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Antisense MDM2 Enhances the Response of Androgen Insensitive Human Prostate Cancer Cells to Androgen Deprivation In Vitro and In Vivo

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Abstract

Background—Antisense MDM2 oligonucleotide (AS-MDM2) sensitizes androgen sensitive LNCaP cells to androgen deprivation (AD) in vitro and in vivo. In this study, we investigated the effects of AS-MDM2 combined with AD on androgen resistant LNCaP (LNCaP-Res) and moderately androgen resistant bcl-2 overexpressing LNCaP (LNCaP-BST) cells.

Methods—The LNCaP-Res cell line was generated by culturing LNCaP cells in medium containing charcoal-stripped serum for more than 1 year. Apoptosis was quantified in vitro by Annexin V staining and caspase 3+7 activity. For the in vivo studies, orthotopic tumor growth was monitored by magnetic resonance imaging (MRI). AS-MDM2 and the mismatch control were given by i.p. injection at doses of 25 mg/kg per day, 5 days/week for 15 days.

Results—LNCaP-Res cells expressed high levels of androgen receptor (AR) and bcl-2, and displayed no growth inhibition to AD. AS-MDM2 caused significant reductions in MDM2 and AR expression, and increases in p53 and p21 expression in both cell lines. AS-MDM2 + AD resulted in the highest levels of apoptosis in vitro and tumor growth inhibition in vivo in both cell lines; although, these effects were less pronounced in LNCaP-BST cells.

Conclusions—AS-MDM2 + AD enhanced apoptotic cell death in vitro and tumor growth inhibition in vivo in androgen resistant cell lines. The action of AS-MDM2 + AD was influenced somewhat by bcl-2 expression as an isolated change (LNCaP-BST cells), but not when accompanied by other molecular changes associated with androgen insensitivity (LNCaP-Res cells). MDM2 knockdown has promise for the treatment of men with early hormone refractory disease.

Keywords

antisense; MDM2; androgen deprivation; prostate cancer

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Introduction

The most common treatment for advanced prostate cancer is androgen deprivation (AD) therapy. Inhibitory tumor growth response is temporary, as the tumor eventually becomes refractory to AD. The options at that point are few. The mainstay of treatment for hormone refractory disease is chemotherapy, which prolongs median survival by 2–3 months [1,2]. Targeted therapy that enhances and prolongs response to AD has the potential to further extend survival.

MDM2 is an oncogene, the protein product of which has a central role in the apoptotic pathway. MDM2 binds to and modulates p53, E2F-1, and pRb, as well as other proteins [3–5]. MDM2 also negatively regulates p21 waf1/cip1 protein independent of p53 [6,7]. Recent studies have demonstrated that MDM2 is involved in the regulation of androgen receptor (AR) degradation by ubiquitination and proteolysis [8,9].

MDM2 is overexpressed in 30–40% of prostate cancers [10–12]. AS-MDM2 inhibits MDM2 expression, and enhances the effects of radiation and chemotherapy on prostate cancer cells [13–15]. We have previously shown that AS-MDM2 sensitizes androgen sensitive LNCaP cells to AD in vitro via an increase in apoptosis and in vivo by tumor growth inhibition [16–18]. In this report, we examine the potential of AS-MDM2 + AD in promoting cell killing in cell lines with increasing resistance to AD. An androgen resistant cell line, LNCaP-Res, was created by growth of LNCaP cells in androgen-deprived medium in vitro for 1 year; these cells overexpress bcl-2 when exposed to AD, which is common in human hormone refractory prostate cancer cells [19–21]. The role of bcl-2 overexpression was further investigated by contrasting the action of AS-MDM2 + AD on LNCaP-Res cells with LNCaP cells that overexpress bcl-2 (LNCaP-BST cells) [22].

Materials and Methods

Cell Culture and Growth Measurements

LNCaP cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium, containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin (complete medium [CM]), as described previously [16]. AD treatment in vitro was carried out by culturing the cells in medium containing 10% charcoal-stripped serum (AD medium). Androgen was replaced by adding the synthetic androgen R1881 (NEN Life Science Products, Boston, MA) at 1×10^{-10} M to AD medium (AD + R1881). This concentration was chosen because prior studies have shown that unaltered (wild-type) LNCaP cell growth was maximally restored when R1881 at 1×10^{-10} M was added to AD medium [23].

LNCaP-Res cells were established by serial passages of LNCaP cells in AD medium for 1 year. LNCaP-Res cells were maintained in AD medium or returned to complete, or AD + R1881 medium for 3 days in the experiments. The early passages (below 20) of LNCaP-Res cells was used for the experiments. LNCaP-BST cells are a clonal cell line derived by stable transfection of LNCaP cells with the neomycin-selectable pSFFV/bcl-2 expression plasmid (kindly provided by Dr. Stanley Korsmeyer, Harvard Medical School, Boston, MA) as described previously [22]. Quantification of cell number over time was accomplished by culturing the cells in 24-well plates at 5×10^4 cells/well in complete, AD or AD + R1881 medium for 3 or more days. The number of viable cells was counted at 24-hr intervals.

Antisense Oligonucleotides

The antisense MDM2 oligonucleotide (AS-MDM2 or AS; GEM[®]240) and its mismatch control oligonucleotide (MM) are 20-mer mixed-backbone oligonucleotides with following sequence

(AS; 5'-UGACACCTGTTCTCACUCAC-3') and (MM; 5'-UGTACCCTTTTTCATUCAC-3'). Both oligonucleotides were comprised of deoxynucleosides and 2'-O-methylribonucleosides at the 5' and the 3' ends (bold face letters) with a phosphorothioate backbone. Synthesis and confirmation of those oligonucleotides was carried out as described previously [24]. The oligonucleotides were provided by Idera Pharmaceuticals, Inc. (Cambridge, MA). They were stored as frozen aliquots at -20°C.

