Radiosensitization of lung cancer by nutlin, an inhibitor of murine double minute 2

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Abstract

p53 plays a critical role in cell cycle arrest and induction of apoptosis. Certain malignancies carry wild-type p53, which is frequently down-regulated by murine double minute 2 (MDM2) overexpression. Availability of a small-molecule inhibitor against MDM2, nutlin, has made it feasible to evaluate the anti-MDM2-based therapeutic strategies. The rationale for the current study is that functional p53 has been linked with improved responses to radiation treatment. Hence, this study evaluates the use of nutlin, a small-molecule inhibitor that blocks the interaction of p53 and MDM2, in sensitizing cancer cells to radiation. Expression of MDM2, p53, and p21 in both p53 wild-type and p53-defective lung cancer cell lines was examined. Clonogenic and 7-amino-actinomycin D studies were used to determine possible mechanisms of cell death. The combined effect of MDM2 inhibition and radiation on cell cycle was also studied. We found that radiosensitization by nutlin occurs in lung cancer cells with wild-type p53. There were increased apoptosis and cell cycle arrest following administration of nutlin and radiation. Furthermore, the combination of nutlin and radiation decreased the ability of endothelial cells to form vasculature, as shown by Matrigel assays. Our data suggest that nutlin is an effective radiosensitizer of p53 wild-type cells. The radiosensitizing effect seems to be at least partially due to induction of apoptosis and cell cycle arrest. In addition, nutlin may be an effective radiosensitizer of tumor vasculature. [Mol Cancer Ther 2006;5(2):411 – 7]

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Introduction

Murine double minute 2 (MDM2) is a protein that binds the tumor suppressor p53, repressing its transcriptional expression and promoting its degradation. p53 is a transcription factor that protects cells from transformation through the mechanisms of cell cycle arrest and apoptosis. These functions of p53 are attenuated by a negative feedback loop with MDM2 (1). MDM2 expression suppresses p53-induced cell cycle arrest, apoptosis, and response to DNA damage (2–5). Therefore, increasing p53 activity through inhibition of MDM2 is a novel therapeutic strategy for cancer treatment.

The role of functional p53 in controlling cancer is important as approximately half of all human tumors have dysfunctional p53 (6). In addition, decreased levels of p53 have been shown to increase resistance to the cytotoxic effects of chemotherapy or radiotherapy (7–9). Accordingly, MDM2 inhibition could be an effective approach to enhance cancer therapy. Recently, small-molecule antagonists of MDM2 have been developed. Nutlin has been shown to activate the p53 pathway in cells and suppress tumor growth in animal models (10).

DNA damaging agents such as radiation activate the p53 pathway, resulting in cell cycle arrest and apoptosis (3, 7). Because MDM2 attenuates the activity of p53, MDM2 inhibition has been suggested as a mechanism to increase radiosensitivity. Using antisense oligonucleotides, MDM2 inhibition has been shown to be an effective method of radiosensitization (11, 12).

The purpose of the present study is to analyze the combined effects of nutlin, a potential small-molecule therapeutic agent for cancer therapy, and radiation on lung cancer cells and vascular endothelial cells. Changes in the p53 pathway, cell cycle, and apoptosis were examined following the combined therapy. To determine whether the interaction between nutlin and radiation is p53 dependent, cell lines that are p53 wild-type and p53 mutant were studied. We found that nutlin enhanced the cytotoxic effects of radiation through a p53-dependent mechanism.

Experimental Procedures

Cell Culture and Drug Treatment. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (Walkersville, MD) and maintained in EBM-2 medium supplemented with EGM-2 MV single aliquots (BioWhittaker, Walkersville, MD). H460 lung carcinoma cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin. Val138 cells were obtained from Fox Chase Cancer Center and maintained in DMEM medium with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.8 gm/L of G418. Val138 cells were grown at 39°C to induce the expression...
of the p53 mutant. Nutlin 3a possessed potent MDM2 binding activity; in contrast, the enantiomer nutlin 3b is 150 times less active than 3a (Hoffmann-La Roche, Inc., Nutley, NJ). Nutlins were dissolved in DMSO and kept as 10 mmol/L stock solutions in small aliquots at −80°C. Irradiation was given by use of a 137Cs irradiator (J.L. Shepherd and Associates, Glendale, CA).

