Cotargeting Androgen Receptor Splice Variants and mTOR Signaling Pathway for the Treatment of Castration-Resistant Prostate Cancer

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Abstract

**Purpose:** The PI3K/Akt/mTOR pathway is activated in most castration-resistant prostate cancers (CRPCs). Transcriptionally active androgen receptor (AR) plays a role in the majority of CRPCs. Therefore, cotargeting full-length (FL) AR and PI3K/Akt/mTOR signaling has been proposed as a possible, more effective therapeutic approach for CRPC. Here, we evaluated the efficacy and mechanism of combination therapy to block mTOR activity together with EPI-002, an AR N-terminal domain (NTD) antagonist that blocks the transcriptional activities of FL-AR and AR-Vs in models of CRPC.

**Experimental Design:** To determine the functional roles of FL-AR, AR-Vs, and PI3K/Akt/mTOR pathways, we employed EPI-002 or enzalutamide and BEZ235 (low dose) or everolimus in human prostate cancer cells that express FL-AR or FL-AR and AR-Vs (LNCaP95). Gene expression and efficacy were examined in vitro and in vivo.

**Results:** EPI-002 had antitumor activity in enzalutamide-resistant LNCaP95 cells that was associated with decreased expression of AR-V target genes (e.g., UBE2C). Inhibition of mTOR provided additional blockade of UBE2C expression. A combination of EPI-002 and BEZ235 decreased the growth of LNCaP95 cells in vitro and in vivo.

**Conclusions:** Cotargeting mTOR and AR-NTD to block transcriptional activities of FL-AR and AR-Vs provided maximum antitumor efficacy in PTEN-null, enzalutamide-resistant CRPC.

Introduction

Androgen deprivation therapy (ADT) is initially effective for most recurring prostate cancers. Unfortunately, the malignancy will eventually begin to grow again to form castration-resistant prostate cancer (CRPC). Transcriptionally active androgen receptor (AR) plays a major role in CRPC in spite of reduced blood levels of androgen (1, 2). AR mechanisms of resistance to ADT include: overexpression of AR (3, 4), gain-of-function mutations of AR LBD (5), intratumoral androgen synthesis (6), altered expression and function of AR coactivators (7, 8), aberrant post-translational modifications of AR (9, 10), and expression of AR splice variants (AR-V) which lack the ligand-binding domain (LBD; refs. 1, 11–13). Androgenins such as bicalutamide and enzalutamide target AR LBD, but have no effect on truncated constitutively active AR-Vs such as AR-V7 (14). Expression of AR-V7 is associated with resistance to current hormone therapies (14, 15). EPI compounds were developed to specifically target the AR amino-terminal domain (NTD) to block the transcriptional activities of full-length (FL)-AR and AR-Vs, which results in antitumor activity in CRPC xenografts (11, 12, 16). EPI-506, an analogue of EPI-002, is in clinical trials for CRPC patients that are resistant to enzalutamide or abiraterone.

The PI3K/Akt/mTOR pathway is a key oncogenic pathway in various cancers (17), and is linked to resistance to ADT in prostate cancers (18). Alterations of components in PI3K/Akt/mTOR pathway occur in 42% of primary prostate tumors and 100% of metastatic tumors (19). The PI3K/Akt/mTOR pathway is constitutively active due to loss of PTEN in the majority of advanced prostate cancers (20). Targeting PI3K/Akt/mTOR is therefore considering a promising approach to treat CRPC (21, 22). However, the effects of inhibiting PI3K/Akt/mTOR signaling on AR are controversial (23–26). There are numerous inhibitors that target PI3K/Akt/mTOR signaling such as rapamycin and its analogues, dual TORC1/2 inhibitors, pan-P13K inhibitors, Akt inhibitors, and dual PI3K/TORC1/2 inhibitors. Here, we used BEZ235, a dual PI3K/TORC1/2 inhibitor, to achieve better blockade of PI3K/Akt/mTOR signaling by inhibiting possible reactivation of PI3K and Akt through mTOR-dependent feedback loops (27, 28). Reciprocal feedback regulation of PI3K and FL-AR signaling in PTEN-deficient prostate cancer has been reported (23). From those studies, concomitant blockade of PI3K/Akt/mTOR and FL-AR signaling pathways were proposed to achieve better antitumoral activity in CRPC (29). To date, there are no reports that examine the impact of such a combination on human prostate cancer cells that are resistant to antiandrogens.
Truncated constitutively active AR-splice variants (AR-V) are correlated to resistance to drugs targeting AR-LBD either directly with antiandrogens or indirectly with approaches that reduce levels of androgen. EPI-002 is an AR N-terminal domain (NTD) antagonist that inhibits transcriptional activities of full-length (FL)-AR and AR-Vs. P110/Akt/mTOR is activated in advanced prostate cancer and accounts, in part, for resistance to androgen deprivation therapy. Here, we characterized a functional role of combination treatment with EPI-002 and mTOR inhibitors in a model of enzalutamide-resistant castration-resistant prostate cancer (CRPC) that is driven by AR-Vs. Our findings suggest that inhibition of mTOR activates FL-AR transcriptional activity, independent of Akt, which has been previously reported to increase expression of AR. Most importantly, inhibition of mTOR decreased levels of expression of AR-V7 target genes, such as UBE2C, which is correlated to CRPC. These results support a rationale for cotargeting the AR-NTD and mTOR for the treatment of CRPC patients.