Western Blot Analysis

Protein levels were analyzed after different treatments using previously described methods [16]. In brief, cells were cultured in complete, AD or AD + R1881 medium for 3 days, and then incubated with 200 nM of AS-MDM2 or MM for 24 hr in the presence of 7 µg/ml Lipofectin (Invitrogen, Carlsbad, CA). Cells were exposed to lysis buffer (50 mM Tris-HCL, pH 6.8, 2% sodium dodecyl sulfate [SDS] with protease inhibitor), and were sonicated for 30 sec on ice. Tumor tissue was homogenized in 10 mM Tris-HCL (pH 7.8), the homogenate centrifuged at 10,000 rpm for 10 min at 4°C, and glycerol added (final concentration 15%) to the supernatant. Protein concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Identical amounts of protein from cell lysates and tumor tissue homogenates were resolved by SDS-PAGE electrophoresis and probed with anti-MDM2, anti-p53, anti-p21, anti-β actin monoclonal antibodies at 1:1,000 dilutions (Calbiochem, San Diego, CA), anti-bcl-2 monoclonal antibody at 1:1,000 dilution (DAKO A/S, Carpinteria, CA), or anti-AR polyclonal IgG at 1:1,000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Membranes were incubated with 1:2,000 diluted sheep antimouse IgG or donkey-rabbit IgG horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hr at room temperature. Immunoreactive proteins were visualized by the enhanced chemiluminescence reagents. The integrated density of protein band was quantified using Sicon Image software from the National Institutes of Health.

Measurements of Apoptosis

Annexin V staining and caspase 3+7 activity assays were used to determine apoptotic cell death, as described previously [16]. For each assay, cells were cultured in complete, AD, or AD + R1881 medium for 3 days. Cells were then incubated with 200 nM AS-MDM2 or MM in the presence of Lipofectin (7 µg/ml) for 30 hr with LNCaP-BST cells or 40 hr with LNCaP-Res cells. All cells (floating and attached) were collected and labeled with Annexin V-Phycoerythrin and 7-amino-actinomycin D (7-AAD) (Guava Technologies, Inc., Burlingame, CA) according to the manufacturer's instructions. The samples were then analyzed by flow cytometry on a GuavaPC personal flow cytometer (Guava Technologies, Inc.). Caspase 3 + 7 activity was determined using the Apo-ONE™ Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI). Harvested cells (2×10^4) in 50 µl culture medium were mixed with 50 µl of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 18 hr. The activity was measured according to the manufacturer's instructions using a fluorescent plate reader (LabSystems, Inc., Franklin, MA).

Animal Tumor Model

Male athymic Balb/c nude mice (4–8 weeks old) were purchased from (Harlan, Indianapolis, IN). All injections and surgical procedures were performed using 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 [18,25]. Nude mice were anesthetized using methoxyflurane obtained from Medical Developments Australia Pty. Ltd (Melbourne, Australia), and a lower midline was made approximately 1.5 cm above the presumed location of the bladder. LNCaP-BST or LNCaP-Res cells (5×10^5) in 20 µl were

injected into the dorsal prostate using a 30-gauge 1-inch needle. The incision was closed in one layer by 2–3 autoclips.

Tumor volume was directly monitored by magnetic resonance imaging (MRI) after orthotopic implantation of the cells. Treatments were initiated when tumor volumes were approximately 10–20 mm³. At this point bilateral orchiectomy was performed under methoxyflurane anesthesia on the animals in the AD groups. After a trans-scrotal incision of the betadine 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 2–3 clips. AS-MDM2 or MM, dissolved in PBS, was administered by i.p. injection at doses 25 mg/kg per day, 5 days/week for 15 days.

Tumor Volume Analysis and Statistics

Once treatment began, tumor volumes were measured weekly by MRI in a vertical wide-bore magnet equipped with a Bruker DRX 300 console at a field strength of 7 Tesla, as described previously [18]. Tumor volumes were determined by outlining tumors using the Paravision software supplied with the spectrometer, and then summing the volumes from all the sections. The images were made with a two-dimensional multi-slice spin echo pulse sequence. Repetition times were in the range of 400–600 msec, the echo time was 13.2 msec, the slice thickness was 0.5 mm, and the in-plane resolution was 0.1 mm. Signal averaging over four acquisitions brought the scan time to 7 min. This protocol allowed us to visualize tumors with volumes as small as 5 mm³. Animal preparation and scout image acquisition brought the total time for the MRI scans to 10–15 min/animal. Prior to imaging, each mouse received an injection of 0.2 ml of the commercial contrast agent Magnevist (Berlex Industries-Montville, NJ) diluted 10:1 with a physiological concentration of saline. During imaging the mouse was anesthetized with a mixture of 1% isoflurane in oxygen. The animals were sacrificed when the MRI-based tumor volume was greater than 300 mm³.

The MRI measurements of tumor growth were reasonably described by first order rate process. The time series for each animal were fitted with a single exponential as follows:

$$S(t)=S_0\exp(Rt)$$

where $S(t)$ is the tumor volume at time t , S_0 is the estimated baseline tumor volume at $t = 0$ and R is the rate of tumor growth. Tumor doubling time was calculated as the $\ln 2/R$. It should be noted that for some of the measurements the exponential fits resulted in a negative rate ($R < 0$). For the purposes of the statistical comparisons, the doubling time in these cases was set to 25 (LNCaP-Res) and 100 (LNCaP-BST) days. The growth of LNCaP-Res tumors was much faster and so a shorter default upper-limit doubling time interval was used. Freedom from tumor volume failure (FFTVF) was defined as an MRI-based tumor volume of ≤ 30 mm³ by 6 weeks for both cell line. Tumor growth response was also assessed by plotting the median tumor volumes at different times, calculated from the fitted data for each animal.

