Pharmacologically Increasing Mdm2 Inhibits DNA Repair and Cooperates with Genotoxic Agents to Kill p53-Inactivated Ovarian Cancer Cells

Alexia M. Carrillo1, Mellissa Hicks1, Dineo Khabele2, and Christine M. Eischen1

Abstract

The Mdm2 oncogene is a negative regulator of the p53 tumor suppressor and recently identified inhibitor of DNA break repair. Nutlin-3 is a small-molecule inhibitor of Mdm2–p53 interaction that can induce apoptosis in cancer cells through activation of p53. Although this is a promising therapy for those cancers with wild-type p53, half of all human cancers have inactivated p53. Here, we reveal that a previously unappreciated effect of Nutlin is inhibition of DNA break repair, stemming from its ability to increase Mdm2 protein levels. The Nutlin-induced increase in Mdm2 inhibited DNA double-strand break repair and prolonged DNA damage response signaling independent of p53. Mechanistically, this effect of Nutlin required Mdm2 and acted through Nbs1 of the Mre11–Rad50–Nbs1 DNA repair complex.

Materials and Methods

Cell culture

p53−/−, p53−/−Mdm2−/−, and Nbs1Δ/Δ murine embryonic fibroblasts (MEF) were cultured as we previously described.
Mol Cancer Res; 13(8) August 2015

Molecular Cancer Research

OF2

with Nutlin (10 μmol/L) or vehicle control (DMSO) for 1 hour. At the intervals indicated cells were harvested and whole cell protein lysates were subjected to Western blot analysis for the protein indicated. Quantification of Mdm2 relative to β-actin was performed by densitometry. B to E, 293T cells (B), p53+/− MEFS (C), p53+/−/Mdm2+/− MEFS (D), and Nbs1+/−/+ Mdm2−/− MEFS (E) were cultured with Nutlin (10 μmol/L) or vehicle control (DMSO). Ectopic Mdm2 overexpression in each cell line served as a positive control. Whole-cell lysates were Western blotted for the indicated proteins. Following 5 Gy of γ-radiation, neutral comet assays were used to evaluate the percentage of cells with a tail moment ≥4 (damaged DNA). All graphs represent a minimum of three independent experiments, and each circle indicates one experimental sample consisting of a minimum of 40 cells analyzed. For E, 120 minutes following γ-radiation is shown. B, **P = 0.001; ∗∗, P = 0.007; D, †, P = 0.0007; ‡, P = 0.0093; E, ‡‡, P = 0.031, all as compared with DMSO, t test.

Chemotherapeutic agents

Nutlin (Sigma) and etoposide (Sigma) were dissolved in DMSO (Sigma) at 17.2 and 50 mmol/L, respectively, from which working stocks were generated. The concentration of Nutlin used refers to the entire mixture, but only half of the total concentration represents the active enantiomer A. Cisplatin (Sigma) was resuspended in 0.9% NaCl at 5 mmol/L from which working stocks were generated.

Western blotting and protein half-life analysis

Following addition of Nutlin (10 μmol/L) or vehicle control (DMSO) for 1 hour, cycloheximide (20 μg/mL; Sigma) was added to cultures of p53+/− MEFS. At intervals, cells were placed on ice and harvested for Western blot analysis. Whole-cell lysates were prepared, subjected to SDS–PAGE, transferred to nitrocellulose, and Western blotted as we previously described (11). Antibodies specific for Mdm2 (2A10 for mouse, Calbiochem; 3G9 for human, Millipore), cleaved caspase-3 (ASP175; Cell Signaling Technology), and β-actin (Sigma) were used. Densitometry for quantification of Mdm2 protein bands was performed using ImageJ software (NIH) and were relative to band intensities of β-actin.

Comet assay

Cells were left untreated or were treated with Nutlin (10 μmol/L) or vehicle control (DMSO) 24 hours before γ-radiation (137Cs source), where indicated. As a control, cells were either transfected or infected with a bicistronic retroviral vector encoding Mdm2 and GFP (10). Neutral comet assays were performed at intervals following γ-radiation, as previously described (10, 11). A minimum of three independent experiments were performed for all analyses. Statistical significance was determined by Student t tests.