Materials and Methods

Cells, reporter assays, and reagents

LNCaP, COS-1, and DU145 cells, plasmids (PSA-luciferase, PB-luciferase, AR3-luciferase, SxCaH4AS-TATA- luciferase, AR1-558Gal4DBD), and transfection protocols have been described previously (7, 11, 12). Cell lines were obtained from the following: LNCaP cells from Dr. Leland Chung (Cedars-Sinai Medical Center, Los Angeles, CA) in September 1993; COS-1 cells from Dr. Rob Kay (British Columbia Cancer Agency, Vancouver, British Columbia, Canada) in April 2000; DU145 cells from Dr. Victor Ling (British Columbia Cancer Agency, Vancouver, British Columbia, Canada) in October 1998; and LNCaP95 cells from Dr. Stephen Plymate (University of Washington, Seattle, WA) in February 2012. LNCaP and COS-1 cells were not authenticated in our laboratory, but were regularly tested to ensure they were mycoplasma-free (VenorTMGeM Mycoplasma Detection Kit, Sigma-Aldrich). LNCaP95 and DU145 cells were authenticated by short tandem repeat analysis and tested to ensure they were mycoplasma-free by DDC Medical in September 2013. All cells were maintained in culture for not more than 10 passages and regularly tested to ensure they were mycoplasma-free by DDC Medical in September 2013. All cells were maintained in culture for not more than 10 passages and regularly tested to ensure they were mycoplasma-free by DDC Medical in September 2013.

Gene expression analysis

LNCaP95 cells (180,000 cells/well) in 6-well plates were serum-starved for 24 hours before treating with vehicle, EPI-002 (35 μmol/L), enzalutamide (10 μmol/L), BEZ235 (15 nmol/L), or its combination for 1 hour prior to the addition of R1881 or EtOH for 48 hours. Total RNA was isolated using PureLink RNA Mini Kit (Life Technologies) and reverse transcribed to cDNA with High Capacity RNA-to-DNA Kit (Life Technologies). Quantitative real-time RT-PCR was performed in triplicates for each biologic sample. Expression levels were normalized to RPL13A housekeeping gene. Primers have been described previously (11, 31).

LNCaP95 FL-AR sequence

Total RNA (3 μg) from LNCaP95 cells was reverse transcribed using Superscript I First Strand Synthesis Kit (Invitrogen) with 5 μmol/L Oligo (dT)20. The coding region of FL-AR was PCR amplified with Platinum DNA Taq Polymerase High Fidelity in 50 μl with 1 U Taq polymerase, 1 mmol/L MgSO4, 0.2 mmol/L deoxynucleotide mix, and 2% DMSO with the AR primers (forward: 5’-AGGGAGAGGCGGTAAAGGAAGA-3’; reverse: 5’-CATGACGTGGGGTGGAATAGGC-3’). The PCR fragment and express AR-Vs. Here, we evaluate the therapeutic efficacy of a combination of EPI-002 and BEZ235 using CRPC models both in vitro and in vivo.

Transfection and luciferase assay

LNCaP95 cells (150,000 cells/well) and LNCaP cells (100,000 cells/well) in 12-well plates were transfected with PSA-luciferase, AR3-luciferase, or PB-luciferase reporters and pretreated the next day with DMSO, EPI-002 (25 μmol/L), enzalutamide (10 μmol/L), BEZ235 (15 nmol/L), or its combination for 1 hour before the addition of R1881 or EtOH under serum-free and phenol red-free conditions. After 48 hours of incubation, cells were harvested and luciferase activities measured and normalized to protein concentration. Transactivation of AR-NTD was measured in LNCaP and LNCaP95 cells as described (7). Transcriptional activity of AR-Vs was performed using AR-negative Cos-1 cells as reported (30). Transient knockdown of p110β or p110δ was performed in LNCaP95 cells using Lipofectamine RNAiMAX.