Mann–Whitney test and Fisher exact test were used to determine the significance between the treatment groups, and $P < 0.05$ was considered significant.

Results

Cell Growth

The growth properties of wild-type LNCaP, LNCaP-BST, and LNCaP-Res cells are compared in Figure 1. The cells were cultured in complete, AD or AD + R1881 medium for 5 days and

the number of viable cells quantified at days 3,4, and 5 after the culture conditions were established. Figure 1A shows that, similar to the results of our previous studies [23], LNCaP cell growth was nearly completely inhibited when cultured in AD medium; this inhibition was partially reversed by adding the synthetic androgen R1881 back to AD medium. In contrast, the growth of LNCaP-Res cells was the same when cultured in complete or AD medium. Moreover, the growth of LNCaP-Res cells was inhibited markedly when restoring R1881 to AD medium (Fig. 1B). Such notable sensitivity to androgen in “androgen resistant” cell lines has been described before [26,27]. The concentration of R1881 used (1×10^{-10} M) was based on titration experiments in wild-type LNCaP cells designed to optimally restore growth [23].

LNCaP-BST cell growth was inhibited when cultured in AD medium as compared to CM, but the effect was less than that seen in the LNCaP line (Fig. 1C). The growth inhibition of LNCaP-BST cells to AD was partially reversed by adding the synthetic androgen R1881 to the medium, a pattern similar to that seen in wild-type LNCaP cells.

Effect of AS-MDM2 on the Expression of Key Proteins in the Apoptotic Pathway

Our previous studies have shown that AS-MDM2 inhibits MDM2 expression and results in a significant increase in p53 and p21 protein levels in LNCaP cells [16,17]. To better understand why LNCaP-Res cells are able to proliferate unabated in AD medium, we examined the expression levels of MDM2, p53, p21, AR, and bcl-2 proteins after cell growth in complete (CM), AD or AD + R1881 medium for 3 days. As displayed in Figure 2A, LNCaP-Res cells displayed upregulated AR and downregulated p21 compared to LNCaP and LNCaP-BST cells. AR and p21 expression were decreased when LNCaP and LNCaP-BST cells were grown in AD medium for 3 days, compared to culture in complete and AD + R1881 medium; little change in these proteins was seen from AD in LNCaP-Res cells. p53 and bcl-2 were upregulated in LNCaP-Res cells grown in AD medium. In contrast, bcl-2 expression was reduced by AD in LNCaP and LNCaP-BST cells. There was also a minimal decline in MDM2 with AD treatment in LNCaP and LNCaP-BST cell lines, with essentially no change in LNCaP-Res cells. The upregulation of bcl-2, p53 and the AR in response to AD in LNCaP-Res cells suggests that these changes had a role in maintaining proliferation in the setting of AD.

Figure 2A also shows the effect of synthetic androgen R1881 on the expression of these proteins. The expression of MDM2, p53 and bcl-2 in LNCaP-Res cells was reduced when R1881 was added to AD medium, relative to that seen in AD medium alone. These changes were not seen in wild-type LNCaP and LNCaP-BST cells. In LNCaP and LNCaP-BST cells, adding R1881 back to AD medium increased AR expression relative to levels seen in CM. In contrast, adding R1881 to AD medium had no effect on AR expression in LNCaP-Res cells. These molecular changes from androgen restoration with R1881 occurred with the growth inhibition of LNCaP-Res cells and growth promotion of wild-type LNCaP and LNCaP-BST cells. LNCaP-BST responded to AD + R1881 like wild-type LNCaP cells, but less in degree. The alterations in LNCaP-Res cell proliferation (Fig. 1) under the conditions of complete, AD and AD + R1881 paralleled fluctuations in bcl-2 and p53 expression, which may or may not be the key etiologic factors.

We then analyzed the effects of AS-MDM2 on protein expression in LNCaP-Res (Fig. 2B) and LNCaP-BST cells (Fig. 2C). Consistent with our previous results in LNCaP cells [16,17], AS-MDM2 almost completely abrogated MDM2 expression, and increased the levels of p53 and p21 in all media conditions. The MM control had no consistent effect on p53 and p21 expression levels. There was a decrease in AR levels after AS-MDM2 treatment in complete and AD medium in LNCaP-Res cells. AR expression in LNCaP-BST cells was inhibited by AD and appeared to be slightly lower with the addition of AS-MDM2. AS-MDM2 caused no consistent change in bcl-2 levels in LNCaP-Res and LNCaP-BST cells.

Effect of AS-MDM2 on Apoptotic Cell Death

Previous results on wild-type LNCaP cells [16,17] revealed that when AS-MDM2 and AD were combined, apoptotic cell death was greater than when either treatment was administered individually. Figure 3A,B shows that the combined treatment of AS-MDM2 with AD also promoted enhanced apoptosis in LNCaP-Res cells, even though LNCaP-Res cells overexpress bcl-2 and are not growth inhibited in AD medium. Early apoptotic cell death measured by Annexin V staining was significantly higher when LNCaP-Res cells were exposed to AS-MDM2 + AD, as compared with other groups (Fig. 3A). Annexin V staining was 27.7% from AS-MDM2 + AD, 14.7% from AS-MDM2 alone and 11.2% from AD + R1881 + AS-MDM2. The difference between AS-MDM2 and MM was also significant. For LNCaP-BST cells, the trends were similar (Fig. 3C), but the level of apoptosis achieved with AS-MDM2 + AD was much lower, compared to that seen in LNCaP-Res cells.

