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ANTISENSE-MDM2 SENSITIZES LNCaP PROSTATE CANCER CELLS TO ANDROGEN DEPRIVATION, RADIATION, AND THE COMBINATION *IN VIVO*

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Abstract

Purpose—To test the effects of antisense (AS)-MDM2 alone and with androgen deprivation (AD), radiotherapy (RT), and AD + RT on wild-type LNCaP cells in an orthotopic *in vivo* model.

Methods—Androgen-sensitive LNCaP cells were grown in the prostates of nude mice. Magnetic resonance imaging–based tumor volume and serum prostate-specific antigen (PSA) measurements were used to assess effects on tumor response. Tumor response was measured by biochemical and tumor volume failure definitions and doubling time estimates from fitted PSA and tumor volume growth curves. Expression of MDM2, p53, p21, and Ki-67 was quantified using immunohistochemical staining and image analysis of formalin-fixed tissue, analogous to methods used clinically.

Results—Antisense-MDM2 significantly inhibited the growth of LNCaP tumors over the mismatch controls. The most significant increase in tumor growth delay and tumor doubling time was from AS-MDM2 + AD + RT, although the effect of AS-MDM2 + AD was substantial. Expression of MDM2 was significantly reduced by AS-MDM2 in the setting of RT.

Conclusions—This is the first *in vivo* investigation of the effects of AS-MDM2 in an orthotopic model and the first to demonstrate incremental sensitization when added to AD and AD + RT. The results with AD underscore the potential to affect micrometastatic disease, which is probably responsible for treatment failure in 30–40% of men with high-risk disease.

Keywords

Antisense-MDM2; Prostate cancer; *In vivo* tumor model; Radiation; MRI

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INTRODUCTION

The reasons for studying MDM2 as a target are many. MDM2 is an oncogene, is overexpressed in 30–40% of prostate cancers (1–3), regulates p53 expression through a negative feedback loop involving p53 ubiquitination (4–7), is a negative regulator of p21waf1/cip1 independently of p53 (8), with p53 is a key regulatory protein in responses to genotoxic stress (9–12), and mediates ubiquitination and proteolysis of the androgen receptor (13,14) and E2F1 (15). In addition, p53 has been implicated in the apoptotic response of prostate epithelial cells to androgen deprivation (AD) (16–20). These multiple direct and indirect effects suggest that the suppression of MDM2 would enhance the response of prostate cancer cells to local treatment with radiation and systemic treatment with AD.

Mixed backbone antisense oligonucleotides offer a means for the highly specific targeting of genes that contribute to the dysregulation of tumor cells. An effective method for ablating MDM2 expression is through antisense-MDM2 (AS-MDM2) (21–26). Prior studies from our group have shown that AS-MDM2 + AD results in increased apoptosis over that seen from these treatments given individually, as well as from mismatch (MM) controls (27). We have also reported that AS-MDM2 sensitizes cultured prostate cancer cells to AD + radiotherapy (RT), a combination frequently used to treat intermediate- and high-risk clinically localized prostate cancer.

In the study reported here, we tested whether AS-MDM2 will sensitize androgen-sensitive prostate cancer cells to AD and/or RT in an orthotopic *in vivo* model. Use of an orthotopic model is essential in prostate cancer because of tumor–stromal interactions (28,29), which are particularly important to consider when treatments in combination with AD are being evaluated. This is the first *in vivo* investigation of the effects of AS-MDM2 on response to AD. If AS-MDM2 sensitizes prostate cancer cells to AD, then it has the potential to affect micrometastatic disease, which is probably responsible for treatment failure in 30–40% of men with high-risk disease.

METHODS AND MATERIALS

Antisense oligonucleotides

The antisense-MDM2 oligonucleotide (AS-MDM2) and its mismatch control oligonucleotide (MM) are 20-mer mixed-backbone oligonucleotides with the following sequence: AS-MDM2: 5'-UGACACCTGTTCTCACUCAC-3'; MM: 5'-UGTCACCCTTT TTCATUCAC-3'. The oligonucleotides were provided by Idera Pharmaceuticals (Cambridge, MA). They were stored as frozen aliquots at –20°C. Second-generation, mixed-backbone oligonucleotides of this type have an elimination half-time of more than 2 days, with detectable intact oligonucleotide in the blood for more than 12 h (30,31).

Cell culture and transfection

LNCaP cells were cultured in Dulbecco's modified Eagle's medium F12 medium, containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin–streptomycin (complete medium), as described previously (32). LNCaP-MST cells are a clonal cell line derived by stable transfection of LNCaP cells with the neomycin-selectable pCMV-mdm2 expression plasmid (G418; Life Technologies, Gaithersburg, MD) (27).

