likely results in part from an increase in apoptosis, since caspase-3 activity of the cultures was significantly higher following the Nutlin-3/doxorubicin combination treatment than the individual treatments (Figure 8B). The full pro-apoptotic action of the Nutlin-3/doxorubicin combination likely requires a normal p53 protein since HCT116 cells that lack p53 were not responsive to the combination treatment (Figure 8C) and HT29 cells (with a mutated p53) showed a more modest degree of caspase-3 enhancement when Nutlin-3 was included with doxorubicin (Figure 8D).

To determine whether growth inhibition by Nutlin-3 and doxorubicin was reversible, HCT116 cells were treated individually or in combination with these drugs for 24 hours. Cells were then passed into new medium without drug, and viable cell counts were made at 24, 48 and 96 hours. Fewer cells from the cultures treated with the drug combination survived the procedure (Figure 9A), and those that did had an altered, rounded morphology (Figure 9B). The growth inhibition by doxorubicin and the Nutlin-3/doxorubicin combination was not reversed over the 96-hour time period, although growth arrest by Nutlin-3 alone was reversible. In general, these data further support the potent growth-inhibitory activity of the Nutlin-3 doxorubicin combination.

Discussion

Patients with colon tumors that possess a functional p53 gene have a better prognosis, suggesting that p53 plays an important role in restraining lesion growth and progression [46,47]. Enhancing p53 activity pharmacologically with Mdm2 inhibitors may therefore be an effective adjuvant to present treatment strategies. To assess the therapeutic potential of Mdm2 inhibitors on colon cancer, we determined the activity of Nutlin-3 in the mouse AOM model. This model is well-suited to study the pharmacological activities of Mdm2 inhibitors since tumors are typically p53-normal. Cancer cell lines are also available for this model, allowing a rapid assessment of growth responses following drug exposure.

Using a cell culture system to recapitulate in vivo dosing, we found that daily exposure of an AOM-induced mouse colon cancer cell line to Nutlin-3 induced a growth arrest response after three days. Cell cycle arrest at G1 and G2/M was responsible for the reduced cell numbers in the culture, without a significant increase in apoptosis. Although there was a statistically significant increase in the number of senescent cells following 4 daily exposures to Nutlin-3, the fraction of cells entering senescence was low (approximately 5%), indicating that the response to Nutlin-3 in the mouse colon cancer cells was primarily a reversible cell cycle arrest, as has been reported for other cell lines [48]. Using this 4-day exposure protocol, the response of AOM-induced tumors in vivo was tested and found to be similar to that seen with the cell line; four daily treatments with Nutlin-3 induced a proliferation arrest without significantly increasing apoptosis. Both in vivo and in vitro, the growth arrest was accompanied by accumulation of p53. In mouse colon cancer cells and liver tissue, p53 stabilization corresponded with the expression of a shorter p53-stabilizing Mdm2 isoform [40]. Our findings demonstrate that colon cancers are responsive to p53 activation and growth arrest by a pharmacological Mdm2 inhibitor, similar to what has been reported for cell culture and xenograft models [49–51]. However, the lack of an apoptotic response in the AOM-induced tumors indicates that Mdm2 inhibitors may have limited application as stand-alone chemotherapeutic agents.

Adjacent normal tissue in the colon displayed a significant decrease in both proliferation and apoptosis following four Nutlin-3 doses, consistent with a general reduction in cell turnover. Although a prolonged reduction in cell turnover in the colon could have drawbacks, the lack

of an apoptotic response within the normal tissue is consistent with a low-toxicity of Mdm2 inhibitors [22,51]. This finding also supports the potential application of Mdm2 inhibitors as pre-conditioning agents for preparing patients for chemo- or radiation therapy. The concept here is that activating growth arrest in p53-normal tissue with an Mdm2 inhibitor would slow their proliferation and increase their resistance to chemotherapy [24,52]. Such an approach would also in theory be useful for patients with p53-mutated cancers, since the cancer cells would not be protected by pre-treatment with Nutlin-3. Our data demonstrate the ability of Mdm2 inhibitors to slow cell turnover in normal colon tissue and supports the ability of Mdm2 inhibitors to function as “chemoprotective” agents [53].