Apoptosis was also measured by caspase 3+7 activity assay. Treatment with AS-MDM2 + AD caused a significant increase in caspase 3 + 7 activity, as compared to all other groups, in both LNCaP-Res and LNCaP-BST cells (Fig. 3B,D). Although AS-MDM2 alone caused an increase in caspase 3 + 7 activity over that from MM-control alone in LNCaP-Res cells, the difference was not significant. In LNCaP-BST cells, caspase 3+7 activity was significantly increased from AS-MDM2 over that from MM control.

Effect of AS-MDM2 on Tumor Growth In Vivo

Recently, we reported that MDM2 knockdown by AS-MDM2 oligonucleotide enhanced antitumor activity when combined with AD in the androgen sensitive LNCaP tumor model [18]. To evaluate whether AS-MDM2 combined with AD resulted in enhanced tumor growth inhibition in cell lines with increasing resistance to AD, the antitumor activity of AS-MDM2 + AD was tested in LNCaP-Res and LNCaP-BST cells in vivo. The tumors were grown orthotopically in the prostates of nude mice since tumor–stromal interactions contribute to the overall response of prostate cancer cells to AD. Accounting for tumor–stromal interactions is essential when judging the potential of a strategy to sensitize prostate cancer cells to AD. Tumor volume was directly measured by MRI after treatment was initiated. MRI resulted in excellent images of tumors, allowing for accurate determination of tumor volumes (Fig. 4). The coronal MRI images from a mouse with an orthotopically grown LNCaP-Res tumor were taken 6 weeks apart. Clear depiction of the tumor borders and anatomic structures are apparent. The younger tumor had a total volume of 10 mm³ (Fig. 4A) and the older tumor 190 mm³ (Fig. 4B).

AS-MDM2 was given by daily intraperitoneal injections for 15 days. There was a strong relationship between the MRI and caliper measured tumor volume (Fig. 4C, $R^2 = 0.90$, $P < 0.001$), and MRI measurements versus tumor weight (Fig. 4D, $R^2 = 0.90$, $P < 0.001$). The linear regression analyses were performed on the pooled data from both cell lines; there were no differences between the cell lines. The relationship between caliper measured tumor volume and tumor weight ($R^2 = 0.97$, $P < 0.001$) was also significant (data not shown).

The changes in tumor volume following the different treatments for both cell lines are shown in Figure 5A,B. The graphs represent the group median values over the course of 10 weeks, calculated from the fitted data for each animal. AS-MDM2 alone did not result in tumor growth inhibition in both LNCaP-Res and LNCaP-BST cells. The combination of AS-MDM2 + AD was more effective at reducing tumor volume relative to the other treatment groups (MM, AS, and MM + AD) in both cell lines. However, the action in LNCaP-BST cells was much less than that seen in LNCaP-Res cells. To confirm the effect of AS-MDM2 on MDM2 expression in vivo, the protein levels of MDM2 from pooled LNCaP-Res and LNCaP-BST tumors was analyzed by Western blot. As shown in Figure 5C,D, AS-MDM2 markedly inhibited MDM2 expression in both cell line tumors.

Table I displays the average tumor doubling times and tumor volumes calculated from the fitted MRI tumor volume data. The mean tumor doubling time from the combination of AS-MDM2 + AD was longer than for all of the other treatment groups in both cell lines; however, the difference was only significant for LNCaP-Res cells. In LNCaP-BST tumors, the tumor volume doubling time was significantly longer in both of AS-MDM2 + AD and MM+AD treatments relative to AS-MDM2 and MM alone. The trends were similar for the calculated mean tumor volumes at 6 weeks; there was a smaller tumor volume from the combination of AS-MDM2 + AD than for all of the other treatment groups (MM, AS-MDM2 and MM+AD) in both cell lines. The difference was significant for AS-MDM2 + AD versus MM in LNCaP-Res tumors, and for AS-MDM2 + AD versus AS-MDM2 in LNCaP-BST tumors.

Table I also shows that AS-MDM2 + AD resulted in the highest percentage of FFTVF as compared to all of the other treatments in both cell lines. The FFTVF parameter was designed to mimic threshold PSA parameters that are commonly used in prostate cancer assessments clinically, as we have described previously [18]. While the greatest inhibition was seen with AS-MDM2 + AD, the effect was marginally significant for LNCaP-Res tumors ($P = 0.09$), but not for LNCaP-BST tumors. Overall, the in vivo results are concordant with the in vitro findings.

Discussion

Although AD is initially effective at inducing prostate cancer tumor regression by promoting apoptosis and a shift from cell proliferation to quiescence, tumor growth eventually resumes [28,29]. Hormone refractory prostate cancer is resistant to AD in terms of growth inhibition. Some clinical [30–34] and animal [35] studies have shown that despite tumor progression while receiving AD, the continued administration of AD is beneficial, presumably because some of the tumor cells still respond to AD, albeit at a reduced level. With continued AD, some tumor cells (cell lines) grow at a slower rate, while the growth of others is completely independent of the influence of AD [35–37]. The LNCaP-Res model described here seems to fall somewhere in-between; there is no obvious effect of AD on growth, yet molecular alterations in response to AD predispose the cells to an increased apoptotic response to the combination of AS-MDM2 + AD.

