Radiosensitization by Antisense Anti-MDM2 Mixed-Backbone Oligonucleotide in in Vitro and in Vivo Human Cancer Models

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ABSTRACT

Purpose: The MDM2 oncogene, amplified or overexpressed in many human cancers, has been suggested to be a novel target for cancer therapy. We have demonstrated a second-generation antisense antihuman-MDM2 oligonucleotide to have antitumor activity when administered alone or in combination with cancer chemotherapeutic agents. In the present study, we investigated the effect of the antisense oligonucleotide on radiation therapy.

Experimental Design: The in vitro radiosensitization activity was determined in cell lines of human cancers of prostate (LNCaP and PC3), breast (MCF-7 and MDA-MB-468), pancreas (PANC-1), and glioma (U87-MG and A172) and its in vivo radiosensitization activity in xenograft models of LNCaP, PC3, MCF-7, MDA-MB-468, and PANC-1.

Results: In cells containing at least one functional p53 allele (LNCaP, U87-MG, and A172), after specific inhibition of MDM2 expression, p53 and p21 levels were elevated. In LNCaP cells, the Bax level was increased, and Bcl-2 and E2F1 levels were decreased. In PC3 cells that are p53 null, after inhibition of MDM2 expression, Bax and p21 levels were elevated, and E2F1 levels were decreased. On the basis of in vitro clonogenic assay, the antisense oligonucleotide, in a sequence-specific manner, significantly increased radiation-induced antiproliferation effects. It also increased radiation-induced inhibitory effects on tumor growth in SCID or nude mice bearing LNCaP, PC3, MCF-7, MDA-MB-468, and PANC-1 xenografts.

Conclusions: These results suggest that MDM2 has a role in radiation therapy of human cancers, regardless of p53 status, providing a basis for future development of MDM2 inhibitors, such as antisense oligonucleotides, as radiosensitizers.

INTRODUCTION

The MDM2 oncogene was first cloned as an amplified gene on a murine double-minute chromosome in the 3T3DM cells (1). The MDM2 protein contains a p53-binding domain at the NH2 terminus, nuclear localization signal, central acidic domain, and three COOH-terminal zinc-finger motifs (Ref. 1 and reviewed in Ref. 2). MDM2 overexpression confers the tumorigenicity (1, 3). The connection between MDM2 and cancer has also been shown in human cancers (2, 4, 5). The MDM2 gene is amplified and/or overexpressed in a number of human tumors, including human cancers of breast (6–9), prostate (10), pancreas (11), and glioma (12–14). More importantly, MDM2 amplification and/or overexpression have been indicated as a diagnostic factor in cancer patients (8–17). In addition, studies have demonstrated that MDM2 overexpression is associated with advanced cancer disease states, such as invasive (18–20) and high-grade/late stage tumors (21–23), recurrence (24, 25), and metastasis (26–30).

In general, mutations in MDM2 and P53 genes do not occur within the same tumor, and most MDM2 amplification-positive tumors have wild-type p53, indicating overexpression of MDM2 is a means of inactivating p53 (2, 4, 5). The expression of MDM2 is induced by p53 (31), and MDM2 binds to p53 with high affinity and inhibits its transcriptional activity (32), indicating that MDM2 functions as a negative feedback regulator of p53. In addition, MDM2 overexpression abrogates the ability of p53 to induce cell cycle arrest and apoptosis (33, 34). MDM2 also enhances the degradation of p53 (35, 36), suggesting that it can regulate p53 functions through multiple mechanisms. The MDM2-p53 interaction is modulated by the tumor suppressor ADP ribosylation factor (p14ARF; Refs. 37 and 38).

It has been demonstrated that many cancer therapeutic agents exert their cytotoxic effects through activation of wild-type p53. However, the activation of p53 by DNA damage after cancer chemotherapy and radiation treatment may be limited in cancers with MDM2 expression, especially those with MDM2 overexpression. Therefore, inactivation of the MDM2-negative feedback loop may increase the magnitude of p53 activation after DNA-damaging treatment, thus enhancing the therapeutic effectiveness of DNA-damaging drugs and radiation therapy.