Immunofluorescence

Cells were cultured on coverslips and, where indicated, treated for 24 hours with the specified drug. Following γ-radiation, cells were fixed and γH2AX and pS/TQ foci were detected by immunofluorescence and analyzed as previously described (11, 14). Antibodies against pSer139-H2AX (Millipore), pSer1841-ATM (Rockland), and secondary antibodies Alexa Fluor 594 and Alexa Fluor 488 (Invitrogen) were used. The number of γH2AX and pS/TQ foci per cell for at least 40 cells per individual condition was quantified. A minimum of two to three independent experiments were performed for these analyses. Statistical significance was determined using a confidence interval of 95%. The assays determining the percentage of γH2AX-positive cells 24 hours following removal of cisplatin or etoposide were performed two times as previously published (15), except Nutlin (10 μmol/L) was added to the samples indicated. Cells that had five or more foci were considered positive; statistical significance was determined using a Student t test.

Proliferation, viability, and apoptosis analysis

For MT assays, cells (5 × 10^3–1 × 10^4) were cultured in 96-well plates and treated in quadruplicate with chemotherapeutic agents (Nutlin, etoposide, cisplatin, or a combination) at varying concentrations for 72 hours. At intervals, MT assays (Sigma) were performed according to the manufacturer’s

Figure 1.

Nutlin inhibits DNA break repair independent of p53 and through Mdm2 and Nbs1. A, cycloheximide (CHX) was added to p53−/− MEFs pretreated with Nutlin (10 μmol/L) or vehicle control (DMSO) for 1 hour. At the intervals indicated cells were harvested and whole cell protein lysates were subjected to Western blot analysis for the protein indicated. Quantification of Mdm2 relative to β-actin was performed by densitometry. B to E, 293T cells (B), p53+/− MEFs (C), p53+/−/Mdm2−/− MEFs (D), and Nbs1+/−/+ Mdm2−/− MEFs (E) were cultured with Nutlin (10 μmol/L) or vehicle control (DMSO). Ectopic Mdm2 overexpression in each cell line served as a positive control. Whole-cell lysates were Western blotted for the indicated proteins. Following 5 Gy of γ-radiation, neutral comet assays were used to evaluate the percentage of cells with a tail moment ≥4 (damaged DNA). All graphs represent a minimum of three independent experiments, and each circle indicates one experimental sample consisting of a minimum of 40 cells analyzed. For E, 120 minutes following γ-radiation is shown. B, **P = 0.001; ∗∗, P = 0.007; D, †, P = 0.0007; ‡, P = 0.0093; E, ‡‡, P = 0.031, all as compared with DMSO, t test.

Proliferation, viability, and apoptosis analysis

For MT assays, cells (5 × 10^3–1 × 10^4) were cultured in 96-well plates and treated in quadruplicate with chemotherapeutic agents (Nutlin, etoposide, cisplatin, or a combination) at varying concentrations for 72 hours. At intervals, MT assays (Sigma) were performed according to the manufacturer's...
protocol. To evaluate apoptosis, cells were treated with chemotherapeutic agents for 48 hours, photographed, and harvested. Whole-cell protein lysates were prepared as described above. Cleaved caspase-3 was evaluated by Western blot analysis (see above).

Results

Nutlin increases Mdm2 protein stability independent of p53

It was previously reported that cell lines treated with Nutlin can lead to increased levels of Mdm2 as detected by Western blot analysis, and this can occur in the absence of p53 (6, 8). To determine whether Nutlin influences Mdm2 protein stability, we evaluated Mdm2 protein levels following addition of the protein synthesis inhibitor, cycloheximide. To exclude effects on Mdm2 from p53 activation due to Nutlin treatment, we used p53−/− MEFs. We also only exposed the MEFs to Nutlin for 1 hour to evaluate direct effects of Nutlin and eliminate any secondary effects that may occur with prolonged treatment. Mdm2 protein remained present for longer in cells with Nutlin compared with vehicle control–treated cells (Fig. 1A). Specifically, there were high levels of Mdm2 protein remaining after 20 minutes with cycloheximide in the Nutlin-treated cells, whereas in the vehicle control–treated cells, Mdm2 was barely detectable. These data indicate increased stability of Mdm2 protein in the presence of Nutlin, resulting in elevated levels of Mdm2.

