Preclinical Development

Reduced Expression of the Androgen Receptor by Third Generation of Antisense Shows Antitumor Activity in Models of Prostate Cancer

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Abstract

The androgen receptor (AR) is a member of a unique class of transcription factors because it contains a ligand-binding domain that, when activated, results in nuclear translocation and the transcriptional activation of genes associated with prostate cancer development. Although androgen deprivation therapies are effective initially for the treatment of prostate cancer, the disease eventually relapses and progresses to castration-resistant prostate cancer (CRPC). Nonetheless, the AR still plays a critical role because late-stage investigational agents that deplete testosterone (abiraterone) or block ligand binding (MDV3100) can still control tumor growth in patients with CRPC. These findings indicate that downmodulation of AR expression may provide a complementary strategy for treating CRPC. In this article, we describe a novel, locked, nucleic acid–based antisense oligonucleotide, designated EZN-4176. When administered as a single agent, EZN-4176 specifically downmodulated AR mRNA and protein, and this was coordinated with inhibition of the growth of both androgen-sensitive and CRPC tumors in vitro as well as in animal models. The effect was specific because no effect on growth was observed with a control antisense oligonucleotide that does not recognize AR mRNA, nor on tumors derived from the PC3, AR-negative, tumor cell line. In addition, EZN-4176 reduced AR luciferase reporter activity in a CRPC model derived from C4-2b cells that were implanted intratibially, indicating that the molecule may control prostate cancer that has metastasized to the bone. These data, together with the continued dependency of CRPC on the AR signaling pathway, justify the ongoing phase I evaluation of EZN-4176 in patients with CRPC. Mol Cancer Ther; 10(12); 2309–19. ©2011 AACR.

Introduction

Prostate cancer is the second leading cause of cancer-related deaths among men in the United States (1). For locally advanced, recurrent, or metastatic prostate cancer, initial systemic therapy is based on blocking activation of the androgen receptor (AR) by androgen deprivation therapy (ADT), including surgery (orchiectomy) or chemical methods (luteinizing hormone-releasing hormone agonists/antagonists or AR antagonists such as bicalutamide; ref. 2). Because the AR contains a ligand-binding domain that leads to nuclear localization and transactivation, ADT directly inhibits AR-induced activation of genes that stimulate prostate cancer growth (3). Current ADT in patients with advanced disease leads to remissions lasting 3 to 4 years. However, for virtually all patients, the disease progresses to castration-resistant prostate cancer (CRPC). As patients with ADT-refractory prostate cancer gain limited benefit from cytotoxic therapies such as taxanes or mitoxantrone combined with prednisone (3–5), therapies that further inhibit AR activation have been considered.

Compelling evidence shows that the AR remains active despite ADT and promotes further cancer progression (2, 6). Late-stage investigational agents such as abiraterone acetate (a CYP17 enzyme inhibitor that effectively blocks the synthetic pathway of testosterone; ref. 7) and MDV3100 (a second generation of AR antagonist; refs. 8, 9) reduce prostate-specific antigen (PSA) levels and control tumor growth in CRPC, thereby underscoring the importance of the AR signaling pathway in the castration-resistant setting. A complementary therapy capable of downregulating the mRNA encoding the AR would be a novel therapeutic strategy in this setting.
Locked nucleic acid–based antisense oligonucleotides (LNA-ASO), which downregulate mRNA levels, have overcome several issues of older antisense technologies and are likely to translate into applications with clinical benefits. In particular, LNA-ASOs have shown very high binding affinity to mRNA, excellent potency for target mRNA downmodulation, improved resistance to nuclease digestion, and excellent stability in plasma and tissues in preclinical studies (10). These features allow LNA-ASOs simplistically prepared in saline to be highly effective in vitro and in vivo (11, 12). The LNA technology has been used to design antisense molecules to hypoxia-inducible factor-1α (HIF-1α; ref. 13) and survivin (14) for the control of cancer as well as a microRNA (miRNA) antagonist that mimics miRNA-122 for the control of cholesterol levels (15) and hepatitis C infection (16). We have designed an LNA-ASO, designated EZN-4176, for ARs and it specifically binds within exon 4 of the AR mRNA and consequently downmodulates AR expression in vitro and in vivo. We explored the antitumor activity of EZN-4176 in both androgen-sensitive and castration-resistant tumor models.