Western Immunoblots

Cells were incubated with 8 μmol/L nutlin 3a and 3b for 8 hours as described by Grunbaum et al. (11), then rinsed and irradiated with either 0 or 5 Gy. Cells were collected and protein extracts were made. Equal amounts of protein were loaded into each well and separated by 10% SDS-PAGE gel. The blots were then incubated with antibodies against MDM2 (Oncogene, Boston, MA), p53 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), and p21 (1:1,000; Santa Cruz Biotechnology) overnight at 4°C. Immunoblots were developed by using the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) according to the protocol of the manufacturer and autoradiography.

In vitro Clonogenic Assay

H460 or Val138 cells were diluted serially to appropriate concentrations and plated out into 25-cm² flasks in 5 mL medium in triplicate. Cells were treated with 3 μmol/L nutlin 3a and 3b and then immediately irradiated with 0 to 6 Gy as indicated. Cells were incubated for 48 hours before nutlins were washed off. Cells were irradiated using a 137Cs irradiator (J.L. Shepherd and Associates) at room temperature. The dose rate was 1.8 Gy/min and dose range was 0 to 6 Gy. After treatment, cells were returned to 37°C or 39°C and maintained for 8 days. Cells were then fixed for 15 minutes with 3:1 (methanol/acetic acid) and stained for 15 minutes with 0.5% crystal violet (Sigma, St. Louis, MO) in methanol. After staining, colonies were counted by the naked eye with a cutoff of 50 viable cells.

Measurement of Apoptosis

7-Amino-actinomycin D is a fluorescent DNA-binding stain usually used as a single agent to detect apoptotic cells by flow cytometry. Percent apoptosis was measured by using 7-amino-actinomycin D (Molecular Probes, Eugene, OR) with flow cytometry. Cells were treated with 1 μmol/L nutlin 3a and 3b for 48 hours. Cells, 1 × 10⁶, were centrifuged and stained in PBS plus 7-amino-actinomycin D. They were then fixed in PBS plus 1% paraformaldehyde and analyzed by FACSscan. Cells with intermediate levels of 7-amino-actinomycin D staining were scored as apoptotic.

Cell Cycle Analysis

Cells were labeled with 20 μmol/L bromodeoxyuridine (Sigma) and fixed with 70% ethanol. Anti-bromodeox- yuridine FITC-conjugated monoclonal antibody (20 μL; Becton Dickinson, San Jose, CA) was added and incubated for 30 minutes in the dark at room temperature. Bromodeoxyuridine incorporation was analyzed by dual-color FACSscan. Cell number in each phase of the cell cycle was determined and calculated as a percentage of the total cell population.

Endothelial Cell Morphogenesis Assay: Tube Formation

HUVECs were treated with nutlins for 8 hours. Medium was changed and cells were treated with 3 Gy of γ irradiation. Cells were trypsinized and counted. They were seeded at 48,000 per well on 24-well plates coated with 300 μL of Matrigel (BD Biosciences, San Jose, CA). These cells undergo differentiation into capillary-like tube structures and were periodically observed by microscope. One day later, cells were stained with H&E and photographs were taken via microscope. The average number of tubes for three separate microscopic fields (100×) and representative photographs were taken.

Results

Response to Nutlin 3a–Induced MDM2 Inhibition Is Dependent on p53 Status

To determine how tumors with different p53 status respond to nutlin in the absence and presence of radiation treatment, expression levels of MDM2, p53, and p21 were determined by Western blot analysis. Cells were incubated with DMSO vehicle (untreated control), nutlin 3b, or 3a for 8 hours and were subsequently irradiated with 0 or 5 Gy. As shown in Fig. 1A, H460 (p53 wild-type) cells treated with nutlin 3b showed little to no induction of MDM2, p53, or p21 expression. In H460 cells treated with 5 Gy, there was a slight increase in MDM2, p53, and p21 expression. However, H460 treated with nutlin 3a plus or minus radiation had significant induction of all three proteins. In contrast, protein levels in p53-defective Val138 cells showed a relatively constant level of expression of MDM2, p53, and p21 across all four treatment groups as shown in Fig. 1B.