Animal tumor model

Male athymic nude mice (4–8 weeks old) were purchased (Harlan, Indianapolis, IN). All injections and surgeries were performed according to methods approved by the institutional animal care and use committee. Aseptic techniques were used for injections and implantation

of prostate tumor cells in the prostates of nude mice (33). Nude mice were anesthetized with methoxyflurane. A lower midline incision was made approximately 1.5 cm above the presumed location of the bladder. Tumor cells (5×10^5 to 3×10^6 in $24 \mu\text{L}$) were injected into the dorsal prostate lobes using a 30-gauge 1-in needle. The experiments described were carried out with LNCaP cells. Attempts to grow MDM2-overexpressing LNCaP-MST cells (27,34) orthotopically as well are described in the Results section.

Experimental design

Beginning at 3 weeks after orthotopic implantation of cells, weekly tail vein bleedings to obtain $70 \mu\text{L}$ of blood for serum prostate-specific antigen (PSA) measurements were initiated. When the PSA level was in the 3–8-ng/mL range, mice were assigned at random to one of eight groups. The MM and AS-MDM2 oligonucleotide treatments consisted of oligonucleotide alone, oligonucleotide + RT (5 Gy to the pelvis), oligonucleotide + AD, and oligonucleotide + RT + AD.

At this point we performed bilateral orchiectomy on the animals in the AD groups. The procedure was carried out under methoxyflurane anesthesia. After a transscrotal incision of the povidone-iodine-sterilized area, the testes were withdrawn from the scrotal sac and the vessels clamped. The vessels were tied off proximal to the clamps and the testes removed. The incision was sealed with clips. Injection of AS-MDM2 or MM at 25 mg/kg i.p. was performed once daily (5 days per week) for 15 days in $150 \mu\text{L}$ phosphate-buffered saline (see Results for details regarding determination of dose and length of treatment). On the 5th day of this treatment, animals assigned to receive RT received a single-fraction irradiation at 5 Gy (see Results for details regarding determination of the RT dose). For RT, the animals were restrained under anesthesia in the supine position with surgical tape in a jig to deliver radiation (Cesium 137 irradiator, model 81-14; J. L. Shepherd and Associates, San Fernando, CA) to the prostate while shielding the lungs, abdomen, and legs. Daily AS-MDM2 or MM injections (5 days/week) were continued for another 10 days. In the final experimental design, the oligonucleotides were injected for a total of 15 days over 3 weeks.

Magnetic resonance imaging

Tumor volumes by MRI and serum PSA levels were obtained weekly after the beginning of treatment. All imaging studies were performed in the Small Animal Imaging Facility at Fox Chase Cancer Center. Imaging was performed at a field strength of 7 T in a vertical wide-bore (10 cm) magnet using a Bruker DRX spectrometer (Bruker Biospin, Karlsruhe, Germany) with microimaging accessory, including a 3-cm-diameter birdcage coil. Animals were anesthetized with a mixture of 1% isoflurane in oxygen. Animals received an i.m. injection in the shoulder of 0.2 mL gadopentetate dimeglumine diethylenetriamine pentaacetic acid (Gd-DTPA) (Magnevist; Berlex Laboratories, Hamilton, NJ) diluted 10:1 with $1 \times$ phosphate-buffered saline. After recording a series of scout images in the axial orientation, a scan was proscribed in the coronal orientation for tumor detection and volumetric measurement. The number of slices recorded depended on the tumor volume and varied from 10 to 14. For tumors smaller than approximately 50 mm^3 , parameters for the coronal scan were as follows: slice thickness = 0.5 mm, repetition time = 400–600 ms (depending on the number of slices), echo time = 13.2 ms, in-plane resolution = 0.1 mm, field of view (FOV) = 2.56 cm (superior–inferior) $\times$ 1.28 cm (left–right), with four averages. Outer volume suppression was used to reduce the FOV and number of phase encode steps in the left–right direction. For larger tumors ($>50 \text{ mm}^3$), a coronal scan with a slice thickness of 0.75 mm, in-plane resolution of 0.2 mm, a square FOV of 2.56 cm , and two averages was used. The total scan time (excluding animal preparation) was approximately 10 min, allowing a skilled operator to scan 4 to 5 animals per hour. The procedure was well tolerated by the animals, with fewer than 1 in 500 examinations resulting

in animal mortality. Tumor volume (TV) was measured by standard planimetry using the Paravision software supplied with the DRX spectrometer.