The overexpression of bcl-2 after AD has been correlated with progression to androgen insensitivity [19–21,38]. LNCaP-Res cells are representative in that they overexpress bcl-2 when cultured in AD medium in vitro. Other common features of LNCaP-derived androgen-insensitive cells after long-term culture in AD medium are increased AR activity and growth inhibition by relatively low concentrations of androgens [26,27,39–41]. LNCaP-Res cells have these attributes.

Our prior studies have shown that knockdown of MDM2 using a second generation antisense MDM2 oligonucleotide with a mixed backbone results in a significantly increased apoptotic and antitumor response of androgen-sensitive LNCaP cells to AD. The results described here extend these observations, showing that AS-MDM2 promotes increased cell death when combined with AD in AD resistant LNCaP-Res cells. The Annexin V and caspase 3 + 7 assay responses of LNCaP-Res cells to the combination of AS-MDM2 + AD were greater than that seen with AS-MDM2 alone, AD alone, AD + R1881, and the mismatch control groups. Moreover, the apoptotic response of LNCaP-Res cells to AS-MDM2 + AD was proportionally much greater in magnitude than that seen in LNCaP-BST cells, even though LNCaP-BST cells continue to be inhibited by AD.

Androgens regulate the differentiation, survival and function of normal prostate epithelial cells, as well as the proliferation and survival of prostate cancer cells [28,42]. Androgens cause

growth stimulation at low concentrations in androgen-sensitive cells. Our results showed that androgen replacement using R1881 (AD + R1881) at 1×10^{-10} M reduced the growth inhibition by AD in LNCaP and LNCaP-BST cells, while the growth of LNCaP-Res cells was substantially decreased (Fig. 1). Replenishment of androgen with R1881 in the culture medium of LNCaP cells reversed the effects of AS-MDM2 combined with AD on apoptotic cell death and clonogenic survival [16,17]. LNCaP-Res cells also showed a non-significant trend for reduced apoptosis in the presence of R1881 (Fig. 3A,B); however, the effect of androgen restoration with R1881 on LNCaP-Res cells is consistent with pronounced tumor cell growth inhibition, as opposed to the growth stimulatory action seen in LNCaP and LNCaP-BST cells. The molecular mechanisms of how androgens regulate the proliferation and apoptosis in LNCaP-Res cells need to be further investigated.

The apoptotic response of the prostate tumor cells lines to AS-MDM2 + AD in vitro paralleled tumor growth inhibition in vivo. All cell lines displayed enhanced MRI-based tumor growth inhibition when treated with AS-MDM2 + AD, as compared to the other possible combinations. However, the action of AS-MDM2 + AD was notably less in LNCaP-BST cells.

We have shown previously that AS-MDM2 inhibits MDM2 expression and results in significant increase in p53 and p21 protein levels in LNCaP cells [16,17]. The expression of p53 and p21 were likewise enhanced after MDM2 inhibition in vitro in both LNCaP-Res and LNCaP-BST cells. Antisense-MDM2 caused a substantial reduction in AR expression level when LNCaP-Res cells were cultured in complete and AD medium. We have also found that AS-MDM2 causes a reduction in the AR level in LNCaP cells (data not shown). The AR is a determinant of response to AD, with overexpression associated with the development of androgen-insensitivity [43,44]. Direct knockdown of AR expression by AR antisense, small-interfering RNA or neutralizing AR antibodies [45–48], or the indirect inhibition of AR protein expression by other agents [49], usually leads to increased apoptotic cell death and tumor growth inhibition in prostate cancer cells. The downregulation of AR expression by AS-MDM2 suggests that the AR signaling pathway has a role in the sensitization of LNCaP-Res cells to AD. There is evidence that MDM2 is involved in the response of prostate cancer to androgens and has a role in AR-mediated transcription [50]. The AR is subject to ubiquitin-dependent proteolysis, which is catalyzed by a trimeric reaction that includes MDM2, an E3 ubiquitin ligase [8,9]. MDM2 may also indirectly affect AR-mediated signaling through p53 regulation since MDM2 inhibition results in a significant increase in p53 expression. Recent studies have shown that the expression and activity of AR is regulated by p53 [51,52]. The molecular mechanisms of how AR activity is modulated by AS-MDM2 are under further investigation by our group.

To our knowledge, this is the first report of continued modulation of response to targeted therapy by AD in an AD resistant prostate cancer model. The clinical implications are that even men who have evidence of progression while receiving hormone therapy may continue to benefit from AD when targeted therapy is used. In our models, AS-MDM2 + AD was more effective at cell killing in vitro and tumor growth inhibition in vivo than either treatment given individually in AD resistant LNCaP-Res cells and moderately androgen sensitive LNCaP-BST cells. LNCaP-BST cells were less affected by the cell killing and tumor growth inhibitory actions of AS-MDM2 + AD, suggesting that bcl-2 overexpression as an isolated change reduces the impact of AS-MDM2 + AD. Another consideration is that the level of bcl-2 overexpression was much higher in LNCaP-BST cells in vitro and in vivo (data not shown) over that seen in LNCaP-Res cells. In contrast, bcl-2 overexpression in the background of other molecular changes associated with androgen insensitivity (LNCaP-Res cells), did not dampen the response to AS-MDM2 + AD.