Radiosensitization of p53 Wild-Type Lung Cancer Cells by Nutlin

To further determine how nutlin and radiation affect cancer cell survival, clonogenic assays were done using both H460 and Val138 lung cancer cells. H460 cells were treated with vehicle (DMSO), nutlin 3b, or nutlin 3a, followed by radiation doses from 0 to 6 Gy, as shown in Fig. 2A. H460 treated with either vehicle or nutlin 3b showed no difference in colony-forming ability across the various radiation doses. However, H460 cells treated with nutlin 3a plus or minus radiation had significant induction of all three proteins. In contrast, protein levels in p53-defective Val138 cells showed a relatively constant level of expression of MDM2, p53, and p21 across all four treatment groups as shown in Fig. 1B.

p53 Wild-Type Lung Cancer Cells Treated with Nutlin 3a and Radiation Have Increased Levels of Apoptosis

To determine the mechanism of cell death that contributes to decreased cell viability and colony-forming ability following nutlin and radiation, apoptosis was measured...
untreated control. H460 cells treated with nutlin 3b and Mol Cancer Ther 2006;5(2). February 2006

14% of cells. Val138 cells were kept at 39°C to induce apoptosis in the greatest increase in apoptosis was seen in cells treated from 2% at baseline to 6% and 4%, respectively. However, showed an increase in the proportion of apoptotic cells Fig. 3A, H460 cells treated with 2 Gy or nutlin 3a alone apoptotic cells among treatment groups. As shown in apoptotic cells. Shown in Fig. 3 are the percentages of Gy. Flow cytometry was used for quantification of with vehicle, nutlin 3b, or nutlin 3a, followed by 0 or 2 Gy. The percentage of cells in each stage treatment, HUVEC cells were studied. Western blots probed for MDM2, p53, and p21 showed that the response of HUVEC was similar to that of H460 cells. There was little MDM2, p53, or p21 expression in response to nutlin 3b alone; however, there was a slight induction of expression of all three proteins in response to radiation. Treatment with nutlin 3a induced a robust expression of all three proteins and the addition of 5 Gy to nutlin 3a treatment resulted in a slight increase in expression of MDM2 and p53, although p21 levels seemed to be relatively constant, as shown in Fig. 5A.

Nutlin 3a Is an Effective Radiosensitizer of Vascular Endothelial Cells

To determine how normal tissue and, specifically, endothelial cells respond to nutlin 3a and radiation treatment, HUVEC cells were studied. Western blots probed for MDM2, p53, and p21 showed that the response of HUVEC was similar to that of H460 cells. There was little MDM2, p53, or p21 expression in response to nutlin 3b alone; however, there was a slight induction of expression of all three proteins in response to radiation. Treatment with nutlin 3a induced a robust expression of all three proteins and the addition of 5 Gy to nutlin 3a treatment resulted in a slight increase in expression of MDM2 and p53, although p21 levels seemed to be relatively constant, as shown in Fig. 5A.

To further study the effects that nutlin 3a may have on vasculature, Matrigel assays were used. HUVEC cells were plated, grown to confluence, and treated with one of the nutlins plus or minus 5 Gy. The cells were then allowed to form tubular structures, which is indicative of endothelial cell mobility. As shown in Fig. 5B, HUVEC treated with nutlin 3a and 5 Gy had markedly reduced capacity of forming vascular tubules.

Discussion

It has previously been shown that cytotoxic stimuli, such as radiation, activate p53 pathway, which leads to the expression of p21 and other downstream targets, resulting in cell cycle arrest and apoptosis (3, 7, 13, 14). Similarly, recent studies with novel small-molecule inhibitors of MDM2 increased expression of p53 and p21, inducing cell cycle arrest and apoptosis (1, 10, 15). This may be important because decreased levels of p53 and p21 in malignancies have been shown to increase cancer cell resistance to the cytotoxic effects of chemotherapy or radiotherapy (3, 16–19). Under normal conditions, p53 expression is controlled by MDM2 through feedback inhibition (8, 20). MDM2 expression suppresses p53, attenuating p53 functions in cell cycle arrest, apoptosis, and response to DNA damage (2, 4, 9, 21). Because p53 is involved in multiple cell death pathways, there is still some disagreement about how defective p53 affects radiosensitivity (5, 22, 23).