General monitoring of the mice was performed daily. Mice showing signs of morbidity (cachexia, hunched posture, lethargy, abdominal fluid build-up, or tumor bulging/ulceration) were killed within 24 h. Animals were also killed if the MRI-assessed TV reached 200 mm³ and/or the serum PSA level exceeded 80 ng/mL. The mice were killed by CO₂ treatment. When removed, tumors were immediately excised and prepared by fixation in 10% formalin, 60% ethanol, or liquid nitrogen freezing for further analysis. The tumor volume (measured with calipers) and weight were recorded.

Gene expression by immunohistochemistry

A separate study was performed for the analysis of gene expression by immunohistochemistry. Mice were separated into two main categories, AS-MDM2 and MM, and further subdivided at random into three treatment groups: oligonucleotide alone, oligonucleotide + RT, and oligonucleotide + AD. Androgen deprivation by orchiectomy was initiated and after 3 days of oligonucleotide (AS-MDM2 or MM) administration was begun (daily i.p. injections at 25 mg/kg/day). Gamma irradiation to 5 Gy was administered to the RT group on the 9th day (after 7 days of AS-MDM2/MM) and tumor tissue removed on day 10. Treatment with oligonucleotide was for a total of 8 days. Control tissue was collected from tumors grown in untreated LNCaP mouse xenografts.

Tumor volume and PSA analysis

Tumor volume and PSA measurements were collected weekly for each mouse after initiation of treatment. The efficacy of the treatment was assessed by estimating failure according to definitions modeled after freedom-from-failure assessment in patients with prostate cancer. Two parameters were used: freedom from biochemical failure (FFBF) and freedom from tumor volume failure (FFTVF). Freedom from biochemical failure was defined as a PSA level of <1.5 ng/mL 6 weeks after treatment (33). Freedom from tumor volume failure was defined as an MRI-based TV of <30 mm³ 10 weeks after treatment.

Statistical analysis of freedom-from-failure data addressed two questions: (1) does AS-MDM2 result in an increased effect on tumor growth relative to MM? and (2) does AS-MDM2 result in an increased inhibitory effect when combined with AD, RT, or AD + RT? First, the outcomes from the experimental pairs MM and AS-MDM2 for each of the treatments (alone, RT, AD, AD + RT) were placed in 2 × 2 contingency tables and tested for significance using the chi-square test. This type of analysis was also used for the pooled AS-MDM2 and MM data. To address the second question, all possible pairs of treatment outcomes within a group (MM or AS-MDM2 by RT, AD, and AD + RT) were analyzed. The chi-square test was applied to determine the cases in which the treatment affected the freedom-from-failure rates significantly.

We also modeled analytically the longitudinal TV and PSA data to characterize tumor growth kinetics. Because tumor growth by serum PSA and TV are reasonably described by first-order rate kinetics, both in their decline after treatment and in their rise when it occurs, the serial PSA levels and TVs of each mouse were fitted to one of two exponential models. For each of the mice whose time series showed either straight growth or decline, the data were fitted to the following equation:

$$S(t)=S_0 \times \exp(R \times t),$$

where $S(t)$ is the TV or PSA level at time t , S_0 is the estimated baseline value at $t = 0$, and R is the rate of tumor/PSA growth ($R > 0$) or decline ($R < 0$). Prostate-specific antigen and TV doubling time is calculated as $\ln 2/R$ in cases when $R > 0$. In cases of a negative R , the doubling times for TV and PSA were assumed to be 100 days. For each mouse whose PSA level or TV declined or maintained a very slow growth pattern for the initial time period but then rose, the data were fitted to this equation:

$$S(t) = S_1 \times \exp(R_1 \times t) + S_2 \times \exp(R_2 \times t).$$

In this model, $(S_1 + S_2)$ is the estimated baseline TV or PSA value for $t = 0$. The first term of the equation describes the initial drop ($R_1 < 0$) or slow tumor growth ($R_1 > 0$), and the second term describes the subsequent rise. Doubling time in this model is defined as $\ln 2/R_2$.

Modeling the data allows calculation of a series of parameters related to TV and PSA, including baseline values, doubling times, and values at 6 and 10 weeks. Tumor volume and PSA values for animals with rapidly growing tumors, sacrificed before the 6th week, were assumed to be 500 mm³ and 125 ng/mL. Student's t test was applied to each of these parameters to compare MM and AS-MDM2 for each of the four treatments (alone, RT, AD, AD + RT).