The MDM2 oncoprotein has been shown to have p53-independent activity. MDM2 binds to and interacts with pRB (39), E2F1 (40), ribosomal protein L5 (41), and RNA (42). The biological consequences of these activities are not clear but may be associated with transforming properties of MDM2. In addi-
Radiosensitization by Antisense Oligonucleotides

**MATERIALS AND METHODS**

MDM2 has been shown to be responsible for drug resistance and radioresistance of human cancers (48–57), which may be associated with the increase in MDR-1 expression (50), loss of p53 function (55, 56), and transforming growth factor-β1 resistance (57). Therefore, inhibition of p53-independent activity of MDM2 may be as important as the inactivation of its p53-dependent activity. It is also possible to overcome some drug resistance in tumors with dysfunctional p53 by MDM2 inhibition. Recently, we have successfully designed anti-MDM2 antisense oligos that inhibit MDM2 expression in tumor cells *in vitro* and *in vivo* (58–65). Antitumor activity of the antisense oligo has been shown in various human cancer models, including sarcoma (59), breast cancer (58, 60, 61), colon cancer (64), prostate cancer (65), and glioma (63) after administration of the oligo alone or in combination with cancer chemotherapeutic agents, such as 5-fluorouracil (61), camptothecins (61, 64), and paclitaxel (63, 65). The present study was undertaken to determine whether the antisense oligo has radiosensitization activity in both *in vitro* and *in vivo* human cancer models and to characterize the underlying mechanisms.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Minimum Essential Medium Eagle with Earle’s salts, DMEM, Ham’s F-12, RPMI 1640, trypsin, HEPES buffer, sodium pyruvate, nonessential amino acids, and trypsin were acquired from Mediatech (Herndon, VA). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). PBS was purchased from the Comprehensive Cancer Center Media Preparation Shared Facility of the University of Alabama at Birmingham (Birmingham, AL). Lipofectin was purchased from Life Technologies (Rockville, MD). Anti-β-actin, anti-p53, anti-p21, antiretinoblastoma, anti-Bcl-2, anti-Bax, and anti-β-actin at 1:5000 dilution and anti-p53, anti-p21, antiretinoblastoma, anti-Bcl-2, anti-Bax, and anti-E2F1 at a 1:1000 dilution and anti-β-actin at 1:5000 dilu.

**Antisense Oligonucleotides.** The anti-MDM2 antisense oligonucleotide, Oligo AS, a 20-mer mixed-backbone oligonucleotide (5'-UGACACCTGGTCTCCAC'3'), and its mismatch control (Oligo ASM, 5'-UGACACCTGGTCTCCCTAC-3') were synthesized, purified, and analyzed as described previously (61, 66). Two nucleosides at the 5'-end and four nucleosides at the 3'-end are 2'-O-methylribonucleosides (represented by boldface letters); the remaining are deoxynucleosides. The italicized nucleosides of Oligo ASM are the sites of the mismatch controls compared with Oligo AS. For both mixed-backbone oligos, all internucleotide linkages are phosphorothioate. The purity of the oligos was shown to be >99% by capillary gel electrophoresis and PAGE, with the remainder being n-1 and n-2 products (61). The integrity of the internucleotide linkages was confirmed by 31P NMR.

**Cells and Culture.** The human prostate cancer cell lines LNCaP and PC3, the breast cancer cell lines MCF-7 and MDA-MB-468, glioma cell lines U87-MG and A172, and pancreatic cancer cell line PANC-1 were obtained from the American Type Tissue Culture Collection (Rockville, MD) and cultured as per American Type Tissue Culture Collection protocol. All culture media contained 10% fetal bovine serum and 1% penicillin-streptomycin. LNCaP cells were cultured with RPMI 1640 supplemented with 4.5 grams/liter glucose, 1% L-glutamine, 1.5 grams/liter sodium bicarbonate, 1% HEPES buffer, and 1% sodium pyruvate. The medium for PC3 consisted of Ham’s F-12 medium, supplemented with 1.5 grams/liter sodium bicarbonate, and 1% L-glutamine. U87-MG cells were cultured with Essential Medium Eagle with Earle’s salts, supplemented with 1% sodium pyruvate, and 1% nonessential amino acids. The medium for A172 consisted of DMEM supplemented with 4.5 grams/liter glucose. MCF-7 cells were cultured with Essential Medium Eagle with Earle’s salts, supplemented with 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10 μg/ml bovine insulin; MDA-MB-468 cells were cultured with DMEM:F12 media (50:50), supplemented with 10% fetal bovine serum. PANC-1 cells were cultured with RPMI 1640 containing 1 mM HEPES buffer, 25 μg/ml gentamicin, 1.5 grams/liter sodium bicarbonate, and 0.25 μg/ml amphotericin B.