Nutlin inhibits DNA break repair through Mdm2 and Nbs1, independent of p53

We have previously shown that increased levels of Mdm2 protein inhibit double-strand DNA break repair independent of p53 (10, 11, 14). To determine whether the increase in Mdm2 levels caused by Nutlin would result in an inhibition in double-strand DNA break repair, we first tested this by neutral comet assay in 293T cells, which have inactivated p53 due to expression of SV40 large T antigen. We detected that 60.1% (±1.57%) of the cells exposed to Nutlin had DNA damage remaining 60 minutes after γ-radiation, compared with only 29.7% (±2.35%) of DMSO-treated cells (Fig. 1B). The reduction in repaired DNA caused by Nutlin was comparable with cells that ectopically overexpressed Mdm2. To more fully assess whether this effect of Nutlin is independent of p53, we evaluated DNA repair in p53−/− MEFs in the presence of Nutlin. Sixty minutes after γ-radiation, p53−/− MEFs with Nutlin had reduced repair of double-strand DNA breaks, resulting in significantly more cells harboring damaged DNA compared with cells treated with vehicle control (Fig. 1C). Again, the inhibition of DNA repair by Nutlin was comparable with cells overexpressing Mdm2 from a vector.

To determine the requirement of Mdm2 for this effect on DNA repair of Nutlin, we exposed p53−/− Mdm2−/− MEFs to Nutlin or DMSO vehicle control. We observed at both 60 and 90 minutes following γ-radiation, Nutlin-treated cells repaired their DNA to a similar extent as DMSO control (Fig. 1D), whereas MEFS overexpressing Mdm2 had significantly reduced DNA repair. These results demonstrate the Nutlin-induced inhibition of double-strand DNA break repair is mediated by Mdm2.

The inhibition in DNA break repair induced by Mdm2 is mediated through its interactions with Nbs1 of the MRN complex (11). To evaluate the requirement of Nbs1 for the effects of Nutlin on DNA break repair, we used MEFS where Nbs1 has been mutated resulting in instability and severely reduced Nbs1 expression (Nbs1ΔRΔR; ref. 16). DNA break repair is already delayed in
Nutlin inhibits the DNA damage signal through Mdm2

To determine whether the reduction in DNA break repair caused by Nutlin is due to a delay in DNA damage signaling that occurs when Mdm2 is overexpressed (10, 11), we evaluated γH2AX foci formation and resolution. H2AX is rapidly phosphorylated (γH2AX) upon DNA damage and returns to an unphosphorylated form upon repair (17). Assessment of γH2AX foci formation showed that 10 minutes following γ-radiation, p53−/− MEFs in the presence of Nutlin formed 31% fewer foci than vehicle-treated cells (Fig. 2A and Supplementary Fig. S1A). These results were comparable with cells overexpressing Mdm2 from a retroviral vector. At 150 minutes after γ-radiation, there were significantly increased numbers of γH2AX foci in the Nutlin-exposed MEFs compared with DMSO-treated controls, indicating a delay in foci resolution. To determine the role of Mdm2, we measured foci in p53−/− Mdm2−/− MEFs. When Mdm2 was not present, Nutlin no longer inhibited the formation or the resolution of γH2AX foci compared with DMSO-treated cells (Fig. 2B and Supplementary Fig. S1B). These results indicate that Nutlin treatment has a comparable inhibitory effect on γH2AX foci formation and resolution as Mdm2 overexpression, and this effect is independent of p53, but requires Mdm2.

γ-Radiation induces DNA breaks, which activates ATM, the DNA damage–induced kinase, that then phosphorylates motifs with a serine or threonine followed by a glutamine (S/TQ; ref. 18). Another means to measure DNA damage signaling is to evaluate these phosphorylated S/TQ (pS/TQ) sites. Following γ-radiation, pS/TQ foci were assessed in p53−/− MEFs cultured with Nutlin or vehicle control. Cells with Nutlin had 28% fewer foci immediately after γ-radiation, and 62% more foci at 240 minutes after γ-radiation compared with controls (Fig. S2A). When p53−/− Mdm2−/− MEFs were used, Nutlin had neither an impact on the formation nor the resolution of the pS/TQ foci (Fig. S2D and Supplementary Fig. S2B). In both cell types, pS/TQ foci formation and resolution was impaired when Mdm2 was ectopically overexpressed, as we previously reported (11). Therefore, Nutlin inhibits the initial DNA damage response signal leading to prolonged signaling, and this requires Mdm2 but is independent of p53.