Western blot analysis

LNCaP95 cells (180,000 cells/well) in 6-well plates were serum-starved for 24 hours, followed by treatment with DMSO, EPI-002 (25 μmol/L), BEZ235 (15 nmol/L), or a combination for 1 hour prior to the addition of R1881 or EtOH for 48 hours. Antibodies used were: AR (1:1,000; Santa Cruz Biotechnology), AR-V7 (1:400; Precision), p110α (1:500; BD Biosciences), p110β (1:1,000; Abcam), p110δ (1:1,000; Abcam), PTEN (1:1,000), pS6 (1:2,000), pAktThr308 (1:1,000), pAktSer473 (1:2,000), p4EBP1 (1:1,000), total-Akt (1:1,000), total-S6 (1:1,000), total-4EBP1 (1:1,000), pERK/MAPK (1:1,000), and total-ERK/MAPK (1:1,000) from Cell Signaling Technology. β-Actin (1:10,000, Abcam) was used as a loading control.

Cell proliferation BrdUrd immunoassay

LNCaP95 cells (8000 cells/well) were seeded in a 96-well plate and incubated for 48 hours in RPMI with 10% charcoal-stripped serum before pretreating for 1 hour with DMSO, EPI-002 (25 μmol/L), enzalutamide (10 μmol/L), BEZ235 (15 nmol/L), and a combination of EPI-002 (25 μmol/L) and BEZ235 (15 nmol/L) in serum-free conditions prior to the addition of 0.1 nmol/L R1881 or EtOH. Bromodeoxyuridine (BrdUrd) incorporation was measured after 2 days using BrdU ELISA Kit (Roche Diagnostics).

Translational Relevance

Truncated constitutively active AR-splice variants (AR-V) are correlated to resistance to drugs targeting AR-LBD either directly with antiandrogens or indirectly with approaches that reduce levels of androgen. EPI-002 is an AR N-terminal domain (NTD) antagonist that inhibits transcriptional activities of full-length (FL)-AR and AR-Vs. P110/Akt/mTOR is activated in advanced prostate cancer and accounts, in part, for resistance to androgen deprivation therapy. Here, we characterized a functional role of combination treatment with EPI-002 and mTOR inhibitors in a model of enzalutamide-resistant castration-resistant prostate cancer (CRPC) that is driven by AR-Vs. Our findings suggest that inhibition of mTOR activates FL-AR transcriptional activity, independent of Akt, which has been previously reported to increase expression of AR. Most importantly, inhibition of mTOR decreased levels of expression of AR-V7 target genes, such as UBE2C, which is correlated to CRPC. These results support a rationale for cotargeting the AR-NTD and mTOR for the treatment of CRPC patients.
was gel purified and cloned into PCR 2.1 TOPO cloning vector using TOPO TA cloning kit from Invitrogen and transformed into chemically competent TOP10 cells. FL-AR plasmids were sequenced at the NAPS core Unit at The University of British Columbia (Vancouver, BC, Canada; http://naps.msl.ubc.ca).

**Animal studies**

Six- to 8-week-old male NOD-SCID mice were maintained in the Animal Care Facility in the British Columbia Cancer Research Centre. All animal experiments were approved by the University of British Columbia Animal Care Committee. Mice were castrated two weeks before inoculating LNCaP95 cells (5 million cells/tumor) subcutaneously, and divided into four groups: vehicle control [N-Methyl-2-pyrrolidone: polyethylene glycol 400 (10/90, v/v), n = 10], EPI-002 (100 mg/kg body weight, n = 8), BEZ235 (5 mg/kg body weight, n = 10), and a combination of EPI-002 (100 mg/kg body weight) and BEZ235 (5 mg/kg body weight; n = 8). Fresh solutions were prepared daily and application volume was 5 mL/kg body weight/dose. When the tumors reached approximately 80 mm^3 in size, animals were treated by oral gavage, every day, for 2 weeks. Body weight was measured every day and tumor volumes were measured twice a week using a caliper by the formula length × width × height. Tumors were harvested 1 hour after the last treatment and prepared for Western blot analyses, gene expression assays, and IHC.

**IHC**

Sections (5 μm thick) were processed and endogenous peroxidase blocked. Incubations with anti-pS6 (1:200; Cell Signaling Technology), anti-Ube2C (1:200; Boston Biochem), and anti-Ki-67 (1:50; Dako) were done at 4°C overnight. Antigen was detected with 3,3'-diaminobenzidine and counterstained with hematoxylin. For TUNEL staining, ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore) was used.

**Results**

**EPI-002 inhibited AR-V7, mTOR, and blocked BEZ235-induced FL-AR transcriptional activity**

A combination of PI3K inhibitor with antiandrogen is suggested to provide potential therapeutic advantage for the treatment of CRPC (23, 29). Constitutively, active AR-Vs lack LBD to which antiandrogens bind. Thus, cells that express these variants may be resistant to antiandrogens. No studies are reported that examine the combination of PI3K/Akt/mTOR inhibitor plus antiandrogen, or an AR-NTD antagonist, on prostate cancer cells that express constitutively active AR-Vs.