**In Vitro Radiation Treatment and Cell Survival Assay.** Human cancer lines MCF-7, MDA-MB-468, PANC-1, A172, LNCaP, or PC3 were plated at varying cellular concentrations and transfected with oligonucleotides in the presence of Lipofectin (7 μg/ml). After 24 h, cells were placed in a 66Co Picker unit irradiator (1.56 Gy/min) and exposed to γ-irradiation. Immediately after irradiation, serum-low media (used for oligo transfection) were replaced with normal medium-containing serum. After 8–14 days, fixative (one part glacial acetic acid: seven parts methanol) was added to the media for 10 min. The fixative/media mixture was discarded and replaced with sufficient fixative to cover each plate for 15 min. Plates were then washed twice, with water and deionized water, and exposed to crystal violet (0.2 grams/liter) for 20 min. After a final wash, the colonies on each plate were counted with 50 cells being the requirements for scoring as a colony. Relative levels of cell survival were calculated by comparison with control without radiation (e.g., AS alone versus AS + 4 Gy).

**Western Blot Analysis.** The protein levels of MDM2, p53, p21, Bcl-2, Bax, E2F1, and β-actin were assessed using methods described previously (58, 61–65). In *in vitro* studies, cells were incubated with Oligos AS or ASM at various concentrations for 24 h, in the presence of Lipofectin (7 μg/ml), followed by γ-irradiation. Cell lysates, collected at various times, were fractionated with identical amounts of protein by SDS-PAGE and transferred to Bio-Rad trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose membrane was then incubated in blocking buffer (Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk) for 1 h at room temperature. The membrane was then incubated with the appropriate primary antibody (anti-MDM2, anti-p53, anti-p21, antiretinoblastoma, anti-Bcl-2, anti-Bax, and anti-E2F1 at a 1:1000 dilution and anti-β-actin at 1:5000 dilu.
tion) overnight at 4°C or 2 h at room temperature with gentle shaking. The membrane was then washed thrice with the washing buffer (Tris-buffered saline containing 0.1% Tween 20) for 15 min and then incubated with 1:3000 diluted goat antimouse IgG-horseradish peroxidase-conjugated antibody (Bio-Rad) for 1 h at room temperature. After repeating the washes in triplicate, the protein of interest was detected by enhanced chemilumines-

### RESULTS

#### Oligo AS Modified the Expression of Multiple Proteins

*In Vitro Oligo Chemistry and Radiation Treatment.* Mice bearing LNCaP, PC3, MCF-7, MDA-MB-468, or PANC-1 xenografts were randomly divided into multiple treatment groups in addition to a control group (five mice per group). Oligo AS or ASM dissolved in sterile physiological saline (0.9% NaCl) was given by i.p. injection (volume, 5 μl/gm body weight) at a dose of 25 mg/kg, five times per week. Mice in radiation groups were first anesthetized with a 70–100-μl mixture of ketamine (20 mg/ml) and xylazine (20 mg/ml) at a 6:7:1 ratio and then placed under a specially designed lead shield so that only the tumors were exposed to the radiation beam. γ-Irradiation was administered by a 60Co Picker unit irradiator (1.56 Gy/min; JL Shepard Co., Glendale, CA). Animals received 3 Gy of radiation twice for 1 week (LNCaP), four times for 1 week (PC3), or three times on days 2, 4, and 9 (MCF-7, MDA-MB-468, and PANC-1). Mice in oligo/radiation combination groups were pretreated with oligo 4 h before γ-irradiation.

#### Data Analysis

To quantify the density of protein bands in Western blot analysis, all values were expressed as a percentage of Lipofectin control and normalized by the corresponding β-actin levels. To illustrate the synergistic effects between antisense oligonucleotides and radiation therapy in vitro, cell survival data were analyzed by using a computer program (Cellsur 2.4), provided by Dr. J.A. Bonner, to fit the raw data (67). Tumor monitoring and body weight data were expressed as mean and SD of tumor mass, and the significance between various treatment groups were analyzed by ANOVA.