Materials and Methods

Oligonucleotides
Oligonucleotides were synthesized as described previously (13). EZN-4176 (5′-ACCAGTTTTCtACGC-3′) is a fully phosphorothioated oligonucleotide complementary to residues in exon 4 of the AR. Capital letters denote LNA monomers and lowercase letters denote DNA monomers. 5′-Cy5.5-labeled EZN-4176 LNA/DNA gapmer and a mismatched control oligonucleotide (mismatched bases are indicated in italics), designated as EZN-4176-MM (5′-ACCaagtttcttcACGC-3′), as well as a scrambled control, designated as EZN-3046 (5′-CGCAgattagaaACCt-3′), were also synthesized. G3139 (5′-tcctcccagcgtgcgccat-3′), a fully mismatched control oligonucleotide (mismatched bases indicated in italics), was obtained from TriLink. ODN1826 (5′-tcctcccagcgtgcgccat-3′) was obtained from InvivoGen.

Growth inhibition
LNCaP cells were plated at a density of 1,000 cells per well in 24-well plates in culture medium and incubated overnight. The medium was replaced with phenol red-free medium containing 5% charcoal-stripped serum the next day. On the third day, the medium was replaced with charcoal-stripped serum medium containing compounds or without dihydroxytestosterone (DHT). On day 7, 100 μL MTS was added to the culture and incubated until the desired OD540 was achieved.

mRNA downmodulation in tumors
Twenty-four hours after the last dose, tumors were harvested. Five to 10 mg of each tumor sample was transferred to 1.5 mL Lysing Matrix D tubes containing 1 mL lysis/binding solution and then homogenized in FastPrep (MP Biomedicals). RNA purification, cDNA synthesis, and quantitative real-time PCR were carried out as previously described (13). AR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were quantified using TaqMan Assay Kits (Applied Biosystems) according to the manufacturer’s instructions. TaqMan Assay Kits Hs00171722_m1, 4326317E, Hs01105076_m1, and Hs00237175_m1 were for use in human AR, GAPDH, PSA, and transmembrane protease serine 2 (TMPRSS2), respectively.

Western blot analysis
Tumor cells and tissue samples were analyzed as previously described (12). AR protein and α-tubulin were probed with an antibody against AR (SC-815; Santa Cruz Biotechnology) and α-tubulin (T5168; Sigma), respectively.

Generation of reporter line expressing AR-responsive luciferase
LNCaP or C4-2b cells were infected according to the manufacturer’s instructions with lentiviral particles containing firefly luciferase gene under the control of a minimal (m) CMV promoter and tandem repeats of the AR transcriptional response element (TRE; SABiosciences). Infected cells were treated with 1 mg/mL puromycin and selected for more than 2 weeks.

Human tumor xenografts
CWR-22 tumors were established by subcutaneous injection of CWR-R22 tumors of a brie, equivalent to approximately 0.1 g tumor, into the right axillary flank of 6- to 8-week-old male noncastrated nude mice (e.g., 500 mg of tumor tissue minced with scalpel to produce brie, which was mixed with 500 μL of 100% Matrigel, and used for injection into 5 mice). The growth of the tumors requires implantation of a slow-release DHT pellet (60-day pellet; Innovative Research of America), into the flank of mice. Tumors were measured in 2 dimensions with calipers, and tumor volume was calculated as length × width²/2.

C4-2b and LuCaP35V tumors were established by subcutaneous injection of 0.1 mL brie (equivalent to ~0.1 g tumor in 100% Matrigel) into the right axillary flank of 6- to 8-week-old castrated, male severe combined immunodeficiency (SCID) mice. PC3 tumors were established by subcutaneous injection of 5 × 10⁶ cells per mouse. Tumor sizes and body weights were monitored biweekly, and the animals were euthanized when individual tumor volumes reached approximately 1,680 mm³. Animal experiments were conducted in the animal facility of the University of Medicine and Dentistry of New Jersey in accordance with the current guidelines for animal welfare and Institutional Animal Care and Use Committee protocols.

Bone model
The model was established by injection of 2 × 10⁶ C4-2b-AR-Luc cells per mouse suspended in 0.01 mL of media into the right tibia of male C57BL/6J mice. After 4 days, bioluminescent images were obtained at baseline.
(predose) as follows: mice were injected intraperitoneally with 150 mg/kg of luciferin; 8 minutes after luciferin injection, mice were anesthetized. Mice were imaged 12 minutes after luciferin injection using the Xenogen IVIS imaging System (Caliper Life Sciences). Mice with a total flux between $2 \times 10^6$ and $9 \times 10^7$ photons/s were randomized into the groups, treated, and imaged to assess the effect of compounds on AR activity.