Immunohistochemical analysis

Xenograft tumors were removed, fixed in formalin, embedded in paraffin, and 5- μ m sections cut onto the slides. The complete antigen retrieval and staining process has been previously described (3,35). The primary monoclonal antibodies used were MDM2 (clone IF2; Zymed, South San Francisco, CA), p21 (clone SX118; Dako, Carpinteria, CA), p53 (clone DO-7; Dako) and Ki-67 (Mib-1; Dako). All dilutions were at 1:100. For the secondary antibody incubation, a biotin/streptavidin (Dako LSAB II Kit) reaction, followed by diaminobenzidine chromogen (Research Genetics, Huntsville, AL) was used to identify stained areas in the cells. The counterstain was hematoxylin (Dako). The percentage of cells with nuclear staining was approximated using an image-analysis system (ACIS II; Clariant, San Juan Capistrano, CA). An individualized color threshold for brown (positive nuclei) and blue (negative nuclei) staining was set for each slide. Thirty random areas in the tissue were quantified using the 40 $\times$ magnification circular tool. A final sample mean percentage index was derived by the proprietary software.

RESULTS

In vivo pilot studies were carried out to optimize the experimental conditions. The rate of tumor formation from several amounts of LNCaP-MST and LNCaP (wild-type) cells was examined. When 3×10^6 LNCaP-MST cells were injected orthotopically, only 15.7% of the mice formed tumors detectable by MRI-based TV. In contrast, 74% of mice injected orthotopically with 5×10^5 LNCaP cells formed tumors. Consequently, the experiments focused on LNCaP cells (5×10^5 cells injected).

Experiments were carried out to determine the optimal RT dose. A total of 16 mice (4 per group) were treated with four different doses of radiation: 2.5, 5.0, 7.5, and 10.0 Gy. Single-fraction irradiation at 2.5 or 5.0 Gy resulted in no detectable effect on FFBF (0 of 4 free of biochemical failure in each group). The FFBF rates were 25% (1 of 4) and 50% (2 of 4) after 7.5 Gy and 10.0 Gy, respectively. These data suggest that 5.0 Gy causes a borderline undetectable response, which is desirable in testing for radiosensitization. Therefore, the subsequent *in vivo* experiments were carried out with this RT dose.

We also conducted pilot studies to optimize AS-MDM2 treatment for sensitization experiments. A total of 69 animals were treated with 12.5 mg/kg/day AS-MDM2 for 10 days, 5 days per week. This treatment alone resulted in no detectable effect on FFBF (0 of 13). Increases in FFBF were seen when AS-MDM2 was combined with 5.0 Gy RT (18%; 4 of 22), AD (33%; 5 of 15), and RT + AD (32%; 6 of 19). We did not think that these increases were sufficient and designed the larger experiment using 25 mg/kg/day of AS-MDM2 for a longer period (15 days, 5 days per week).

The larger experiment consisted of 94 mice, divided into 8 experimental groups. The time-related data collection resulted in a total of 812 MRI scans and 1013 PSA measurements. On average, 8.6 (range, 3–23) MRI scans and 10.8 (range, 3–27) PSA measurements were performed per mouse. The TV and PSA measurements were obtained for 3–27 weeks after completion of AS-MDM2 or MM treatment. The average TV and PSA baseline values were 14.8 mm³ and 4.0 ng/mL, respectively.

The relationships between tumor volume (measured with calipers) and tumor weight and serum PSA level are shown in Fig. 1a and b. There was a strong correlation between tumor weight and TV based on caliper measurements of the excised tumor ($R^2 = 0.98$, $p < 0.001$). Serum PSA level was also significantly correlated with caliper-based TV ($R^2 = 0.57$, $p < 0.001$), although the association was weaker than for that of tumor weight. Serum PSA level and tumor weight were also related ($R^2 = 0.57$, $p < 0.001$) (data not shown).

The relationship of MRI-based TV to caliper-based TV was also robust (Fig. 1c; $R^2 = 0.94$, $p < 0.001$). The correlation between tumor weight and MRI-based TV was high ($R^2 = 0.94$, $p < 0.001$) (data not shown). The association of MRI-based TV to serum PSA was significant but weaker (Fig. 1d, $R^2 = 0.67$, $p < 0.001$). The source of the variability in PSA levels was explored by separating the data into two groups: tumors with and without AD treatment. Androgen deprivation regulates PSA expression (36,37) and may, like other therapeutic methods, alter the relationship of PSA to TV (38,39). Figure 2a shows that AD does not affect the association between MRI and caliper-measured TV; however, the slope of the caliper-based TV to PSA plot is significantly lower ($p < 0.05$) in the setting of AD (Fig. 2b). To our knowledge these are the first data to document this relationship, which has clinical implications in terms of interpreting PSA levels in men with hormone-refractory disease. The changes in tumor volume in the MM and AS-MDM2 treated mice following AD and AD + RT treatments are shown in Fig. 3.