### Animal Xenograft Model

The models of LNCaP, PC3, MCF-7, MDA-MB-468, and PANC-1 xenografts were established using the protocol described previously (61–65). MCF-7, MDA-MB-468, and PANC-1 models used female athymic nude mice (nu/nu, 4–6 weeks old). For the LNCaP in vivo model, male SCID mice (4–6 weeks old) were used, and male athymic nude mice (nu/nu, 4–6 weeks old) were used for the PC3 model. All mice were obtained from Frederick Cancer Research and Development Center (Frederick, MD). Cultured cells were washed with serum-free media and resuspended in the same medium. This suspension (5 × 106 cells, 0.2 ml/mouse) was then injected into the left inguinal area of the mice. BMMx was combined with this suspension before injection at a ratio of 1:1 (LNCaP) or 1:5 (PC3, MCF-7, MDA-MB-468, and PANC-1). The mice were monitored by general clinical observation as well as by body weight and tumor growth. Tumor growth was recorded with the use of calipers by measuring the long and short diameters of the tumor. Tumor mass (in grams) was calculated using the formula 1/2a2b, where “a” and “b” are the long and short diameters (in centimeters), respectively.

#### Oligo AS Modified the Expression of Multiple Proteins after in Vitro Combination Treatment with γ-Irradiation.

The effects of antisense antihuman-MDM2 oligo AS on the levels of several proteins were first determined in prostate cancer LNCaP (Fig. 1, A and B) and PC3 cells (Fig. 1C). The
cells were treated either with 25 nM (LNCaP) or 50 nM (LNCaP and PC3) of Oligo AS or ASM for 24 h followed by γ-irradiation (10 Gy) using a 60Co Picker unit irradiator (1.56 Gy/min) and then incubated for ≤24 h. As shown in Fig. 1A, in LNCaP cells that contain wild-type p53, the MDM2 level was elevated in a time-dependent manner by irradiation alone with its peak at 4 h. On the basis of the results from two test levels of Oligo AS, in a dose-dependent manner, the antisense agent significantly inhibited radiation-induced MDM2 levels [Fig. 1A; MDM2 (I) and MDM2 (II) at the concentrations of 25 nM (I) and 50 nM (II) Oligo AS, respectively]. Oligo ASM had no effect, demonstrating a sequence-specific effect of Oligo AS. The levels of p53 in LNCaP cells were increased modestly after radiation treatment. In contrast, the levels of p53 in LNCaP cells treated with 25 or 50 nM Oligo AS were significantly increased in a dose- and sequence-specific manner (Fig. 1A). The levels of p21 and Bax were also increased after radiation treatment, which were significantly augmented by Oligo AS but not affected by Oligo ASM treatment (Fig. 1B). The levels of Bcl-2, an antiapoptotic protein, were initially increased and then decreased after radiation treatment. The control oligo ASM had no effect on Bcl-2 levels. Oligo AS inhibited Bcl-2 levels, and no significant increase was observed after radiation in the cells treated with Oligo AS. The levels of E2F1 were increased at 4 h after radiation, and Oligo ASM had no effect. Oligo AS almost completely inhibited E2F1 (Fig. 1B).

As illustrated in Fig. 1C, in PC3 cells that are p53 null, after a transient decrease, the MDM2 level was elevated in a time-dependent manner by irradiation alone with its peak at 24 h. At 50 nM, Oligo AS almost completely inhibited radiation-induced MDM2 levels. Oligo ASM had no effect, further demonstrating the specificity of Oligo AS. The levels of p21 and Bax were increased after radiation treatment, which were significantly enhanced by Oligo AS but not affected by Oligo ASM treatment. There was no significant change in the levels of Bcl-2 in all cells, regardless of treatment. The levels of E2F1 were increased after radiation. Although Oligo ASM had no effect, Oligo AS significantly inhibited E2F1.