### Biodistribution of Cy5.5-labeled EZN-4176 in CWR-22 xenograft tumor model

After the tumors reached an average volume of 500 mm$^3$, mice received treatment with Cy5.5-EZN-4176. The retention of Cy5.5-EZN-4176 was examined at different times following a single bolus intravenous injection of 60 mg/kg EZN-4176 containing Cy5.5-EZN-4176, diluted to 0.0475 mg/mL in unlabeled EZN-4176 to be used for the intravenous injection. The tumors were excised at each time point and imaged with the Xenogen Lumina. Fluorescent (Cy5.5 excitation at 675 and emission at 694 nm) images of tumors were acquired and reported as the pixel intensity (total efficiency) with the autofluorescence subtracted using a 3-dimensional (3D) Xenogen IVIS optical imaging system.

### Data analysis

For efficacy studies, the percentage of tumor growth inhibition (TGI) was calculated using the formula $\frac{100 \times (C_1 - C_2)}{C_1}$, where $C_1$ = mean tumor volume of control group at time $t$; $C_2$ = mean tumor volume of control group at time 0; $T_1$ = mean tumor volume of treatment group at time $t$; and $T_0$ = mean tumor volume of treatment group at time 0. Differences between treatments were compared using an unpaired 2-tailed Student $t$ test using the GraphPad/InStat3 computer program.

### Results

#### Effect on target downmodulation and proliferation

Initially, EZN-4176 was tested in vitro in prostate cancer cell lines by lipofection. Under these conditions, downmodulation of mRNA and protein levels of both AR (Supplementary Fig. S1A and S1B) and PSA (Supplementary Fig. S1C and S1D) was observed with an IC$_{50}$ of approximately 5 nmol/L in androgen-dependent (LNCaP) prostate cancer cells. Consequently, cell growth was inhibited (IC$_{50}$<10 nmol/L; Supplementary Fig. S1E). The biologic effects were specific because in a scrambled control LNA-ASO, EZN-3046 was ineffective. Furthermore, the inhibitory effects on growth were not observed with EZN-4176 in an AR-negative cell line (Supplementary Fig. S1F).

Because transfection conditions are highly artificial and do not represent the context of our in vivo experiments in which no transfection systems were used, the remainder of the in vitro studies were conducted without lipofection. This was enabled by LNA technology because LNA-ASOs are not susceptible to nuclease digestion when placed in plasma for more than 4 days (10). In addition, it has been shown that LNA-ASOs used without transfection are highly effective and specific at downregulating target mRNA and protein (11, 12). We first investigated whether EZN-4176 could inhibit the DHT-induced growth of LNCaP cells (17). Under these conditions, DHT alone stimulated approximately 7-fold growth (Fig. 1A).
Significant inhibition of hormone-dependent cell growth was noted in the presence of 2.5 μmol/L EZN-4176. To show that the results were not simply due to an off-target oligonucleotide backbone effect, a mismatched control oligonucleotide, EZN-4176-MM, was designed and tested. Although EZN-4176-MM showed some antiproliferative effect against the cells without DHT, it did not significantly inhibit DHT-induced growth (Fig. 1A).

To confirm that the growth inhibition was due to target inhibition, the effect of EZN-4176 on AR expression was examined. AR mRNA and protein levels were found to be downmodulated by EZN-4176 but not by EZN-4176-MM (Fig. 1B and C). Interestingly, a more profound effect was found at the AR protein level. To validate that the growth inhibition was associated with the AR activity, downmodulation of AR transcription was shown in LNCaP-AR-luc cells. AR activity, measured in luciferase light units, was dramatically induced by DHT at 10 nmol/L (Fig. 1D). Nonetheless, EZN-4176 inhibited DHT-induced AR transcriptional activity by 62%, 69%, and 77% with 2.5, 5, and 10 μmol/L EZN-4176, respectively. A moderate effect was observed after the cells were treated with a suboptimal dose of 1 μmol/L bicalutamide. However, EZN-4176 potentiated the effect of 1 μmol/L bicalutamide (Fig. 1D), indicating that this combination may offer better antitumor activity. The control oligonucleotide EZN-4176-MM alone had no effect on DHT-induced AR transcriptional activity (data not shown).