Ionizing radiation activates p53, causing cell cycle arrest

by 7-amino-actinomycin D staining. Cells were treated with vehicle, nutlin 3b, or nutlin 3a, followed by 0 or 2 Gy. Flow cytometry was used for quantification of apoptotic cells. Shown in Fig. 3 are the percentages of apoptotic cells among treatment groups. As shown in Fig. 3A, H460 cells treated with 2 Gy or nutlin 3a alone showed an increase in the proportion of apoptotic cells from 2% at baseline to 6% and 4%, respectively. However, the greater increase in apoptosis was seen in cells treated with both nutlin 3a and 2 Gy, which induced apoptosis in 14% of cells. Val138 cells were kept at 39°C to induce expression of mutant p53. They were treated in an analogous fashion as the H460 cells described above. There was no appreciable difference in apoptosis as shown in Fig. 3B.

Nutlin 3a–Induced MDM2 Inhibition Results in Greater Cell Cycle Arrest

To determine if inhibition of MDM2 with nutlin 3a affects progression through the cell cycle, both H460 and Val138 cells were treated with vehicle, nutlin 3b, or nutlin 3a, followed by 0 or 2 Gy. The percentage of cells in each stage of the cell cycle was then determined using flow cytometry. Results shown in Fig. 4A indicate that H460 cells treated with nutlin 3b alone had 51.8% of cells in S phase, 34.1% of cells in G0-G1, and 17.1% of cells in G2-M, similar to the untreated control. H460 cells treated with nutlin 3b and radiation showed a marked decrease in the percentage of cells in S phase (26.9%) and an increase in the number of cells in G0-G1 (53.7%) and G2-M phase (19.4%). Nutlin 3a alone showed a decrease in percentage of cells in S phase (23.6%). However, the proportion of cells in G0-G1 (70.6%) was greater with a slight decrease in the percentage of cells in G2-M (5.8%). In H460 cells treated with nutlin 3a and radiation, the percentage of cells in S phase (1.25%) dropped to the lowest among all groups. A marked increase in the percentage of cells in G2-M phase (36.6%) was detected as compared with other groups. However, in similarly treated Val138 cells, there was no appreciable difference in the percentage of cells in each phase of the cell cycle across the two controls and four treatment groups as shown in Fig. 4B.

Molecular response to radiation and nutlin in lung cancer cells. Figure 1. Molecular response to radiation and nutlin in lung cancer cells. H460 (A) and Val138 (B) lung cancer cells were treated with nutlin 3a, nutlin 3b, or vehicle (untreated controls). The treated cells were concurrently irradiated with either 0 or 5 Gy. Twenty-four hours later, the treated cells were collected and analyzed by Western blotting. β-Actin was probed to show equal loading of protein extracts.
and apoptosis. On the other hand, ionizing radiation also activates MDM2 in a p53-dependent fashion, which attenuates p53-induced cell cycle arrest and apoptosis (1, 24, 25). MDM2 activation may play a critical role in preventing uncontrolled cell death caused by p53 in response to radiation (26). MDM2 inhibits p53 activity by binding to p53 to block its ability to activate transcription; additionally, it facilitates the export of p53 from the nucleus and promotes its degradation (10, 27, 28). Previous studies have suggested that restoring the balance between p53 and MDM2, either by inhibiting MDM2 activity or restoring p53 function by transfection with wild-type p53, can enhance radiosensitivity (11). Because of its ability to increase p53 expression, nutlin may have a role in radiosensitization.

In the present study, the effect of p53 status on radiosensitivity was studied using p53 wild-type and p53 mutant lung cancer cells. Although Val138 cells can resume its wild-type p53 status when cultured at 32°C, these cells are difficult to maintain beyond 24 hours and undergo cell death. Therefore, H460 cells were used as a model for p53 intact cancer cells. Because H460 and Val138 may be genetically variable, the genetic differences other than p53 status may also contribute to their different response to nutlins and radiation.