The selected protein expression profiles after radiation were further studied in human glioma cells, U87-MG (p53 wild type), and A172 (p53 heterozygous). As shown in Fig. 2A, in U87-MG cells, after a transient decrease, the MDM2 level was elevated in a time-dependent manner by γ-irradiation alone with its peak at 4 h. At 50 nM, Oligo AS significantly inhibited radiation-induced MDM2 levels. Oligo ASM had no effect, further demonstrating a sequence-specific effect of Oligo AS. As seen with LNCaP cells, the levels of p53 and p21 were increased after radiation treatment, which were significantly augmented by Oligo AS but not Oligo ASM treatment. Similar responses were seen with A172 cells that contain one functional p53 allele (Fig. 2B). The MDM2 level was elevated by irradiation alone with its peak at 4 h. At 50 nM, Oligo AS significantly inhibited radiation-induced MDM2 levels, and the levels of p53 and p21 were significantly increased compared with cells treated with radiation alone or in combination with Oligo ASM. Oligo ASM had no effect, further demonstrating a sequence-specific effect of Oligo AS.

**Oligo AS Sensitized Human Cancer Cells to Radiation Therapy in Vitro.** The in vitro effects of Oligo AS on the antitumor activity of radiation treatment were analyzed by clonogenic assay. As illustrated in Fig. 3, the cell survival was decreased by γ-irradiation in a dose-dependent manner in all cell lines analyzed (Fig. 3A, LNCaP; B, PC3; C, MCF-7; D, MDA-MB-468; E, A172; F, PANC-1). At 10 and 25 nM, Oligo AS treatment significantly increased sensitivity of all of the cell lines to radiation by ~4–60-fold, depending on cell types (Fig. 3; Table 1). The mismatch control Oligo ASM had minimal or no radiosensitization effects, suggesting a sequence-specific mechanism of Oligo AS.

**Oligo AS Sensitized Human Cancer Cells to Radiation Therapy in Vivo.** The in vivo effects of Oligo AS on the antitumor activity of radiation treatment were determined by using human cancer xenograft models. Radiosensitization effects of Oligo AS were first tested in human prostate cancer LNCaP and PC3 models (Fig. 4, A and B; Table 2). The control Oligo ASM alone had minimal effects on tumor growth compared with the controls treated with saline, further confirming the specificity of Oligo AS. In LNCaP model, Oligo AS alone or radiation treatment (RT; 3 Gy/day, days 2 and 3) alone showed antitumor activity. After combination treatment with Oligo AS and radiation, significant synergistic inhibitory effects on tumor growth were observed (P < 0.01; Table 2). Oligo ASM showed no effects on radiation-induced tumor growth inhibition. More interestingly, after cessation of the oligo treatment, the tumor growth remained inhibited in animals with combination treatment of Oligo AS and RT (Fig. 4A). Similar radiosensitization effects by Oligo AS were observed in the PC3 model (Fig. 4B). Oligo AS but not the control Oligo ASM alone showed significant antitumor activity compared with the controls treated with...
Fig. 3 Cell survival after Oligo AS or ASM pretreatment and radiation. Cells lines of human prostate cancer LNCaP (A) and PC3 (B), breast cancer MCF-7 (C) and MDA-MB-468 (D), glioma A172 (E), and pancreatic cancer PANC-1 (F) were transfected with Oligo AS or ASM for 24 h, irradiated at various doses (0–10 Gy), and then cultured for 8–14 days. Cells were fixed and stained, and colonies containing >50 cells were then counted. Values were expressed as a fraction of corresponding control ± SE of three separate experiments in triplicates.

Table 1 Radiosensitization effects of oligo AS based on in vitro clonogenic assays

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<th>Cell line</th>
<th>p53 Status</th>
<th>RT&lt;sup&gt;b&lt;/sup&gt; dose</th>
<th>Survival fraction</th>
<th>Relative ratio&lt;sup&gt;a&lt;/sup&gt;</th>
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<td></td>
<td></td>
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<td>0.0019</td>
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<tr>
<td>Glioma (A172)</td>
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<td>0.0017</td>
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<td>Pancreas (PANC-1)</td>
<td>mt</td>
<td>5 Gy</td>
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<td>0.0030</td>
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* The relative ratio of cell survival fractions ([1]/[2] or [1]/[3]) can be used to illustrate the potential improvement on therapeutic effectiveness of radiation therapy when cells are pretreated with Oligo AS or ASM, compared with radiation therapy alone. When the ratio is >1 with statistical significance, a positive radiosensitization effect is indicated.

<sup>b</sup> RT, radiation treatment.

<sup>c</sup> P < 0.01.

<sup>d</sup> P < 0.05.
saline (P < 0.01). RT alone also showed significant antitumor activity (P < 0.01), which was augmented by combination treatment with Oligo AS (P < 0.01; Table 2). Oligo ASM showed no effects on radiation-induced tumor growth inhibition (Table 2).