Antitumor activity in xenograft model

The in vivo therapeutic efficacy of EZN-4176 was evaluated in an androgen-dependent, AR-positive CWR-22 tumor xenograft model (18). EZN-4176 inhibited the growth of CWR-22 tumors by approximately 66% on day 27, whereas EZN-4176-MM did not inhibit tumor growth (Fig. 2A). The inhibitory effect was similar to that observed with bicalutamide (Fig. 2A). To show that the antitumor effect was associated with the AR status, we treated mice bearing AR-negative PC3 prostate tumors with EZN-4176. EZN-4176 was inactive in this tumor (Fig. 2B), indicating that the antitumor effect observed with the CWR-22 xenograft model was probably due to AR downmodulation.

To confirm that the observed tumor growth inhibition was associated with target downmodulation, the effect of EZN-4176 on AR and its downstream target genes, such as PSA and transmembrane protease, serine 2, TMPRSS2 (19), was tested in a short-term study in the CWR-22 tumor model. EZN-4176, but not EZN-4176-MM, at 60 mg/kg downmodulated 40% of the human AR mRNA in tumors (Fig. 2C). Moreover, EZN-4176 downmodulated mRNA expression of human PSA (Fig. 2D) and TMPRSS2 (Fig. 2E). In contrast, EZN-4176-MM increased the expression of mRNA of AR and its target genes such as PSA and TMPRSS2 (Fig. 2C–E). The upregulation of these genes could be attributable to the phosphorothioate backbone effect of the antisense molecule. To show that EZN-4176 downmodulated AR protein expression in vivo in the CWR-22 model, Western blot analysis was conducted 24 hours after the last dose. Tumors from individual mice in the EZN-4176 60 mg/kg group were compared with those in the saline control group (Fig. 2F, top) or with those in mice with EZN-4176-MM administered at 60 mg/kg (Fig. 2F, bottom). Treatment with EZN-4176 resulted in a significant downmodulation of AR protein level when compared with treatment with either saline or EZN-4176-MM control.

Effect in the castration-resistant model

To explore the potential use of EZN-4176 in CRPC, we tested the effect of EZN-4176 in the AR-positive castration-resistant C4-2b model (20). To verify that EZN-4176 affected the functional activity of the AR, we assessed the effect of the compound in C4-2b-AR-luc cells. EZN-4176 specifically inhibited DHT-induced reporter activation in a dose-dependent manner (Fig. 3A). The activity of EZN-4176 was compared with bicalutamide as well as MDV3100. Comparisons with MDV3100 are particularly relevant because it has shown antitumor activity in patients with CRPC and animal models that are less responsive to bicalutamide (9). Interestingly, treatment with 1.25 μmol/L EZN-4176 resulted in a potent inhibition similar to that seen with 10 μmol/L bicalutamide or MDV3100.

To show the specificity of the effect of EZN-4176 in the C4-2b castration-resistant tumor model, we first determined if EZN-4176 could repress the luciferase activity in C4-2b-AR-luc cells after they form tumors in the flanks of nude mice. An example of images from an animal before and after dosing with EZN-4176 (40 mg/kg) is shown in Fig. 3B (left); measurement of the bioluminescence from all treatment groups is shown in the figure, to the right. EZN-4176, but not EZN-4176-MM, significantly inhibited the signal in all dose groups (P < 0.05). Bicalutamide showed marked but statistically insignificant inhibition. On the basis of these results, efficacy studies were conducted in mice bearing C4-2b tumors. A dose of 20 mg/kg EZN-4176 showed significant TGI (Fig. 3C). The effect was specific because EZN-4176-MM showed no significant TGI. To further show that EZN-4176 may have use in castration-resistant tumors, we treated another CRPC tumor xenograft model, LuCaP35V (21), with EZN-4176. In our previous study, bicalutamide failed to show significant antitumor effect in this model (Supplementary Fig. S3). Interestingly, in this study, EZN-4176 showed antitumor activity comparable with that of MDV3100 (Fig. 3D).

Because prostate cancer frequently metastasizes to bone (22), we investigated the use of EZN-4176 in a bone model established by injecting C4-2b-AR-luc cells into the tibia of SCID mice. Imaging was used to monitor the growth of the tumor, which grew progressively larger over time (Supplementary Fig. S4). Twenty-one days after tumor implantation, the mice were treated with the indicated compounds. Examples of images of bone tumors from an animal before and after dosing with either saline or
EZN-4176 (40 mg/kg) are shown in Fig. 3E, and quantitative analyses from all treatment groups are shown in Fig. 3F. On day 8, EZN-4176 specifically and potently inhibited the signal (P < 0.05), whereas neither EZN-4176-MM nor bicalutamide showed any effect, indicating that EZN-4176 may be superior to bicalutamide in treating prostate cancer bone metastases (Fig. 3F).