One particularly interesting finding in our studies is that Nutlin-3 administration to mice generates a DNA damage response that occurs specifically within the tumors. It is somewhat counterintuitive that an Mdm2 inhibitor would activate a DNA damage response, given that p53 plays such a critical role in maintaining genomic integrity [33]. However, it has been reported that p53 slows homologous recombination repair (HRR) through regulation of the Rad51 strand exchange step [41,54,55]. It has also been reported that p53 acts as a transcriptional repressor of the RAD51 promoter, which reduces Rad51 expression to further suppress HRR [42,56]. The slowing of HRR by p53 is thought to enhance the fidelity of repair by ensuring that only homologous chromosome segments are used in the repair process [57]. We propose that Nutlin-3 stabilized p53 slows HRR [45]. In tumors, this activity may slow the HRR that occurs normally during DNA replication. In fact, it has been reported that the increase in γ H2AX expression in Nutlin-3-treated cells occurs primarily during S phase, and is not associated with apoptotic cells [33]. The fact that tumor cells are more proliferative may contribute to their heightened sensitivity to Nutlin-3-stabilized DNA breakage. We observed a similar response in human colon cancer cells, which likewise show a transient increase in γ H2AX following Nutlin-3 exposure. The subsequent decrease in DNA damage signaling at longer time points of Nutlin-3-exposure may result from the halting of DNA replication (resulting from the p53 activation of cell cycle arrest genes). While the usefulness of Nutlin-3 as a single agent for colon cancer chemotherapy is unclear, the combination of Nutlin-3 with treatments that induce double strand DNA breaks may prove to be effective. Consistent with this possibility, we have found that Nutlin-3/doxorubicin has strong anti-growth and apoptosis-enhancing effects on human colon cancer cells.

Previous reports have indicated that Nutlin-3 induces p53 activity without triggering its phosphorylation at ATM/ATR target sites (indicative of a DNA damage response)[50]. Our present and previously published data are consistent with these findings. However, following the rapid and direct activation of p53 by Nutlin-3, p53 phosphorylation can be detected at later time points in cell lines that stall in S phase [33]. Although our findings are consistent with p53 playing a role in inducing the DNA damage response following Nutlin-3, other Nutlin-3 targets may be contributing to this effect. For example, Nutlin-3 can reduce the expression and activity of the E2F-1 transcription factor [58–60]. Since E2F-1 activates expression of a number of DNA repair proteins (including Rad51), its reduced expression following Nutlin-3 treatment may contribute to the reduced rate of HRR [61,62]. Other cellular targets may also be involved in the mobilization of the DNA damage response by Nutlin-3; it has recently been reported that Nutlin-3 can activate this response even in cells that lack p53 or Mdm2 [63]. Understanding the molecular targets involved in provoking the DNA damage response will likely be important for optimal application of Nutlin-3 (and potentially other Mdm2 inhibitors).

Although much work remains to be done to determine whether combining pharmacological Mdm2 inhibitors with other therapies can improve patient outcome, there is a need for new approaches for treating some forms of colon cancer. Present chemotherapeutic approaches

for colon cancer utilize primarily a 5-fluorouracil-based drug combination. The commonly used FOLFOX regimen includes 5-fluorouracil, oxaliplatin and leucovorin. These agents promote uracil misincorporation and DNA methylation, among other cellular effects [64,65]. Although FOLFOX has shown significant benefit for the treatment of many stage 3 and 4 colon cancer patients, it may be largely ineffective for treating cancers with microsatellite instability (MSI-H)[34]. Colon cancers with a MSI phenotype account for approximately 15–20% of colon cancer [66]. These tumors have a defect in at least one mismatch repair protein and can therefore be resistant to the 5-fluorouracil-induced DNA lesion [67]. However, there have been reports that colon cancer cells with MSI are particularly sensitive to topoisomerase inhibitors [68]. Our data suggest that inclusion of Mdm2 inhibitors with topoisomerase inhibitors may be a highly effective combination for treating MSI cancers, which also tend to be p53-normal [69].

In summary, our work indicates that pharmacological Mdm2 inhibitors induce growth arrest without extensive apoptosis in the in vivo mouse AOM colon cancer model. Although the ability of Mdm2 inhibitors to only transiently induce growth arrest in cancers cells suggest that they may have limited use as stand-alone agents, our finding that these agents can impact DNA breakage repair in colon cancer cells provides framework from which effective combinations of Mdm2 inhibitors with other therapeutic agents might be devised.