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References

1. Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson I, Rosenthal MA, Eisenberger MA. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351(15):1502–1512. [PubMed: 15470213]
2. Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moynour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 2004;351(15):1513–1520. [PubMed: 15470214]
3. Dautat S, Neel H, Piette J. MDM2: Life without p53. *Trends Genet* 2001;17(8):459–464. [PubMed: 11485818]
4. Deb SP. Cell cycle regulatory functions of the human oncoprotein MDM2. *Mol Cancer Res* 2003;1(14):1009–1016. [PubMed: 14707284]
5. Meek DW, Knippschild U. Posttranslational modification of MDM2. *Mol Cancer Res* 2003;1(14):1017–1026. [PubMed: 14707285]
6. Jin Y, Lee H, Zeng SX, Dai MS, Lu H. MDM2 promotes p21 waf1/cip1 proteasomal turnover independently of ubiquitylation. *EMBO J* 2003;22(23):6365–6377. [PubMed: 14633995]
7. Zhang Z, Wang H, Li M, Agrawal S, Chen X, Zhang R. MDM2 is a negative regulator of p21WAF1/CIP1, independent of p53. *J Biol Chem* 2004;279(16):16000–16006. [PubMed: 14761977]
8. Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. *EMBO J* 2002;21(15):4037–4048. [PubMed: 12145204]
9. Gaughan L, Logan IR, Neal DE, Robson CN. Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation. *Nucleic Acids Res* 2005;33(1):13–26. [PubMed: 15640443]
10. Osman I, Drobniak M, Fazzari M, Ferrara J, Scher HI, Cordon-Cardo C. Inactivation of the p53 pathway in prostate cancer: Impact on tumor progression. *Clin Cancer Res* 1999;5(8):2082–2088. [PubMed: 10473090]
11. Leite KR, Franco MF, Srougi M, Nesrallah LJ, Nesrallah A, Bevilacqua RG, Darini E, Carvalho CM, Meirelles MI, Santana I, Camara-Lopes LH. Abnormal expression of MDM2 in prostate carcinoma. *Mod Pathol* 2001;14(5):428–436. [PubMed: 11353053]
12. Khor LY, Desilvio M, Al-Saleem T, Hammond ME, Grignon DJ, Sause W, Pilepich M, Okunieff P, Sandler H, Pollack A. MDM2 as a predictor of prostate carcinoma outcome: An analysis of Radiation Therapy Oncology Group Protocol 8610. *Cancer* 2005;104(5):962–967. [PubMed: 16007688]
13. Zhang Z, Li M, Wang H, Agrawal S, Zhang R. Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proc Natl Acad Sci USA* 2003;100(20):11636–11641. [PubMed: 13130078]
14. Wang H, Yu D, Agrawal S, Zhang R. Experimental therapy of human prostate cancer by inhibiting MDM2 expression with novel mixed-backbone antisense oligonucleotides: In vitro and in vivo activities and mechanisms. *Prostate* 2003;54(3):194–205. [PubMed: 12518324]
15. Bianco R, Caputo R, Caputo R, Damiano V, De Placido S, Ficorella C, Agrawal S, Bianco AR, Ciardiello F, Tortora G. Combined targeting of epidermal growth factor receptor and MDM2 by gefitinib and antisense MDM2 cooperatively inhibit hormone-independent prostate cancer. *Clin Cancer Res* 2004;10(14):4858–4864. [PubMed: 15269162]

16. Mu Z, Hachem P, Agrawal S, Pollack A. Antisense MDM2 oligonucleotides restore the apoptotic response of prostate cancer cells to androgen deprivation. *Prostate* 2004;60(3):187–196. [PubMed: 15176048]
17. Mu Z, Hachem P, Agrawal S, Pollack A. Antisense MDM2 sensitizes prostate cancer cells to androgen deprivation, radiation, and the combination. *Int J Radiat Oncol Biol Phys* 2004;58(2):336–343. [PubMed: 14751501]
18. Stoyanova R, Hachem P, Hensley H, Khor LY, Mu Z, Hammond ME, Agrawal S, Pollack A. Antisense-MDM2 sensitizes LNCaP prostate cancer cells to androgen deprivation, radiation, and the combination in vivo. *Int J Radiat Oncol Biol Phys* 2007;68(4):1151–1160. [PubMed: 17637390]
19. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM, Campbell ML. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res* 1992;52(24):6940–6944. [PubMed: 1458483]
20. Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS, Buttyan R. Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. *Cancer Res* 1995;55(19):4438–4445. [PubMed: 7671257]
21. Apakama I, Robinson MC, Walter NM, Charlton RG, Royds JA, Fuller CE, Neal DE, Hamdy FC. bcl-2 overexpression combined with p53 protein accumulation correlates with hormone-refractory prostate cancer. *Br J Cancer* 1996;74(8):1258–1262. [PubMed: 8883414]
22. Mu Z, Hachem P, Pollack A. Antisense Bcl-2 sensitizes prostate cancer cells to radiation. *Prostate* 2005;65(4):331–340. [PubMed: 16015611]
23. Pollack A, Salem N, Ashoori F, Hachem P, Sangha M, von Eschenbach AC, Meistrich ML. Lack of prostate cancer radiosensitization by androgen deprivation. *Int J Radiat Oncol Biol Phys* 2001;51(4):1002–1007. [PubMed: 11704324]
24. Agrawal S, Zhao Q. Antisense therapeutics. *Curr Opin Chem Biol* 1998;2(4):519–528. [PubMed: 9736926]
25. Cowen D, Salem N, Ashoori F, Meyn R, Meistrich ML, Roth JA, Pollack A. Prostate cancer radiosensitization in vivo with adenovirus-mediated p53 gene therapy. *Clin Cancer Res* 2000;6(11):4402–4408. [PubMed: 11106260]
26. Kokontis J, Takakura K, Hay N, Liao S. Increased androgen receptor activity and altered c-myc expression in prostate cancer cells after long-term androgen deprivation. *Cancer Res* 1994;54(6):1566–1573. [PubMed: 7511045]
27. Kokontis JM, Hay N, Liao S. Progression of LNCaP prostate tumor cells during androgen deprivation: Hormone-independent growth, repression of proliferation by androgen, and role for p27Kip1 in androgen-induced cell cycle arrest. *Mol Endocrinol* 1998;12(7):941–953. [PubMed: 9658399]
28. Pollack A, Joon DL, Wu CS, Sikes C, Hasegawa M, Terry NH, White RA, Zagars GK, Meistrich ML. Quiescence in R3327-G rat prostate tumors after androgen ablation. *Cancer Res* 1997;57(12):2493–2500. [PubMed: 9192831]
29. Joon DL, Hasegawa M, Sikes C, Khoo VS, Terry NH, Zagars GK, Meistrich ML, Pollack A. Supraadditive apoptotic response of R3327-G rat prostate tumors to androgen ablation and radiation. *Int J Radiat Oncol Biol Phys* 1997;38(5):1071–1077. [PubMed: 9276374]
30. Fowler JE Jr, Whitmore WF Jr. The response of metastatic adenocarcinoma of the prostate to exogenous testosterone. *J Urol* 1981;126(3):372–375. [PubMed: 7277602]
31. Taylor CD, Elson P, Trump DL. Importance of continued testicular suppression in hormone-refractory prostate cancer. *J Clin Oncol* 1993;11(11):2167–2172. [PubMed: 8229130]
32. Hussain M, Wolf M, Marshall E, Crawford ED, Eisenberger M. Effects of continued androgen-deprivation therapy and other prognostic factors on response and survival in phase II chemotherapy trials for hormone-refractory prostate cancer: A Southwest Oncology Group Report. *J Clin Oncol* 1994;12:1868–1875. [PubMed: 8083710]
33. Chao D, Harland SJ. The importance of continued endocrine treatment during chemotherapy of hormone-refractory prostate cancer. *Eur Urol* 1997;31(1):7–10. [PubMed: 9032527]
34. Dawson NA. Apples and oranges: Building a consensus for standardized eligibility criteria and end points in prostate cancer clinical trials. *J Clin Oncol* 1998;16(10):3398–3405. [PubMed: 9779719]