Radiosensitization effects of Oligo AS were further investigated in human breast and pancreatic cancer models. In the MCF-7 model, Oligo AS or RT alone showed significant antitumor activity, and the combination of both treatments showed synergistic effects (P < 0.01; Fig. 4C; Table 2). Oligo ASM showed minimal effects on radiation-induced inhibition on tumor growth. (Fig. 4C). The MDA-MB-468 model was sensitive to both Oligo AS and RT treatments alone. Combination of both treatments showed additive effects (Fig. 4D; Table 2). The control Oligo ASM showed no effect on tumor growth when used alone and had no effect on radiation-induced antitumor activity (Fig. 4D; Table 2). In the PANC-1 model, Oligo AS or RT showed limited effects on tumor growth. Combination of both treatments, however, significantly inhibited tumor growth (P < 0.01; Fig. 4E; Table 2).

Combination Treatment with RT and Oligonucleotides Had No Significant Impact on Host Toxicity. To test whether the inhibition of MDM2 and activation of p53 would increase host toxicity after combination therapy, we also monitored clinical toxicity of the treated animals. As illustrated in Fig. 4, the oligos did not significantly alter the host toxicity profile of radiation therapy, as monitored by body weight measurements in tumor-bearing mice (Fig. 4F, LNCaP; G, PC3; H, MCF-7; I, MDA-MB-468; J, PANC-1).

DISCUSSION

In our laboratories, we have been interested in developing novel gene-based cancer therapy, with an emphasis on antisense
technology. Antisense oligos have been shown to be unique research tools in the study of the regulation of gene expression and gene functions (68) and potential therapeutic agents based on rational gene-based drug design (69–72). Antisense oligos may achieve their effects by targeting mRNA with which they can hybridize and specifically block protein expression. Several antisense oligos targeted to various genes, such as Bcl-2, c-myb, protein kinase A, protein kinase C, and C-raf, have been shown to have antitumor effects in clinical trials (69–72). With encouraging results from preclinical studies with anti-MDM2 oligos in various human cancer models (58–65), in the present study, we investigated the role of MDM2 in radiation therapy and potential value of the antisense oligonucleotide as a radiosensitizer.

In the present study, we have demonstrated at least four significant results: (a) the novel anti-MDM2 mixed-backbone oligonucleotide, Oligo AS, specifically blocked radiation-induced MDM2 elevation, resulting in changes in multiple protein levels, such as p53 activation and p21 elevation in cancer cells that express wild-type p53. In prostate cancer LNCaP cells, increase in Bax and decreases in Bcl-2 and E2F1 were also

Table 2  Radiosensitization effects of oligo AS in nude mice bearing human cancer xenografts

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<tr>
<td>Pancreas (PANC-1)</td>
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<td>71</td>
<td>23</td>
<td>65</td>
<td>309d</td>
<td>109</td>
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a T/C (%), percentage of mean tumor mass of treated group compared with the control group treated with saline.

b The relative ratio ([2]/[3] or [2]/[4]) can be used to illustrate the potential improvement on therapeutic effectiveness when AS or ASM was given in combination with radiation therapy compared with radiation therapy alone. When the ratio is >100% with statistical significance, an effect of the oligonucleotide is indicated.

c RT, radiation treatment.

d P < 0.01.

e P < 0.05.
observed. In PC3 cells that are p53 null, increase in Bax and decrease in E2F1 were also observed; (b) in a sequence specific manner, in vitro radiosensitization effects were observed in several tumor cell lines, regardless of p53 status; (c) Oligo AS significantly increased the therapeutic effectiveness of radiation therapy in five human cancer xenograft models, regardless of p53 status; and (d) Oligo AS did not increase host toxicity associated with radiation therapy. These results provide a basis for future investigations of MDM2 inhibitors as a radiosensitizer.