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Abbreviations List

AOM	azoxymethane
5-FU	5-fluorouracil
AML	acute myeloid leukemia
CLL	B-cell chronic lymphocytic leukemia
HRR	homologous recombination repair
PCNA	proliferating cell nuclear antigen
SABG	senescence associated β -galactosidase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
DAPI	4',6-diamidino-2-phenylindole, γ H2AX, p-Histone H2A.X (Ser-139)

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Figure 1.

Nutlin-3 induces expression of target genes in vivo. AOM treated A/J mice were administered a single dose of Nutlin-3 or vehicle control via gavage and sacrificed 4 hrs post dosing. RNA isolated from tumors harvested from the distal colon was used to synthesize cDNA and was analyzed via qPCR for the expression of Mdm2, p21, and Bax. qPCR analysis was performed on cDNA from (A) 3 tumors from each of 4 animals per treatment group and (B) on liver tissue from 4 animals per treatment group. A) ANOVA was performed to determine the significance of gene expression changes. B) Unpaired two-tailed t-tests with 95% confidence intervals were performed to determine the significance of gene expression changes: * $p < 0.05$.

Figure 2.

Nutlin-3 induces cell cycle arrest following multiple daily exposures in vitro. A) Schematic of the exposure protocol. AJ02-NM₀ cells were exposed to Nutlin-3 (20 μ M) beginning 24 hours after passage for 2 hours followed by replacement with fresh media each day for 4 days. Cells were harvested for each time point 4 hours post Nutlin-3 exposure. B) Total viable cell numbers from 4 wells of a 24 well plate were counted for each timepoint. C) Cell cycle analysis was performed on 10,000 cells stained with propidium iodide per time-point. D) AJ02-NM₀ cells were treated with Nutlin-3 beginning 24 hours after passage for two hours daily followed by replacement with fresh media for 3 days or left completely untreated. On the fourth day cells were treated with Nutlin-3 for 0, 3, or 5 hours.

Immunoblotting for p53, Mdm2, and β -Actin (loading control) was performed on cytosolic and nuclear protein extracts. E) AJ02-NM₀ cells were treated with DMSO or Nutlin-3 as described in A for either 1 or 4 days. Cells were stained for senescence-associated β -galactosidase (SABG). For each of the treatment groups, 10 total fields were observed with roughly 300 cells per field. Significant differences were determined by ANOVA; ** $p < 0.01$; *** $p < 0.001$.

Figure 3.

Nutlin-3 induces stabilization of p53 and expression of alternate forms of Mdm2 in vivo. A and B) AOM treated A/J mice were given 4 daily doses of vehicle control or Nutlin-3 via gavage and sacrificed 4 hrs post dosing. Immunoblotting for p53, Mdm2, and β-Actin (control) was performed on protein extracted from the liver (A) or tumors from the distal colon (B). Four animals per group were used to analyze liver proteins (animals 1–8) and five tumors per group were used to analyze tumor proteins (tumors 1–10).

Figure 4.

Multiple doses of Nutlin-3 reduce tumor proliferation in vivo. A) Distal colon tissue sections from AOM treated A/J mice that received 4 daily doses of Nutlin-3 were analyzed by immunofluorescence for PCNA (red). Nuclei were counter stained with DAPI (blue). The top panels show representative images from normal tissue and tumors, as indicated. The fraction of PCNA positive cells was determined for normal or tumor tissue from 4 mice per treatment group, with two to three tumors analyzed from each mouse (ten tumors per treatment group). The significance of differences was determined via unpaired two tailed t-tests: * $p < 0.05$, *** $p < 0.001$. B) Immunoblotting for PCNA was performed on protein extracts from 2 tumors of 3 mice per treatment group. The relative levels of PCNA were

determined by comparison of the integrated density of the PCNA bands relative to the corresponding β -actin bands using ImageJ. Significance of the difference was determined by two-tailed unpaired t-test with a 95% confidence interval.

Figure 5.