**Tumor accumulation, tissue distribution of EZN-4176, and sustained target downmodulation in tumors**

To associate the antitumor effect with the presence of EZN-4176 in the tumors, we evaluated the presence and duration of EZN-4176 in established CWR-22 tumors. We first developed a Cy5.5-labeled EZN-4176 and determined that its activity in AR mRNA downmodulation was equivalent to that of EZN-4176 in LNCaP cells (Supplementary Fig. S2). Tumor-bearing mice were then injected with a single i.v. dose (60 mg/kg) of Cy5.5-labeled EZN-4176. At different time points after the injection of oligonucleotide (range: 4-168 hours), animals were sacrificed and tissues were analyzed by fluorescence imaging.

Rapid and prominent distribution of the oligonucleotide to tumors was evident, with high intensity as early as 4 hours after dosing (Fig. 4A). The uptake of EZN-4176 was equivalent to that of EZN-4176 in LNCaP cells (Supplementary Fig. S2). Tumor-bearing mice were then injected with a single i.v. dose (60 mg/kg) of Cy5.5-labeled EZN-4176. At different time points after the injection of oligonucleotide (range: 4-168 hours), animals were sacrificed and tissues were analyzed by fluorescence imaging.

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Figure 3. Effect of EZN-4176 in castration-resistant models. A, effect on AR transcription activity. C4-2b-AR-luc cells were treated with indicated compounds for 5 days followed by 10 nmol/L DHT treatment. Luciferase activity was determined after 24 hours. Data are mean ± SD (n = 6); *, P < 0.05, treatment groups compared with DHT group. B, mice (n = 8 per group) bearing C4-2b-AR-luc tumors were treated as follows: EZN-4176 (every 3 days, i.v.); EZN-4176-MM (every 3 days, i.v.); and bicalutamide (every day, orally). At baseline (predose) and on day 7, bioluminescence images were captured. Data were normalized values to tumor sizes. **, statistically significant; P < 0.05. C, effect on the growth of C4-2b tumors. Mice (n = 8 per group) were treated with saline, EZN-4176 (every 3 days, i.v.), or EZN-4176-MM (every 3 days, i.v.). **, statistically significant compared with saline group; P < 0.05. ***, statistically significant compared with EZN-4176-MM; P < 0.05. D, effect on the growth of LuCaP35V tumors. Mice were treated with saline, EZN-4176 (every 3 days, i.v.), EZN-4176-MM (every 3 days, i.v.), or MDV3100 (every day, orally); **, statistically significant compared with EZN-4176-MM group; P < 0.05. E, imaging of bone tumors treated with saline or EZN-4176 (every 3 days, i.v.). Color scale: Min = 4.51e4 Max = 2.08e7. **, effect on AR activity in bone tumors. On day 21, mice bearing bone tumors were treated with indicated compounds: EZN-4176 and EZN-4176-MM (every 3 days, i.v.); bicalutamide (every day, orally). Data are mean ± SEM (n = 5 per group); **, statistically significant compared with saline group; P < 0.05.
was graded by the efficiency scale that is proportional to EZN-4176 uptake, intensity of the organ signal, that is harvested and imaged. Fluorescent (postinjection of oligo), tumors were 4176. At the indicated time points (dose (60 mg/kg, i.v.) of Cy5.5-EZN-Mice were injected with a single 4176 in CWR-22 xenograft tumors. A, duration of Cy5.5-labeled EZN-sustained target downmodulation. Residual time in tumors and Figure 4. EZN-4176 showed long residence time in tumors. Figure 5 shows that, in the C4-2b model, the amount of EZN-4176 reached the amount of EZN-4176 in tumors. Figure 5 shows that, (Supplementary Materials and Methods) to measure photography/tandem mass spectrometry (LC/MS-MS) method and precise analysis was done using a liquid chromatog- represent intact drug (23), a quantitative, more reliable relative level of drug over time, and is likely to the top of the scale. B, total intensity (yellow, maximum uptake) no uptake) at the bottom to high intensity (dark red, or background intensity (light red, at the right of each image with low weight. On day 7, EZN-4176 was present in all of the on June 21, 2017. © 2011 American Association for Cancer Research. mct.aacrjournals.org Downloaded from