Figure 3. Delayed DNA repair by Nutlin in ovarian cancer cells with inactivated p53. SKOV-3 (A), OVCAR-5 (B), and OVCAR-8 (C) ovarian cancer cell lines were cultured with Nutlin (10 μmol/L) or vehicle control (DMSO). Whole-cell protein lysates were evaluated by Western blot analysis. Following 5 Gy of γ-radiation, neutral comet assays were used to measure DNA breaks; each bar is an average of two to four independent experiments with a minimum of 40 cells analyzed for each sample in each experiment. A, *, P = 0.0073; **, P = 0.0047; B, *, P = 0.0047; **, P = 0.004; C, *, P = 0.004; **, P = 0.0073, Student t test.

Nutlin inhibits DNA break repair in ovarian cancer cells

Ovarian cancer is a difficult to treat malignancy with the aggressive high-grade serous ovarian carcinoma type harboring mutated or deleted p53 in >90% of the tumors (2). To determine whether Nutlin could increase levels of Mdm2 and inhibit DNA break repair in malignant cells that have inactivated p53, we assessed ovarian cancer cells that had either deleted or mutated p53. SKOV-3 ovarian cancer cells have deleted p53 and showed increased levels of Mdm2 following exposure to Nutlin (Fig. 3A). We then assessed the effect of Nutlin on their ability to repair damaged DNA by exposing the SKOV-3 cells to Nutlin or vehicle control, and performing comet assays following γ-radiation. Only 57.6% of the cells treated with Nutlin were able to repair their DNA damage within 90 minutes, compared with 74.5% of cells exposed to vehicle control (Fig. 3A).

We also evaluated ovarian cancer cells that harbor mutant p53, OVCAR-5 (insertion at codon 224) and OVCAR-8 (deletion of codons 126–132; ref. 19). Nutlin treatment led to increased Mdm2 protein levels in both cell lines (Fig. 3B and C). In addition, both lines exposed to Nutlin had significantly reduced DNA break repair at 60 and 90 minutes after γ-radiation (Fig. 3B and C). These results indicate that Nutlin treatment has a comparable inhibitory effect on γH2AX foci formation and resolution as Mdm2 overexpression, and this effect is independent of p53, but requires Mdm2.

To determine whether the reduction in DNA break repair caused by Nutlin is due to a delay in DNA damage signaling that occurs when Mdm2 is overexpressed (10, 11), we evaluated γH2AX foci formation and resolution. H2AX is rapidly phosphorylated (γH2AX) upon DNA damage and returns to an unphosphorylated form upon repair (17). Assessment of γH2AX foci formation showed that 10 minutes following γ-radiation, p53−/− MEFs in the presence of Nutlin formed 31% fewer foci than vehicle-treated cells (Fig. 2A and Supplementary Fig. S1A). These results were comparable with cells overexpressing Mdm2 from a retroviral vector. At 150 minutes after γ-radiation, there were significantly increased numbers of γH2AX foci in the Nutlin-exposed MEFs compared with DMSO-treated controls, indicating a delay in foci resolution. To determine the role of Mdm2, we measured foci in p53−/− Mdm2−/− MEFs. When Mdm2 was not present, Nutlin no longer inhibited the formation or the resolution of γH2AX foci compared with DMSO-treated cells (Fig. 2B and Supplementary Fig. S1B). These results indicate that Nutlin treatment has a comparable inhibitory effect on γH2AX foci formation and resolution as Mdm2 overexpression, and this effect is independent of p53, but requires Mdm2.

γ-Radiation induces DNA breaks, which activates ATM, the DNA damage–induced kinase, that then phosphorylates motifs with a serine or threonine followed by a glutamine (S/TQ; ref. 18). Another means to measure DNA damage signaling is to evaluate these phosphorylated S/TQ (pS/TQ) sites. Following γ-radiation, pS/TQ foci were assessed in p53−/− MEFs cultured with Nutlin or vehicle control. Cells with Nutlin had 28% fewer foci immediately after γ-radiation, and 62% more foci at 240 minutes after γ-radiation compared with controls (Fig. S2A). When p53−/− Mdm2−/− MEFs were used, Nutlin had neither an impact on the formation nor the resolution of the pS/TQ foci (Fig. S2D and Supplementary Fig. S2B). In both cell types, pS/TQ foci formation and resolution was impaired when Mdm2 was ectopically overexpressed, as we previously reported (11). Therefore, Nutlin inhibits the initial DNA damage response signal leading to prolonged signaling, and this requires Mdm2 but is independent of p53.