The present study is the first study to show the combined effects of nutlin and radiation on p53, p21, and MDM2 levels and how these changes may affect cell cycle arrest, cell viability, and apoptosis. Nutlin 3 has two enantiomers: 3a, the active form, and 3b, which is 150 times less active. We found an increase in MDM2, p53, and p21 levels in irradiated H460 cells, which is consistent with previous studies (3, 29). H460 cells treated with nutlin 3a showed a robust induction of MDM2, p53, and p21. This is expected as MDM2 inhibition leads to decreased degradation of p53, resulting in increased expression of downstream p21. p53 increases the expression of MDM2 by binding to two p53 responsive elements located on the MDM2 gene, promoting MDM2 transcription (20, 30, 31). Antisense oligonucleotides for MDM2 have shown similar results in lung cancer cells (32). On the other hand, treatment with nutlin 3a causes no appreciable change in MDM2, p53, or p21 expression in the Val138 cells. These results are expected as MDM2 inhibition would not exert its p53-dependent effects in this p53 mutant cell line, and hence no effects on downstream p21 should be observed. A high basal level of p53 has been shown to be common in p53-defective cell lines and this may be causing the decrease in the baseline expression of MDM2 (10). These findings suggest that MDM2 inhibition and radiation effects are greatly attenuated in p53 mutant lung cancer cells even with combined therapy.

Although no significant change of p53, p21, and MDM2 levels was detected in H460 cells treated with nutlin 3a in the presence or absence of radiation, apoptosis was found to be much higher in the combined group and the cell cycle analysis also showed marked differences. Therefore, other molecular mechanisms may contribute to these differences at the cellular level.

Cell survival examined by clonogenic assay and cell death examined by staining the apoptotic cells indicated that the radiosensitization by nutlin is p53 dependent. This is in agreement with previous studies that have suggested that p53-defective cells tend to be radio-resistant (33–35). Recent studies to determine the effects of MDM2 inhibition on apoptosis have used terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling staining to show that after 48 hours of exposure to nutlin 3a, terminal deoxyribonucleotidyl

**Figure 2.** Radiosensitization of p53 wild-type lung cancer by nutlin. H460 (A) and Val138 (B) cells were treated with nutlin 3a, nutlin 3b, or vehicle and then immediately irradiated at indicated doses. Forty-eight hours later, medium was changed and cells were incubated for 2 wks. The radiation dose enhancement ratio was calculated as the dose (Gy) for radiation alone divided by the dose (Gy) for radiation plus drugs (normalized for drug toxicity) for a surviving fraction of 0.25. Points, mean of three independent experiments; bars, SE. Dose enhancement ratio for H460 cells was 1.68 and plating efficiency for untreated cell was 75%. Each radiation dose in each group was done in triplicate and colonies of 50 cells were then counted. Points, mean of three counts; bars, SD.
transferase–mediated dUTP nick end labeling–positive staining was found in 45% of SJSA-1 cells (10). The amount of apoptosis is far greater than that seen in the present study in response to MDM2 inhibition. However, the present study addresses the combined effect of radiation and MDM2 inhibition, and therefore a lower dose of nutlin was selected for this study to show the potential synergistic effect of combined therapy. This may be the reason for the difference in the percentage of apoptotic cells between the two studies. Furthermore, recent studies have suggested that the radiosusceptibility of the tissue from which a tumor arises may play an important role in its apoptotic response to radiation. Thus, the use of different tumor cell lines may also explain the difference in apoptotic response (36, 37).

Another major effect of p53 activation in proliferating cells is induction of cell cycle arrest in G₁ and G₂. Previous studies have shown that p53 activation leads to G₁ and G₂ cell cycle arrest, and it seems that p21 plays a major role in inducing this arrest (38). How nutlin-induced inhibition of MDM2 affects the cell cycle has been investigated in previous studies by Vassilev et al. They have shown that in p53-competent cell lines, nutlin induces G₁ and G₂ cell cycle arrest with a decreased fraction of cells in S phase. In p53-defective cell lines, no G₁ and G₂ arrest was observed, indicating that nutlin affects the cell cycle through a p53-mediated pathway. p53 is also known to play an important role in regulating cell cycle arrest in response to radiation (36). p53 status has been shown to be important in predicting whether tissues respond to radiation, through apoptosis or cell cycle arrest, and in directing DNA repair through interactions with ataxia telangiectasia mutated and checkpoint kinase 2 (36). In the present study, H460 cells treated with nutlin 3a showed a

![Figure 3](image-url)

**Figure 3.** Nutlin 3a sensitizes H460 cells (p53 wild-type) to radiation by increasing apoptosis. Lung cancer cells were incubated with nutlin 3a, nutlin 3b, or vehicle as indicated in the histograms. Following this, cells were irradiated with either 0 or 2 Gy. Forty-eight hours later, cells were stained using 7-amino-actinomycin D and the percentage of apoptotic cells was determined by flow cytometry in H460 (A) and Val138 cells (mutant p53; B).