The results of the *in vivo* experiment examining the effect of AS-MDM2 are depicted in Table 1, where the findings of two endpoints (FFBF and FFTVF) are displayed. The pooled data from the two main oligonucleotide treatment categories (MM and AS-MDM2) are displayed in the first two rows. The number of mice free of failure more than doubled with AS-MDM2 relative to MM. The increases in freedom from failure for both endpoints were significant ($p < 0.05$). The outcomes of the individual treatments were also compared. The proportion of failure-free mice by both criteria (FFBF and FFTVF) was higher for AS-MDM2 relative to MM in all four pairs of comparisons (alone, AD, RT, AD + RT). For both endpoints, AS-MDM2 + AD + RT was significantly different from the corresponding MM control (Table 1). Likewise, AS-MDM2 + AD was significantly different from MM + AD for FFBF.

Other statistical comparisons are shown in Table 2. There was no statistically significant difference between AS-MDM2 + AD and AS-MDM2 + AD + RT, although in absolute terms, using FFBF and FFTVF endpoints (Table 1), 77% and 85% were free of failure when all three treatments were used, vs. 54% and 62% for AS-MDM2 + AD. The lack of statistical significance is probably related to the limited power of detecting such a difference with the number of mice used and events recorded. Two lines of evidence support the possibility that

AS-MDM2 + AD + RT has a greater effect than AS-MDM2 + AD. First, AS-MDM2 + AD + RT was more consistently significantly different from AS-MDM2 alone and AS-MDM2 + RT than was AS-MDM2 + AD, for both endpoints tested. Second, the addition of RT to AS-MDM2 + AD resulted in a considerable prolongation of MRI-based TV and serum PSA doubling times, from approximately 57 days to approximately 70 days (Table 3).

The fitted MRI-based TV and PSA measurements were analyzed using one of the two kinetic models described in Methods and Materials. Figure 4 displays examples of four different types of tumor and PSA kinetics with the corresponding fits. The data in Table 3 summarize the fitted tumor growth kinetic results, showing that the most dramatic effects were seen with AS-MDM2 + AD + RT. When these three treatments were administered together, MRI-based TV and PSA doubling times were significantly longer, calculated MRI-based TVs at 6 and 10 weeks were significantly lower, and calculated PSA levels at 6 and 10 weeks were significantly lower than for all of the other groups tested.

The expression of MDM2 and the key related apoptotic pathway proteins p53 and p21 were examined using immunohistochemistry. In addition, the proportion of cells expressing Ki-67 was determined because Ki-67 is a measure of the growth fraction (40,41), which is altered dramatically by AD (42) (Table 4). The most notable effect of AS-MDM2 was a reduction in MDM2 expression in the AS-MDM2 + RT group relative to the MM + RT group ($p < 0.04$). Although MDM2 levels were slightly lower from AS-MDM2 overall (2.1%), as compared with MM (3.0%), the reduction was not significant. There was not the expected increase in p53 from AS-MDM2 treatment, but this may be related to timing of the removal of the tumors relative to the start of AS-MDM2 treatment. Mismatch + RT reduced Ki-67, increased p53, and dramatically increased p21 over MM alone. Minimal changes in Ki-67 and p21 were seen when AS-MDM2 was added to RT, vs. MM + RT. Mismatch + AD resulted in a decrease in the proportion of cells staining positive for Ki-67, MDM2, p53, and p21, relative to MM alone. The addition of AS-MDM2 to AD did not alter these relationships.

DISCUSSION

The use of AD plus RT has emerged as one of the most common treatments for men with intermediate- to high-risk clinically localized prostate cancer. Randomized trials have shown that RT + AD improves survival over RT alone (43–46). The results, however, are far from optimal. Biochemical failure occurs in 30–50% over the long term, even when radiation doses are escalated (47). Failure after RT with or without AD seems to be related to both local persistence of disease (48) and metastasis (49). Newer therapeutic approaches are plainly needed. MDM2 as a target is an ideal candidate in that it has the potential to sensitize prostate tumor cells locally to RT + AD and distantly to AD.