35. Pollack A, Block NL, Stover BJ, Irvin GL III, Fuentes MP, Claflin AJ, Malinin TI. In vivo selection of androgen-insensitive cells in R3327-G rat prostate tumors: Diethylstilbestrol diphosphate treatment versus orchiectomy. *J Natl Cancer Inst* 1983;70(5):907–914. [PubMed: 6573535]
36. Isaacs JT, Coffey DS. Adaptation versus selection as the mechanism responsible for the relapse of prostatic cancer to androgen ablation therapy as studied in the Dunning R-3327-H adenocarcinoma. *Cancer Res* 1981;41(12 Pt 1):5070–5075. [PubMed: 7307008]
37. Pollack A, Irvin GL, Block NL, Lipton RM, Stover BJ, Claflin AJ. Hormone sensitivity of the R3327-G rat prostate adenocarcinoma: Growth rate, DNA content, and hormone receptors. *Cancer Res* 1982;42(6):2184–2190. [PubMed: 7074599]
38. Colombel M, Symmans F, Gil S, O'Toole KM, Chopin D, Benson M, Olsson CA, Korsmeyer S, Buttyan R. Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. *Am J Pathol* 1993;143(2):390–400. [PubMed: 7688182]
39. Umekita Y, Hiipakka RA, Kokontis JM, Liao S. Human prostate tumor growth in athymic mice: Inhibition by androgens and stimulation by finasteride. *Proc Natl Acad Sci USA* 1996;93(21):11802–11807. [PubMed: 8876218]
40. Chuu CP, Hiipakka RA, Fukuchi J, Kokontis JM, Liao S. Androgen causes growth suppression and reversion of androgen-independent prostate cancer xenografts to an androgen-stimulated phenotype in athymic mice. *Cancer Res* 2005;65(6):2082–2084. [PubMed: 15781616]
41. Gao M, Ossowski L, Ferrari AC. Activation of Rb and decline in androgen receptor protein precede retinoic acid-induced apoptosis in androgen-dependent LNCaP cells and their androgen-independent derivative. *J Cell Physiol* 1999;179(3):336–346. [PubMed: 10228952]
42. English HF, Kyprianou N, Isaacs JT. Relationship between DNA fragmentation and apoptosis in the programmed cell death in the rat prostate following castration. *Prostate* 1989;15(3):233–250. [PubMed: 2555799]
43. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL. Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004;10(1):33–39. [PubMed: 14702632]
44. Kokontis JM, Hsu S, Chuu CP, Dang M, Fukuchi J, Hiipakka RA, Liao S. Role of androgen receptor in the progression of human prostate tumor cells to androgen independence and insensitivity. *Prostate* 2005;65(4):287–298. [PubMed: 16015608]
45. Eder IE, Culig Z, Ramoner R, Thurnher M, Putz T, Nessler-Menardi C, Tiefenthaler M, Bartsch G, Klocker H. Inhibition of Lncap prostate cancer cells by means of androgen receptor antisense oligonucleotides. *Cancer Gene Ther* 2000;7(7):997–1007. [PubMed: 10917202]
46. Eder IE, Hoffmann J, Rogatsch H, Schafer G, Zopf D, Bartsch G, Klocker H. Inhibition of LNCaP prostate tumor growth in vivo by an antisense oligonucleotide directed against the human androgen receptor. *Cancer Gene Ther* 2002;9(2):117–125. [PubMed: 11857028]
47. Liao X, Tang S, Thrasher JB, Griebing TL, Li B. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol Cancer Ther* 2005;4(4):505–515. [PubMed: 15827323]
48. Zegar-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res* 2002;62(4):1008–1013. [PubMed: 11861374]
49. Culig Z, Steiner H, Bartsch G, Hobisch A. Mechanisms of endocrine therapy-responsive and -unresponsive prostate tumours. *Endocr Relat Cancer* 2005;12(2):229–244. [PubMed: 15947099]
50. Agus DB, Cordon-Cardo C, Fox W, Drobnjak M, Koff A, Golde DW, Scher HI. Prostate cancer cell cycle regulators: Response to androgen withdrawal and development of androgen independence. *J Natl Cancer Inst* 1999;91(21):1869–1876. [PubMed: 10547394]
51. Shenk JL, Fisher CJ, Chen SY, Zhou XF, Tillman K, Shemshedini L. p53 represses androgen-induced transactivation of prostate-specific antigen by disrupting hAR amino- to carboxyl-terminal interaction. *J Biol Chem* 2001;276(42):38472–38479. [PubMed: 11504717]
52. Cronauer MV, Schulz WA, Burchardt T, Ackermann R, Burchardt M. Inhibition of p53 function diminishes androgen receptor-mediated signaling in prostate cancer cell lines. *Oncogene* 2004;23(20):3541–3549. [PubMed: 15077179]