Figure 3. Delayed DNA repair by Nutlin in ovarian cancer cells with inactivated p53. SKOV-3 (A), OVCAR-5 (B), and OVCAR-8 (C) ovarian cancer cell lines were cultured with Nutlin (10 μmol/L) or vehicle control (DMSO). Whole-cell protein lysates were evaluated by Western blot analysis. Following 5 Gy of γ-radiation, neutral comet assays were used to measure DNA breaks; each bar is an average of two to four independent experiments with a minimum of 40 cells analyzed for each sample in each experiment. A, *, P = 0.0073; **, P = 0.0047; B, *, P = 0.0047; **, P = 0.004; C, *, P = 0.004; **, P = 0.0073, Student t test.

Nutlin inhibits DNA break repair in ovarian cancer cells

Ovarian cancer is a difficult to treat malignancy with the aggressive high-grade serous ovarian carcinoma type harboring mutated or deleted p53 in >90% of the tumors (2). To determine whether Nutlin could increase levels of Mdm2 and inhibit DNA break repair in malignant cells that have inactivated p53, we assessed ovarian cancer cells that had either deleted or mutated p53. SKOV-3 ovarian cancer cells have deleted p53 and showed increased levels of Mdm2 following exposure to Nutlin (Fig. 3A). We then assessed the effect of Nutlin on their ability to repair damaged DNA by exposing the SKOV-3 cells to Nutlin or vehicle control, and performing comet assays following γ-radiation. Only 57.6% of the cells treated with Nutlin were able to repair their DNA damage within 90 minutes, compared with 74.5% of cells exposed to vehicle control (Fig. 3A).

We also evaluated ovarian cancer cells that harbor mutant p53, OVCAR-5 (insertion at codon 224) and OVCAR-8 (deletion of codons 126–132; ref. 19). Nutlin treatment led to increased Mdm2 protein levels in both cell lines (Fig. 3B and C). In addition, both lines exposed to Nutlin had significantly reduced DNA break repair at 60 and 90 minutes after γ-radiation (Fig. 3B and C). These results indicate that Nutlin treatment has a comparable inhibitory effect on γH2AX foci formation and resolution as Mdm2 overexpression, and this effect is independent of p53, but requires Mdm2.

γ-Radiation induces DNA breaks, which activates ATM, the DNA damage–induced kinase, that then phosphorylates motifs with a serine or threonine followed by a glutamine (S/TQ; ref. 18). Another means to measure DNA damage signaling is to evaluate these phosphorylated S/TQ (pS/TQ) sites. Following γ-radiation, pS/TQ foci were assessed in p53−/− MEFs cultured with Nutlin or vehicle control. Cells with Nutlin had 28% fewer foci immediately after γ-radiation, and 62% more foci at 240 minutes after γ-radiation compared with controls (Fig. S2A). When p53−/− Mdm2−/− MEFs were used, Nutlin had neither an impact on the formation nor the resolution of the pS/TQ foci (Fig. S2D and Supplementary Fig. S2B). In both cell types, pS/TQ foci formation and resolution was impaired when Mdm2 was ectopically overexpressed, as we previously reported (11). Therefore, Nutlin inhibits the initial DNA damage response signal leading to prolonged signaling, and this requires Mdm2 but is independent of p53.
results indicate that the Nutlin-induced increase in Mdm2 results in a delay in double-strand DNA break repair in ovarian cancer cells that contain deleted or mutated p53.