MDM2 has a central role in the regulation of apoptosis. Prior studies have shown that knockdown of MDM2 using AS-MDM2 sensitizes cells *in vitro* and *in vivo* to chemotherapy and RT (50,51). Our prior results revealed that AS-MDM2 also sensitizes prostate cancer cells to AD, RT, and the combination *in vitro* (27,34). The experiments described here were designed to test whether AS-MDM2 has similar activity *in vivo*. We chose to test the efficacy of AS-MDM2, AD, and RT in all possible combinations in an orthotopic model so that the effects of tumor–stromal interactions would be incorporated into the overall findings. Another unique aspect of the experimental plan is the use MRI-based tumor volume and serum PSA level as measures of the effects on tumor response and kinetics.

Our results demonstrate that (1) AS-MDM2 has wide-ranging action in inhibiting orthotopically grown prostate cancer (LNCaP) cells, (2) AS-MDM2 significantly sensitizes androgen-sensitive prostate cancer cells to AD, and (3) further inhibition is seen when all three

agents, AS-MDM2 + AD + RT, are given together. A pooled analysis of all mice who received MM vs. those who received AS-MDM2 documented a consistent twofold or greater increase in freedom from failure (FFBF and FFTVF) tested (Table 1). It should be noted that the timing of the assessment for these endpoints is somewhat arbitrary but at least for biochemical failure is analogous to criteria used clinically. The FFBF endpoint was based on the classification of failure at 6 weeks after completion of therapy and is an endpoint we have described previously in this model system (33). We also examined FFBF at 10 weeks, and the results were similar. The FFTVF endpoint has not been described previously. The 10-week assessment was decided upon to allow time for tumor volume changes to become manifest. Similar results were evident at other time points and with other volume cutpoints.

The addition of AS-MDM2 to AD resulted in significantly increased FFBF over that seen with MM + AD (Table 1). Although the effect of AS-MDM2 + AD over AS-MDM2 alone was marginal (Table 2), adding all three agents (AS-MDM2 + AD + RT) together resulted in broader and more significant inhibition.

The use of AS-MDM2 + AD + RT caused a significant increase in FFBF and FFTVF compared with all other groups, except AS-MDM2 + AD. The incremental gain in freedom from failure from the addition of RT was 23% (FFBF: 54% for AS-MDM2 + AD to 77% for AS-MDM2 + AD + RT; FFTVF: 62% for AS-MDM2 + AD to 85% for AS-MDM2 + AD + RT), which was not enough to show significance at the $p < 0.05$ level ($p = 0.216$). To see a difference at the $p < 0.05$ level between AS-MDM2 + AD over AS-MDM2 + AD + RT, three times more mice in each group would be needed. However, two lines of evidence in these *in vivo* studies support our prior *in vitro* work showing that AS-MDM2 + AD + RT results in greater cell-killing/tumor inhibitory effects than AS-MDM2 + AD (34). First, AS-MDM2 + AD + RT was more consistently significantly different from AS-MDM2 alone and AS-MDM2 + RT, as compared with AS-MDM2 + AD, for all of the endpoints tested (Table 2). Second, the addition of RT to AS-MDM2 + AD resulted in a considerable prolongation of MRI-based TV and serum PSA doubling times from approximately 57 to 70 days (Table 3). The cell kinetic results from the fitted tumor volume and PSA curves provide complementary evidence that the difference between AS-MDM2 + AD and AS-MDM2 + AD + RT is meaningful. Not only were the TV PSA doubling times prolonged, but the calculated PSA levels and TVs at 6 and 10 weeks were lower with AS-MDM2 + AD + RT.

Immunohistochemistry was used to measure the expression of MDM2, p21, p53, and Ki-67. The methods used are the same as those we have been applying to archival diagnostic clinical samples (52). Image analysis was used to quantify the expression of these proteins in terms of the proportion of cells showing nuclear accumulation. Another method that shows potential clinically is to measure staining intensity (3). We did not find staining intensity to be a useful tool for measuring the effect of the treatments examined in this model (data not shown).

Antisense-MDM2 reduced the proportion of cells with immunohistochemically detectable MDM2 expression most significantly when combined with RT. Radiation causes an increase in p53, as well as a consequential increase in MDM2. Under these conditions AS-MDM2 resulted in a drop in MDM2 expression by immunohistochemistry from 28% to 10%, which was statistically significant (Table 4). There was no statistically significant reduction in the proportion of cells expressing MDM2 in the absence of RT. We believe that the low levels of expression of MDM2 and p53 in unperturbed tumors (no radiation) is the reason for not detecting changes in the expression levels of these proteins after antisense MDM2 treatment. A relative drop in MDM2 from 3% to 2% was not significant under these conditions, nor was the consequential increase in p53 from 2.1% to 2.4% (Table 4). Nonetheless, the finding that AS-MDM2 does reduce MDM2 and also reduced p53 when combined with RT confirms the activity of AS-MDM2 at the molecular level in tumors grown orthotopically in the prostates

of nude mice. Although not all of the molecular changes seen can be explained by the p53 pathway, the reduction in MDM2 as a result of AS-MDM2 treatment when RT was administered illustrates the main point of these studies.