Fig. 1. Cell growth in complete (CM), AD, or AD + R1881 medium. LNCaP (**A**), LNCaP-Res (**B**), and LNCaP-BST (**C**) cells were cultured in complete, AD, or AD + R1881 medium for 3 days and the number of viable cells counted at the indicated time.

Fig. 2.

A: Immunoblot assays of key related proteins in LNCaP, LNCaP-Res, and LNCaP-BST cells grown for 3 days in complete, AD, or AD + R1881 medium. **B,C:** Effects of AS-MDM2 on the expression of proteins in LNCaP-Res and LNCaP-BST cells. Cells were cultured in complete, AD or AD + R1881 medium for 3 days and transfected with 200 nM AS-MDM2 or MM for 24 hr. The number under each band is expressed as a percentage of complete (CM) medium in LNCaP cell (A) or Lipofectin control (LC) (B,C), normalized by the corresponding β-actin levels.

Fig. 3.

Effect of AS-MDM2 alone or in combination with AD $\pm$ R1881 on apoptotic cell death in LNCaP-Res and LNCaP-BST cells. Cells were cultured in complete (CM), androgen deprivation (AD) or AD + R1881 medium for 3 days, and then incubated with 200 nM of AS-MDM2 or MM for 30 or 40 hr in the presence of Lipofectin (LC). **A,C:** Percentages of Annexin V positive cells were measured by flow cytometric assay. **B,D:** Caspase 3 + 7 activity (RFLU, relative fluorescent units) was measured by fluorometric assay. The data shown represent the average values ($\pm$ SEM) from three independent experiments. *** $P < 0.0001$ (LNCaP-Res cells), $P < 0.01$ (LNCaP-BST cells) compared with all other groups, ** $P < 0.01$ compared with MM control in the same medium group, * $P < 0.05$ compared with MM control in the same medium group (one-way ANOVA, LSD test).

Fig. 4. Coronal views of two MRI scans taken 6 weeks apart on a mouse LNCaP-Res tumor grown orthotopically. The tumor had volume of 10 mm³ (A) and 190 mm³ (B). Correlation between the MRI and caliper measured tumor volume ($R^2 = 0.90$, $P < 0.001$) (C). Correlation between the MRI measurements and tumor weight ($R^2 = 0.90$, $P < 0.001$) (D). The data were pooled from both cell line tumors for statistical analysis.

Fig. 5. Effect of AS-MDM2 alone or in combination with AD (castration) on tumor growth of LNCaP-Res(A) and LNCaP-BST (B) xenografts. Treatments were initiated when tumor volumes were approximately 10 – 20 mm³. AS-MDM2 or MM was given by i.p. injection at doses of 25 mg/kg per day, 5 days/week for 15 days. The median tumor volume values were measured at the time points indicated. Western blot analysis of MDM2 expression from pooled LNCaP-Res (C) and LNCaP-BST (D) tumors. Mice were treated by i.p. injection with AS-MDM2 or MM (25mg/kg/perday), and saline (PBS) for 7 consecutive days when tumor volumes were approximately 60 – 100mm³.

TABLE I
Calculated Tumor Doubling Times, Volumes at 6 Weeks and Freedom From Tumor Volume Failure

Mice	Group	LNCaP-BST			LNCaP-Res		
		Tumor doubling time, M±SEM (days)	Tumor volume at 6 weeks, M±SEM (mm ³)	FFTVF	Tumor doubling time, M±SEM (days)	Tumor volume at 6 weeks, M±SEM (mm ³)	FFTVF
Normal	MM	17±9	230±34	10% (1/10)	8±1	298±2	0% (0/8)
	AS	10±1	266±18	0% (0/11)	9±2	263±37	12% (1/8)
Castrated (AD)	MM	40±13 ^c	186±45	30% (3/10)	10±3	257±43	14% (1/7)
	AS	48±14 ^c	161±44 ^a	40% (4/10)	17±3 ^d	134±59 ^b	57% (4/7) ^e

AS = antisense MDM2; MM = antisense mismatch.
FFTVF = freedom from MRI-based tumor volume failure: tumors ≤30 mm³ at 6 weeks.
Tumor doubling times and average tumor volume were calculated from fitted data.
^aCompared to AS (*P* < 0.05).
^bCompared to MM (*P* < 0.05).
^cCompared to AS and MM (*P* < 0.05).
^dCompared to AS, MM, and MM + AD groups (*P* < 0.05).
^e*P* < 0.1 compared to AS, MM, and MM + AD groups.