Delay of DNA damage signal in ovarian cancer cells caused by Nutlin

To assess the DNA damage signal in ovarian cancer cells, we evaluated γH2AX foci formation and resolution. Ten minutes after γ-radiation, there were 29% fewer γH2AX foci in the Nutlin-treated p53-deleted SKOV-3 cells compared with vehicle control (Fig. 4A and Supplementary Fig. S3A). At 240 minutes after γ-radiation, the Nutlin-treated SKOV-3 cells had 46% more foci remaining compared with DMSO-treated cells. Similar results were obtained with the p53-mutant ovarian cancer cell lines, OVCAR-5 and OVCAR-8 (Fig. 4B and C, respectively, and Supplementary Fig. S3B and S3C). Therefore, Nutlin inhibits DNA damage response signals in ovarian cancer cells with inactivated p53, analogous to what was observed in MEFs.

Inhibition of ovarian cancer cell expansion with combined treatment of Nutlin and genotoxic agents

Cisplatin, used in first-line therapy for ovarian cancer, is a genotoxic agent that causes DNA double-strand breaks (20). To determine whether inhibition in DNA repair by Nutlin would cooperate with genotoxic chemotherapeutic agents, we evaluated the combination effect of Nutlin and cisplatin. SKOV-3 cells were treated with Nutlin alone, cisplatin alone, or Nutlin and cisplatin in combination. SKOV-3 cells treated with 1 μmol/L of cisplatin alone or 10 μmol/L of Nutlin alone resulted in a maximum of 12.3% ± 0.43% reduction in cell growth at 72 hours compared with vehicle-treated control (Fig. 5A and Supplementary Fig. S4A). However, treatment with both 1 μmol/L of cisplatin and 10 μmol/L of Nutlin resulted in a 26.9% ± 0.62% reduction in cell growth at 72 hours compared with vehicle-treated cells (Supplementary Fig. S4A). This cooperative effect was more apparent between Nutlin and 5 μmol/L cisplatin, which combined inhibited growth by 51.5% ± 0.49%, whereas 5 μmol/L cisplatin alone only inhibited 35.0% ± 1.08% (Fig. 5A). Nutlin enhanced the negative effect of 5 μmol/L cisplatin on cell expansion over that of the 10 μmol/L cisplatin alone. Similar results were obtained with the OVCAR-5 and OVCAR-8–mutant p53 ovarian cancer cells (Fig. 5A and Supplementary Fig. S4A). These results indicate that pharmacologically increasing Mdm2 with Nutlin enhanced the lethality of cisplatin in ovarian cancer cells with inactivated p53.

Etoposide also results in double-strand DNA breaks and is used to treat relapsed ovarian cancer, following platinum resistance (5). We evaluated the combination effect of Nutlin with etoposide in SKOV-3, OVCAR-5, and OVCAR-8 ovarian cancer cell lines. For all three cell lines, treatment with either Nutlin or 1 μmol/L etoposide had a very slight effect on the expansion of the cells, but when combined, cell growth was significantly inhibited (35.9%–56.2%; Supplementary Fig. S4B). Similarly, when treated with Nutlin and 5 μmol/L etoposide, cells were inhibited by 60.2% to 75.3% compared with only 49% to 53% when 5 μmol/L etoposide alone was used (Fig. 5B). The effects of Nutlin with 5 μmol/L etoposide was similar to treating the cells with two to four times the amount of etoposide alone (Fig. 5B). Therefore, ovarian cancer cells treated with both Nutlin and etoposide were significantly impaired in their ability to expand, and this was more than the effect of either drug alone, indicating cooperation.

Lethal combination for ovarian cancer cells of Nutlin and genotoxic agents

To determine whether cell death caused the observed reduction in cell growth, SKOV-3, OVCAR-5, and OVCAR-8 ovarian cancer cell lines were cultured with Nutlin (10 μmol/L) or vehicle control (DMSO), and following γ-radiation, γH2AX foci were detected by immunofluorescence. The number of foci per cell was quantified. The mean of two or three independent experiments is graphed; error bars, SEM; and significance (*) determined using a confidence interval of 95% (Nutlin compared to DMSO).

Figure 4.

Nutlin inhibits DNA damage signaling in ovarian cancer cells. SKOV-3 (A), OVCAR-5 (B), and OVCAR-8 (C) ovarian cancer cell lines were cultured with Nutlin (10 μmol/L) or vehicle control (DMSO), and following γ-radiation, γH2AX foci were detected by immunofluorescence. The number of foci per cell was quantified. The mean of two or three independent experiments is graphed; error bars, SEM; and significance (*) determined using a confidence interval of 95% (Nutlin compared to DMSO).