These are the first results to demonstrate that AS-MDM2 significantly sensitizes androgen-sensitive prostate cancer cells to AD and AD + RT *in vivo*. Antisense-MDM2 should substantially enhance the response of prostate tumors locally to AD + RT and has the potential to promote cell killing distantly by potentiating the apoptotic action of AD.

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Fig. 1. Relationships of tumor volume, measured with calipers (a–c) and magnetic resonance imaging (MRI) (c, d), to tumor weight, serum prostate-specific antigen (PSA) level, and each other were derived from mice in the antisense-MDM2 experiments. Linear regression lines and R^2 values are shown.

Fig. 2.

Effects of androgen deprivation (AD) treatment on the relationship of caliper-based tumor volume to (a) MRI-based tumor volume and (b) serum prostate-specific antigen (PSA) level. Linear regression was used to fit the data of tumors grown in intact mice (solid circles) and AD (castrated; open circles). The linear regression lines are indicated on the plot with solid (for intact mice) and broken (for AD) lines.

Fig. 3.

Tumor volume time-related changes in the mismatch (MM) (left panels) and antisense-MDM2 (AS-MDM2) (right panels) treated mice after androgen deprivation (AD) (upper) and AD + radiotherapy (RT) (lower) treatments.

Fig. 4.

(a) Tumor volume and (b) prostate-specific antigen (PSA) level of two tumors: one with no growth delay from treatment (open diamonds) and one with considerably delayed growth (solid triangles). (c, d) Examples for two other tumors: one with an initial decline and subsequent increase in growth (open triangles) and the other with no subsequent increase (solid diamonds).

Table 1

FFBF and FFTVF in LNCaP cells grown orthotopically in the prostates of nude mice

Group	FFBF	FFTVF
MM (pooled)	17 (8/48)	33 (16/48)
AS-MDM2 (pooled)	43 (20/46)*	59 (27/46)*
MM	8 (1/12)	25 (3/12)
AS-MDM2	20 (2/10)	40 (4/10)
MM + RT	9 (1/11)	27 (3/11)
AS-MDM2 + RT	10 (1/10)	40 (4/10)
MM + AD	17 (2/12)	33 (4/12)
AS-MDM2 + AD	54 (7/13)*	62 (8/13)
MM + AD + RT	31 (4/13)	46 (6/13)
AS-MDM2 + AD + RT	77 (10/13)*	85 (11/13)*

Abbreviations: FFBF = Freedom from biochemical failure (prostate-specific antigen <1.5 ng/mL at 6 weeks; FFTVF = freedom from MRI-based tumor volume failure (tumor volume <30 mm³ at 10 weeks); MM = mismatch; AS-MDM2 = antisense MDM2; RT = radiotherapy; AD = androgen deprivation.

Values are percentage (number).

* Statistically significant difference ($p < 0.05$) compared with the group above.

Table 2
Probabilities estimated by chi-square test from comparisons among the treatments in Table 1

	MM	MM + RT	MM + AD	AS-MDM2	AS-MDM2 + RT	AS-MDM2 + AD
FFBF						
MM + RT	0.949					
MM + AD	0.537	0.590				
MM + AD + RT	0.161	0.192	0.409			
AS-MDM2 + RT				0.531		
AS-MDM2 + AD				0.099	0.029	
AS-MDM2 + AD + RT				0.007	0.001	0.216
FFTVF						
MM + RT	0.901					
MM + AD	0.653	0.752				
MM + AD + RT	0.271	0.341	0.513			
AS-MDM2 + RT				1		
AS-MDM2 + AD				0.305	0.305	
AS-MDM2 + AD + RT				0.026	0.026	0.184

Abbreviations as in Table 1.