be approximately 8 days (13). Furthermore, we harvested various organs and analyzed the amount of EZN-4176 at 72 hours post dosing. To compare the relative amount of oligonucleotides present in each organ, the total efficiency (total signal) of each organ was normalized to organ weight. On day 7, EZN-4176 was present in all of the organs examined (Fig. 4C), with high quantities present in the liver and kidney. Interestingly, the amount of labeled EZN-4176 in the tumor was approximately 50% and 75% of the quantities in the kidneys and liver, respectively. The duration of residence of the compound in the tumor provided additional support to the findings of AR mRNA and protein downmodulation (Fig. 2C and F). The imaging results were further supported by the data that show significant AR mRNA downmodulation until at least day 5 (Fig. 4D).

Pharmacokinetic and pharmacodynamic analyses of EZN-4176 in xenograft model

Although Cy5.5-labeled EZN-4176 can be used to assess the relative level of drug over time, and is likely to represent intact drug (23), a quantitative, more reliable and precise analysis was done using a liquid chromatography/tandem mass spectrometry (LC/MS-MS) method (Supplementary Materials and Methods) to measure the amount of EZN-4176 in tumors. Figure 5 shows that, in the C4-2b model, the amount of EZN-4176 reached 1.6 μmol/L after administration of a single dose of 40 mg/kg. The concentration of EZN-4176 almost doubled after a second dose (Fig. 5). The concentration continued to increase after the third and fourth doses, albeit at a slower rate. The concentration of EZN-4176 in CWR-22 tumors was also determined. After 2 and 4 doses (scheduled every 3 days), the concentration of EZN-4176 reached

![Figure 4](image-url)  
*Figure 4. EZN-4176 showed long residence time in tumors and sustained target downmodulation. A, duration of Cy5.5-labeled EZN-4176 in CWR-22 xenograft tumors. Mice were injected with a single dose (60 mg/kg, i.v.) of Cy5.5-EZN-4176. At the indicated time points (postinjection of oligo), tumors were harvested and imaged. Fluorescent intensity of the organ signal, that is proportional to EZN-4176 uptake, was graded by the efficiency scale to the right of each image with low or background intensity (dark red, no uptake) at the bottom to high intensity (yellow, maximum uptake) at the top of the scale. B, total intensity divided by organ weight. D, downmodulation of AR mRNA lasted up to 5 days after the treatment of EZN-4176 (every 3 days × 4, i.v.). , statistically significant; P < 0.05.

![Figure 5](image-url)  
*Figure 5. Pharmacokinetic/pharmacodynamic assessment of EZN-4176 in C4-2b CRPC. C4-2b tumor-bearing mice were treated with 40 mg/kg EZN-4176 with indicated dosing schedule. Twenty-four hours after the last dose, mice were sacrificed and tumors harvested. Effect on AR mRNA downmodulation (gray bars) and the concentration determination of EZN-4176 in the tumors (line) were measured. Data are mean ± SEM (n = 5). qd, every day; q3d, every 3 days.
1.78 and 3.85 μmol/L, respectively. Collectively, our results show that, in tumors, EZN-4176 administrated intravenously reached efficacious concentrations (Fig. 1) after a schedule where one or more doses were administered every 3 days.

Analysis of the effect of EZN-4176 on plasma cytokine levels in mice

Oligonucleotide-based strategies including siRNAs and ASOs are known to have off-target effects through immune stimulation (24, 25), which may contribute to the antitumor activity (26). To rule out this possibility, we examined the effect of EZN-4176 on the activation of Toll-like receptors (TLR) in HEK293 cells transfected with 7 different TLRs. Supplementary Figure S5 shows that each individual TLR in the transfected cells was activated by the treatment of a corresponding ligand. However, there was no evidence of TLR-stimulated activation in HEK293 cells treated with EZN-4176. Furthermore, we tested the effect of EZN-4176 on a panel of cytokines, including IFN-γ, TNFα, interleukin-10, and keratinocyte chemoattractant (KC), cytokine levels in the plasma were measured with a multiplex cytokine kit (Meso Scale Discovery) at indicated time points. Data are mean ± SEM (n = 5).