Multiple doses of Nutlin-3 do not increase apoptosis significantly in tumor or normal tissue. A) Distal colon tissue sections were analyzed for apoptosis via fluorescent TUNEL staining (green). Nuclei were counter stained with DAPI (blue). A minimum of 5 fields at 40X magnification section from 4 animals per group were analyzed. Only areas of tumor with a high epithelial cell content were included in the quantification. The upper panels show representative tissue images, and the bottom graphs are the quantified staining results. Significance of differences was determined by two-tailed unpaired t-test: ** $p < 0.01$. B) Caspase 3 activity in tumor extracts prepared from Nutlin-3-treated and control mice. Six tumors from each group were analyzed, using a fluorogenic DEVD substrate for caspase 3.

The difference between the two groups was not significant as determined by a two-tailed unpaired t-test.

Figure 6.

Nutlin-3 induces a DNA damage response after a single dose that is attenuated following multiple doses. Distal colon tissue sections were analyzed by immunofluorescence for γ H2AX (green). Nuclei were counter-stained with DAPI (blue). A) Representative fields of tumor tissue for single and quadruple vehicle control and Nutlin-3 treatments. B) A minimum of 5 fields from 4 animals per group was quantified for γ H2AX staining. ANOVA was performed to determine the significance of changes in the γ H2AX staining: $*p < 0.05$.

Figure 7.

The effect of Nutlin-3 and doxorubicin on DNA damage and the DNA damage response. A) The human HCT116 colon cancer cell line was treated with Nutlin-3 (20 μ M) and doxorubicin (Dox; 1 μ M), both alone and in combination, for 24 or 48 hours, as indicated. A histone protein fraction was prepared and analyzed for γ H2AX levels by immunoblotting. Histone proteins were Coomassie stained to assess gel loading. B) HCT116 cells were treated as described in 7A, after which cells were incorporated into agarose plugs for cell lysis and proteinase treatment. Agarose plugs were loaded onto agarose gels and DNA fragments were resolved by field inversion gel electrophoresis. C) HCT116 p53-null cells were treated similarly to the p53-normal HCT116 cells described in 7A. A histone protein fraction was prepared and analyzed for γ H2AX levels by immunoblotting. Histone proteins were Coomassie stained to assess gel loading.

Figure 8.

A) Effect of Nutlin-3 and doxorubicin on HCT116 cell viability. HCT116 cells were treated with Nutlin-3 and doxorubicin (Dox), alone or in combination, for 24 or 48 hours. The relative number of viable cells in the cultures were then determined. The Nutlin-3/doxorubicin combination at 48 hours significantly reduced cell viability relative to cells treated with Nutlin-3 or doxorubicin alone ($***p < 0.001$). B) HCT116 cells were treated as described in 8A, but cells were processed for caspase-3 activity determination using the DEVD-AMC peptide as a substrate. The Nutlin-3/doxorubicin combination significantly increased caspase activation at 24 and 48 hours relative to cells treated with Nutlin-3 or doxorubicin alone ($***p < 0.001$). C) HCT116 cells with or without a functional p53 gene

were treated with Nutlin-3 and/or doxorubicin (Dox) as indicated for 24 hours. Cells were processed for caspase-3 activity determination as described in 8B. The Nutlin-3/doxorubicin combination significantly increased caspase activation only in HCT116 cells that possessed an intact p53 gene ($***p < 0.001$). D) HT29 cells (with a mutant p53 gene) were treated as the HCT116 cells were in 8B, and tested for their level of caspase 3 activity. There was a modest, but significant increase in caspase 3 activity at 48 hours when Nutlin-3 was included with doxorubicin ($**p < 0.01$).

Figure 9.

Irreversible growth repression by the Nutlin-3/doxorubicin combination. A) HCT116 cells were incubated with Nutlin-3 (20 μ M), doxorubicin (1 μ M) or a Nutlin-3/doxorubicin combination for 24 hours. Cells were then passed to a fresh plate and the relative number of viable cells was determined 24, 48 and 96 hours after passage. The Nutlin-3/doxorubicin combination significantly reduced the number of viable cells in the culture, relative to all other treatments ($***p < 0.001$). Growth arrest following the combination treatment was also irreversible in the 96 hour period following passage. B) A phase contrast image of the HCT116 cells following drug treatment and passage. Cells treated with both Nutlin-3 and doxorubicin (Nutlin/Dox) display a rounded phenotype 24 hours after passing.