Downloaded from mcr.aacrjournals.org on March 30, 2017. © 2015 American Association for Cancer Research.
Fig. S5) as well as Nutlin and etoposide (Fig. 6B and Supplementary Fig. S5) in comparison with either drug alone. These results suggest that the ovarian cancer cells are dying, but to test whether apoptosis was occurring, we assessed cleaved caspase-3. Very little or no cleaved caspase-3 was detected in Nutlin or vehicle control–treated cells (Fig. 6C and D). However, cleaved caspase-3 was readily apparent in ovarian cancer cells treated with the combination of Nutlin and 5 μmol/L cisplatin, and was similar to the amount of cleaved caspase-3 observed in cells treated with 10 μmol/L cisplatin alone (Fig. 6C). Moreover, Nutlin combined with 5 μmol/L of etoposide resulted in increased cleaved caspase-3 to levels similar to 10 μmol/L of etoposide alone (Fig. 6D). Comparable results were obtained in all three ovarian cancer cell lines with both genotoxic drugs (Fig. 6C and D). These results indicate that Nutlin has a cooperative effect with cisplatin and etoposide in ovarian cancer cells with inactivated p53, resulting in apoptosis.

One established method to measure effects of drugs on ovarian cancer cells at the DNA level is to evaluate γH2AX foci after a prolonged time (i.e., 24 hours) after removal of the genotoxic drug; this is a measure of damage remaining and indicative of cells that are likely to die (15). Using this method, we observed little change or an increased percentage of ovarian cancer cells that were γH2AX positive after incubation with Nutlin or cisplatin alone, respectively, but significantly more when cells were treated with both Nutlin and cisplatin (Fig. 6E). Analogous results were obtained when etoposide was used (Fig. 6F). Combined, the data indicate that inhibition of double-strand DNA break repair resulting from Nutlin-induced increased Mdm2 levels cooperates with genotoxic agents, such as cisplatin or etoposide, to cause apoptosis of ovarian cancer cells lacking functional p53.

**Discussion**

Since the development of Nutlin, scientists have been using it as a research tool to investigate the involvement of the p53 pathway in various settings and for determining the chemotherapeutic potential of activating p53 to treat cancer (6, 7). Although studies have extensively demonstrated p53-dependent properties of Nutlin, p53-independent effects of Nutlin have also emerged. One observed p53-independent effect of Nutlin is the increase in Mdm2 protein levels (6, 8), which our data indicate is due to
Nutlin and genotoxic agents are a lethal combination for ovarian cancer cells that have inactivated p53. The indicated ovarian cancer cell line was treated with the indicated drug(s) or vehicle control (DMSO). Nutlin was always at 10 μmol/L and cisplatin (A, C, and E) or etoposide (B, D, and F) was at 5 μmol/L. For C and D, 10 μmol/L cisplatin or etoposide, respectively, was also used. After 48 hours, representative pictures (A and B) were taken, and whole-cell protein lysates were evaluated by Western blot analysis (C and D) for cleaved caspase-3 and β-actin. E, immunofluorescence for γH2AX was performed 24 hours following removal of cisplatin or etoposide from SKOV-3 cells incubated with Nutlin or DMSO; error bars, SEM; *, P < 0.01, t test.
Mdm2 protein stabilization. We had previously shown that Mdm2 overexpression inhibits DNA break repair and promotes genome instability independent of p53 (10, 11). Here, our data reveal that stabilization of Mdm2 by Nutlin resulted in an inhibition in the DNA damage signal and delayed DNA break repair. This effect of Nutlin was dependent on Mdm2 and its interactions with Nbs1. When Nutlin was combined with genotoxic agents in ovarian cancer cells, a significant increase in DNA damage and apoptosis independent of p53 was induced. These results provide insight into a novel p53-independent, Mdm2-dependent mechanism of Nutlin. Importantly, our data show the therapeutic benefit of pharmacologically increasing Mdm2 in conjunction with genotoxic agents as a means of inducing synthetic lethality in cancers with inactivated p53, such as ovarian cancer.