Discussion

Recent preclinical and clinical trial results have shown that the AR plays an important role in the biology of CRPC. Therefore, new agents with novel mechanisms of interfering with the activity of the AR, including a small-molecule inhibitor that blocks transactivation of the AR (27) or an ASO-mediated specific downregulation of AR expression, may be beneficial in patients who fail to respond to the available therapies targeting the AR.
Previous studies have indicated that downregulation of the AR by siRNAs or first-generation ASOs against AR reduce AR expression and decrease cell growth in both androgen-sensitive and castration-resistant cell lines and animal tumor models (28–31). However, the use of siRNA or early generations of ASOs to treat patients with cancer has been hampered by the instability of these compounds, lack of ability to penetrate into cells, as well as their potential side effects, including immune activation.

Therefore, in this study, we used third-generation LNA oligonucleotide technology, where 6 of the 16 complementary oligonucleotide residues are composed of ribose sugars that are locked in a conformation that provides much higher binding affinity to the complementary mRNA than conventional DNA and 2′-MOE (2′-O-methoxyethyl)-based oligonucleotides (10). Such LNA oligonucleotides have low single-digit nanomolar or high picomolar IC50 values for mRNA downmodulation that have been achieved in cell culture for LNA-ASOs against AR as well as HIF-1α (13) and survivin (14, 23) when used with transfection reagents. Thus, LNA-ASOs have potencies similar to those of siRNAs but without the inherent instability of siRNAs. In addition, the LNA-ASOs are resistant to nuclease digestion and are not degraded even when incubated in plasma for 4 days at 37°C (23).

Because of these improved features, LNA-ASOs reconstituted in saline have shown target inhibition and anti-tumor activities in preclinical models without the use of any delivery agent (12, 32), a feature that distinguishes them from siRNAs that require delivery systems. The long residence time in tumors, sustained target inhibition, and TGI observed following the administration of EZN-4176 to mice in multiple tumor models further highlight the benefit of the LNA-ASOs.

One of the potential drawbacks of using an antisense approach pertains to the recent findings regarding AR splice variants (33–37), some of which may not contain the complementary binding site for EZN-4176. For example, one such variant designated AR3 (also known as AR-V7) has a deletion of exon 4. Because this exon encodes the complementary binding mRNA to EZN-4176, EZN-4176 has a deletion of exon 4. Because this exon encodes the complementary binding site for EZN-4176, EZN-4176 would not downregulate the expression of this AR variant. In contrast, a newly found variant, designated ARv567es, contains the target sequence for EZK-4176 and is a frequently detected AR variant important for cellular survival and growth (35). Therefore, ARv567es has the potential to be downmodulated by EZN-4176. Furthermore, it has been shown recently that the full-length AR is required for the variants to function (36), indicating that targeting full-length native AR is, perhaps, sufficient to inhibit tumor growth. A more complete understanding of AR variants in the biology of prostate cancer will provide insight regarding how to use EZN-4176 effectively.

The data show that EZN-4176 inhibits AR-mediated transcriptional activity and tumor growth similar to Casodex or MVD-3100. Because these agents block AR-mediated activity by different modalities compared with EZN-4176, combination of EZN-4176 with such inhibitors of AR may be superior to agents used alone. Preliminary data indicate that EZN-4176 has synergistic activity with MDV3100 in xenograft models (38) and will be the subject of future communications. The combination approach may also be extended to additional targets because prostate cancer development is governed by many factors. Targeting these key players may provide effective treatment for prostate cancer. A good example will be to target c-myc oncogene, which is amplified in almost 30% of prostate tumors and critical driver for prostate tumors (39–42). Targeting c-myc with LNA-based antisense in combination with EZN-4176 is an attractive and logical approach. Furthermore, numerous studies of AR and its associated target genes have provided a plethora of targets (43–55). Validation of these targets in relevant preclinical models and development of therapeutic drugs against them will provide opportunities for effective and safe treatment options for prostate cancer.

To conclude, EZN-4176, an LNA-ASO antisense molecule, showed significant downregulation of AR mRNA and protein. This effect was correlated with the ability of EZN-4176 to inhibit AR-dependent prostate tumor growth in vitro and in vivo, including models that are resistant to castration. These data justify ongoing phase I studies of EZN-4176 in patients with CRPC.

Disclosure of Potential Conflicts of Interest

All authors, except R.L. Vesole, were employees of Enzon Pharmaceuticals, Inc., when the presented studies were conducted.

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References


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