Table 3
Average values ($\pm$ SEM) of tumor parameters, calculated by modeling of longitudinal data from MRI-based tumor volume and serum PSA measurements

Group	Tumor doubling time (days) [*]	PSA doubling time (days) [*]	Tumor volume at 6 wk (mm ³)	PSA at 6 wk (ng/mL)	Tumor volume at 10 wk (mm ³)	PSA at 10 wk (ng/mL)
MM	25 $\pm$ 9	27 $\pm$ 10	208 $\pm$ 61	64 $\pm$ 17	276 $\pm$ 68	77 $\pm$ 17
AS-MDM2	39 $\pm$ 13	34 $\pm$ 11	75 $\pm$ 27	37 $\pm$ 13	230 $\pm$ 72	80 $\pm$ 27
MM + RT	18 $\pm$ 5	23 $\pm$ 10	173 $\pm$ 65	41 $\pm$ 14	250 $\pm$ 68	70 $\pm$ 16
AS-MDM2 + RT	22 $\pm$ 3	22 $\pm$ 3	26 $\pm$ 8 ^{†‡}	15 $\pm$ 6 ^{†‡}	79 $\pm$ 36 ^{†‡}	34 $\pm$ 13 ^{†‡}
MM + AD	34 $\pm$ 14	22 $\pm$ 9	183 $\pm$ 54	52 $\pm$ 15	308 $\pm$ 66	77 $\pm$ 17
AS-MDM2 + AD	57 $\pm$ 14	58 $\pm$ 13 [†]	103 $\pm$ 51	25 $\pm$ 13	180 $\pm$ 66 [§]	48 $\pm$ 17 [§]
MM + AD + RT	43 $\pm$ 11	39 $\pm$ 12	61 $\pm$ 20 ^{//}	28 $\pm$ 14 ^{//}	146 $\pm$ 54 ^{//}	41 $\pm$ 15 ^{//}
AS-MDM2 + AD + RT	70 $\pm$ 11 ^{†‡}	71 $\pm$ 11 ^{†‡}	10 $\pm$ 5 ^{†‡}	2 $\pm$ 1 ^{†‡}	22 $\pm$ 11 ^{†‡}	6 $\pm$ 4 ^{†‡}

Abbreviations: PSA = prostate-specific antigen. Other abbreviations as in Table 1.

* Tumor and PSA doubling times were calculated from fitted data for mice in which tumor volume/PSA exhibits an upward trend after treatment. For the rest of the animals 100 days is assumed.

[†] $p < 0.05$ compared with the group above.

[‡] $p < 0.05$ compared with the AS-MDM2 group.

[§] $p < 0.1$ compared with the group above.

^{//} $p < 0.05$ compared with the MM group.

Table 4

Immunohistochemistry staining of LNCaP xenografts for MDM2, p21, p53, and Ki-67 expression

Treatment	MDM2	p21	p53	Ki-67
CT	3.0 ± 0.2	6.0 ± 0.0	0.9 ± 0.4	65.5 ± 6.2
MM	3.0 ± 1.2	11.5 ± 2.8*	2.1 ± 0.7	72.8 ± 7.6
AS-MDM2	2.1 ± 0.6	13.2 ± 2.8	2.4 ± 0.8	70.8 ± 4.0
MM + AD	0.7 ± 0.2*	4.8 ± 1.1*	1.2 ± 0.4	33.6 ± 4.2*
AS-MDM2 + AD	0.9 ± 0.3	4.8 ± 1.4	2.0 ± 0.6	43.7 ± 4.6
MM + RT	27.9 ± 8.3*	78.7 ± 5.1*	7.5 ± 1.0*	50.2 ± 5.8
AS-MDM2 + RT	10.0 ± 4.6*	70.8 ± 11.2	4.2 ± 1.4*	38.4 ± 7.4

Abbreviations: CT = control (LNCaP cells alone). Other abbreviations as in Table 1.

The average (± SEM) values shown are representative of the percentage of tumors cells stained for the expression of each biomarker.

* $p < 0.05$, Compared with group above, t test (CT: $n = 2$; all treatment groups: $n = 6$, except AS-MDM2 + RT: $n = 5$). Other significant comparisons:

- For MDM2: MM vs. MM + AD and MM + RT; MM + RT vs. MM + AD; AS-MDM2 + RT vs. AS-MDM2 + AD.
- For P21: MM vs. MM + AD and MM + RT; MM + RT vs. MM + AD; AS-MDM2 vs. AS-MDM2 + AD and AS-MDM2 + RT; AS-MDM2 + RT vs. AS-MDM2 + AD.
- For P53: MM + RT vs. MM and MM + AD.
- For Ki67: MM vs. MM + AD and MM + RT; MM + RT vs. MM + AD; AS-MDM2 vs. AS-MDM2 + AD and AS-MDM2 + RT.