Numerous studies have described a cooperative effect between Nutlin and genotoxic agents in the context of wild-type p53. Our data suggest that in addition to p53 activation, Nutlin is also stabilizing Mdm2 causing an increase in the levels of Mdm2, which dampens the p53 response to DNA damage, resulting in increased sensitivity to genotoxic drugs (21, 22). A small number of studies have also explored the effect of Nutlin in combination with genotoxic drugs in cancer cells with mutated or deleted p53, but the mechanism for the observed cooperation between the two in this context was poorly understood. For example, a cooperative effect between Nutlin and topoisomerase II inhibitors in pancreatic cancer cells with inactivated p53 has been described previously (23). They reported Nutlin caused an increase in DNA damage response signals 3 hours following genotoxic insults. Consistent with this, we observed an increase in DNA damage response signals 4 hours after γ-radiation, but we also detected a reduction in the early DNA damage response signal. Our results indicate that Nutlin delays the DNA damage signal leading to more DNA damage signaling at later times after the genotoxic drug. Another study proposes that Nutlin cooperates with cisplatin and doxorubicin by activating a p53 family member, p73, leading to E2F1 activation to induce apoptosis (24–27). Mdm2 can bind and regulate p73, and both p73 and E2F1 can be activated following DNA damage (28). Our data established that in the absence of functional Nbs1, Nutlin was unable to inhibit double-strand DNA break repair. Thus, if p73 and E2F1 are involved, it appears that they do not contribute to the delay in DNA break repair induced by Nutlin.

Half of all human cancers have directly inactivated p53 and many cancers that progress or relapse from therapies have inactivated p53 (1). Therefore, there is much need for identifying new chemotherapeutic approaches for those malignancies that have inactivated p53, such as ovarian cancer. Current standard chemotherapeutic treatment for ovarian cancer involves the platinum-based genotoxic agents cisplatin or carboplatin (4). Our results reveal Nutlin used in combination with cisplatin increases the efficacy of cisplatin, showing that in the presence of Nutlin, 2-fold lower concentrations of cisplatin are effective. Relapse in ovarian cancer often results from platinum resistance and requires the need to use combination therapy with other genotoxic agents, such as etoposide (5). Our data demonstrate that Nutlin also cooperates with etoposide to increase its efficacy, resulting in 2- to 4-fold lower concentrations of etoposide being equally effective. Our results have significant therapeutic implications, as they reveal that pharmacologically modulating Mdm2 levels could be used to more effectively treat malignancies that have inactivated p53, as well as allow lower concentrations of toxic chemotherapeutic drugs to be used. This study also emphasizes the importance of assessing compounds that lead to increased Mdm2 levels for therapeutic purposes. Moreover, because increasing Mdmx levels also leads to an inhibition of DNA break repair (14), compounds that stabilize Mdmx or both Mdm2 and Mdmx are also likely to have similar results.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.M. Carrillo, C.M. Eischen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Carrillo, M. Hicks, D. Khabele, C.M. Eischen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Carrillo, M. Hicks, D. Khabele, C.M. Eischen
Writing, review, and/or revision of the manuscript: A.M. Carrillo, M. Hicks, D. Khabele, C.M. Eischen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.M. Eischen
Study supervision: D. Khabele, C.M. Eischen

Acknowledgments
The authors thank Pia Arrate, Robert Duszyński, Kristin Peterson, Jeanette Saskowski, and Andrew Wilson for technical assistance.

Grant Support
This work was supported by F31CA150546 (to A.M. Carrillo), R01CA181204 (to C.M. Eischen), T32CA093240 (to M. Hicks), and K08CA148887 (to D. Khabele).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 17, 2015; revised May 1, 2015; accepted May 2, 2015; published OnlineFirst May 11, 2015.

References
Molecular Cancer Research

Pharmacologically Increasing Mdm2 Inhibits DNA Repair and Cooperates with Genotoxic Agents to Kill p53-Inactivated Ovarian Cancer Cells

Alexia M. Carrillo, Mellissa Hicks, Dineo Khabele, et al.

Mol Cancer Res Published OnlineFirst May 11, 2015.

Updated version

Access the most recent version of this article at:

doi:10.1158/1541-7786.MCR-15-0089

Supplementary Material

Access the most recent supplemental material at:

http://mcr.aacrjournals.org/content/suppl/2015/05/12/1541-7786.MCR-15-0089.DC1

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.