![Figure 4](image-url)

**Figure 4.** Alteration of cell cycle following treatment of nutlin and radiation. Lung cancer cells were treated with nutlin 3a, nutlin 3b, or vehicle, with or without radiation (2 Gy), followed by incubation with 20 μmol/L of bromodeoxyuridine. Staining with propidium iodide/anti-bromodeoxyuridine antibody was used to determine cell cycle distribution. Columns, mean percent of cells in each phase of the cell cycle for the various treatment groups for H460 (A) and Val138 (B) lung cancer cells. Each group was done in triplicate.
significant decrease in cells in S phase and an increase in G2-M cells. This may translate into radiation sensitization by reducing radioresistant cells in S phase and increasing radiosensitive cells in G2-M.

Radiation increased the proportion of cells in G0-G1, as well as cells in G2-M, whereas nutlin 3a predominantly increased the number of cells in G0-G1 phase. These findings are not unexpected in that previous studies have shown that radiation or nutlin can cause both G0-G1 and G2-M cell cycle arrest; however, the main effect of radiation is through DNA damage and the resulting activation of DNA repair enzymes has been linked to G2 cell cycle arrest (10, 12, 36, 39–41). Hence, it is possible that radiation could induce a greater G2 arrest when combined with nutlin as compared with nutlin used as a monoagent. This may also explain why there is a greater cytotoxic effect in response to nutlin 3a with radiation compared with nutlin 3a alone, although the difference in expression of p53 is minimal in these two treatment groups. It may be that the overall response of p53 is roughly equivalent in the two treatment groups but that radiation induces greater cell cycle arrest due to increased DNA damage, resulting in the greater reduction in cell viability seen in the 7-ADD and clonogenic assays. The present study also showed no significant change in the proportion of p53-defective Val138 cells in each phase of the cell cycle across all four treatment groups, which is in agreement with previous studies (10).

In addition to studying the effects of nutlin 3a and radiation on lung cancer cell lines, this study attempted to address these treatment effects on host endothelial cells. Previous studies have investigated the effects of nutlin 3a on normal host tissues in human and mouse fibroblasts. These studies have found that nutlin 3a induced decreased proliferation of both lines of fibroblasts, but these cells retained their viability up to a week later, implying a cytostatic, rather than cytotoxic, effect of MDM2 inhibition on normal host cells (10). The present study is the first to look at the effects of nutlin on normal endothelial cells, which is p53 competent, and how nutlin 3a effects on vascular endothelium may be augmented by radiation. This is consistent with our study using antisense approach (42). MDM2 inhibition may have antiangiogenic effects because of its induction of p53 expression, which has been shown to induce the expression of angiogenesis inhibitors, such as thrombospondin-1, and to decrease the expression of vascular endothelial growth factor (43–47).

In conclusion, MDM2 inhibitors have great potential as a monotherapy and as a tumor and vascular radiosensitizer. However, the cytotoxic and antiangiogenic effects of nutlin are limited to p53 wild-type cells. Other defects in p53 pathway may also decrease the efficacy of nutlin. Tumor vasculature presents an attractive target due to its homogeneous expression of wild-type p53. On the other hand, because normal tissues are also p53 intact, minimizing normal tissue toxicity through the precise delivery of radiation will be of great importance to minimize radiosensitization of normal host tissues. Therefore, further studies in animal models may provide additional information about the feasibility of combining radiotherapy and nutlins.

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Figure 5. Effects of nutlin 3a and radiation on vasculature. HUVEC cells were treated with nutlin 3a, nutlin 3b, or vehicle, followed by either 0 or 5 Gy. A, 24 h later, HUVECs were collected and analyzed by Western blot. Western blots from the two control and four treatment groups probed for MDM2, p53, p21, and β-actin. B, HUVEC cells were treated the same way. Cells were trypsinized and plated on 24-well plates coated with Matrigel. After 6 h, cells were fixed and stained with H&E. The slides were examined by microscopy (×100). Stained tubules were then counted in three separate, randomly selected fields; representative fields. Columns, mean; bars, SD.


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