The role of MDM2 in radiation therapy has not been fully understood. As a negative regulator of p53, MDM2 plays an important role in tumor formation and growth. In tumors with wild-type p53, MDM2 binds to and interacts with p53, negatively regulating its transcriptional function (5, 32). On DNA damage treatment, p53 is activated, resulting in apoptosis, cell cycle arrest, and facilitated DNA repair. Several downstream genes, such as p21, Bax, and MDM2, are activated by p53. Elevated MDM2, in return, inhibits p53 function, forming an autoregulatory feedback loop (5, 32), which limits the therapeutic effectiveness of DNA-damaging treatment, such as radiation therapy. Therefore, blocking MDM2 elevation will be a powerful tool to maintain functional p53 at higher levels after irradiation. In the present study, we provide direct evidence supporting this hypothesis. Results from cells expressing wild-type p53 demonstrated that MDM2 levels were elevated after radiation and inhibition of MDM2 resulted in significant increases in radiation-induced p53, p21, and Bax levels. Interestingly, Bcl-2, an antiapoptotic protein, was shown to be elevated initially and then decreased after radiation. MDM2 inhibition resulted in continued Bcl-2 inhibition. E2F1, a potent facilitator of DNA synthesis by activating the expression of a number of genes involved in S phase, has been shown to be activated by MDM2 directly (73). In the present study, E2F1 was elevated after radiation, presumably associated with MDM2 activation. Antisense inhibition of MDM2 resulted in abrogation of E2F1 expression. These results are in agreement with in vitro and in vivo radiosensitization effects of MDM2 inhibition in several human cancer models.

The results from the present study further demonstrated that MDM2 has p53-independent activity. In in vitro and in vivo studies with the PC3 model that is p53 null, inhibition of MDM2 expression resulted in decreased survival fraction in vitro and increased sensitivity to irradiation in vivo. The elevation of p21 and Bax and reduction of E2F1 were also observed, which is independent of p53 status. There are several reports demonstrating p21 induction independent of p53 by various stimuli (10, 74, 75), p21, a potent inhibitor of cyclin-dependent kinases, is a negative regulator of the cell cycle, inducing either p53-mediated G1 arrest or apoptosis. Induction of p21 correlates with inhibition of G2 transition (75). Lack of p21 expression has been shown to correlate with poorer clinical outcome in patients with bladder, colon, and heptocellular cancers, and down-regulation of it was shown to be involved in the development of androgen independence in prostate cancer (76). In the present study, we have shown p21 induction was both p53 dependent and independent, which is consistent with our previous reports (61–65). Although the underlying mechanisms are not clear, the role of MDM2 in p21 regulation is indicated. Additional studies to examine the relationship and interaction between p21 and MDM2 are under way.

Results from the present study also demonstrated MDM2 inhibition resulted in Bax activation, regardless of p53 status. It is well documented that the expression of Bax is enhanced at the transcriptional level by p53 (76). However, Bax can be induced by p53-independent mechanisms, such as transforming growth factor-β1 (77), prostate secretory protein (78), and proteasome inhibitors (79). Bax is an important mediator of p53-dependent apoptosis and a suppressor of oncogenic transformation (80), with its decreased expression leading to protection of radiation-induced apoptosis (81). Therefore, activation of Bax as seen in the present study, regardless of p53 status, may partially account for radiosensitization effects of the anti-MDM2 oligonucleotide. More importantly, it is believed that the relative ratio between Bcl-2: Bax and the formation of their heterodimers ultimately determine the susceptibility of a cell to undergo apoptosis (82). In the present study, we showed that MDM2 inhibition resulted in not only Bax activation but also Bcl-2 inactivation, which favors the Bcl-2:Bax ratio toward apoptosis.

In the present study, E2F1 was shown to be activated by radiation, and MDM2 inhibition resulted in blockade of E2F1 elevation induced by irradiation, regardless of p53 status. In addition to promoting cell cycle progress, E2F1 was also shown to be correlated with increased tumor cell invasiveness and metastatic progression (83). In addition, it has been reported that radiation-induced cell cycle arrest and cell death were inhibited by the transfection of the E2F1 gene (84). Therefore, reduction of radiation-induced E2F1 levels may be in part responsible for the radiosensitization effects of MDM2 inhibition. In summary, our study demonstrated that inhibition of MDM2 expression is a potentially effective radiosensitizer in human cancer in vitro and in vivo. Several possible underlying mechanisms, including p53-dependent and -independent pathways, were indicated. This study provides a basis for broad therapeutic applicability of the MDM2-based antisense approach in modulating cancer therapy.

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Radiosensitization by Antisense Anti-MDM2 Mixed-Backbone Oligonucleotide in *in Vitro* and *in Vivo* Human Cancer Models


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