LNCaP95 human prostate cancer cells are androgen independent and enzalutamide resistant (32, 33). Proliferation of LNCaP95 cells is driven by truncated AR-Vs in spite of endogenous expression of functional FL-AR (32, 33). FL-AR from LNCaP95 cells was sequenced and no unique mutations that would confer resistance to enzalutamide were detected (data not shown) relative to the parental LNCaP FL-AR sequence (accession J03180; ref. 34). The only differences detected between parental LNCaP FL-AR and LNCaP95 FL-AR were in polymorphic regions in the NTD with LNCaP95 FL-AR having six extra glutamines and two less glycines. The T877A mutation originally detected in parental LNCaP FL-AR was maintained in the LNCaP95 FL-AR. EPI-002 is an antagonist of AR activation function 1 (AF-1) that blocks the activity of both full-length and truncated AR species (11, 12, 33). LNCaP and LNCaP95 cells are PTEN null and express p110β and p110γ isoforms, albeit LNCaP95 have lower levels of p110γ compared with LNCaP (Fig. 1A, left). Using siRNA to p110γ or p110β revealed that phosphorylation of Akt (pAkt) in LNCaP95 cells depends predominantly upon p110γ (Fig. 1A, right). BEZ235 is a dual PI3K/mTOR inhibitor and in cell-free assays has the following IC₅₀s: p110α, 4 nmol/L; mTOR (p70S6K), 6 nmol/L; p110β, 7 nmol/L; ATR, 21 nmol/L; and p110β, 75 nmol/L (35, 36). At higher concentrations, BEZ235 inhibits EGFR/Erb1 >8.5 μmol/L and many more kinases at >10 μmol/L including Akt1, IGF1R, and CDK1 (35). However, the previously reported concentration of 500 nmol/L BEZ235 that was used to inhibit pAkt in LNCaP cells (23) also inhibited pAkt in LNCaP95 cells but was associated with enormous cytotoxicity making it difficult to interpret the data. Titration experiments revealed a nontoxic concentration of 15 nmol/L BEZ235 that was subsequently used in all experiments, but at this concentration it did not impact pAkt (Fig. 1B, left). In LNCaP95 cells, BEZ235 (15 nmol/L) inhibited phosphorylation of S6 (pS6) ribosomal protein, an mTOR-regulated protein, but not p4EBP1 levels (Fig. 1B, right). Consistent with the previous reports, BEZ235 increased protein levels of FL-AR but unexpectedly decreased the levels of UBE2C at 48 hours, which is an AR-V7 target gene (Fig. 1B, left). Everolimus, an mTOR inhibitor, reduced pS6 at 10 nmol/L and in the absence of androgen also reduced levels of UBE2C (Fig. 1B, left).

Combination experiments with BEZ235 (15 nmol/L) were examined in LNCaP95 cells compared with parental LNCaP cells. EPI-002 reduced pS6 levels regardless of androgen status (Fig. 1C, left). In the absence of androgen, BEZ235 increased levels of FL-AR and AR-V7. EPI-002, but not enzalutamide, also reduced the expression of UBE2C, consistent with previous reports (12, 33). In the presence of androgen, EPI-002 had no effect on levels of FKBP5, a gene transcriptionally regulated by FL-AR (Fig. 1C, right). Protein levels of prostate-specific antigen (PSA) were undetectable in LNCaP95 cells.

LNCaP cells do not express constitutively active AR-Vs and are androgen sensitive with proliferation dependent on AR. No studies have been reported using a concentration of 15 nmol/L BEZ235 in LNCaP cells. BEZ235 had no effect on pAkt at this concentration. Combinations of BEZ235 with enzalutamide or EPI-002 were substantially better than BEZ235 monotherapy in blocking pS6. (Fig. 1D, left). In the absence of androgen, BEZ235 increased levels of FL-AR (Fig. 1D, right). Consistent with results obtained with LNCaP95 cells, EPI-002 was a poor inhibitor of androgen-induced FKBP5 in spite of being comparable with enzalutamide in blocking androgen-induced levels of PSA (Fig. 1D, right). PSA was increased with BEZ235 regardless of androgen status. Thus, although BEZ235 increased protein levels of FL-AR, AR-V7, and possibly downstream target genes of FL-AR (PSA), these increases were partially attenuated by EPI-002 or enzalutamide.

**BEZ235 increased FL-AR transcriptional activity in LNCaP95 and inhibited transactivation of AR-NTD in LNCaP cells**

PSA-, ARR3-, and PB-luciferase are three well-characterized androgen-induced AR-driven reporter gene constructs. BEZ235 (15 nmol/L) significantly increased PSA-, ARR3-, and PB-luciferase activities in LNCaP95 cells treated with androgen which...
were blocked by both enzalutamide and EPI-002 (Fig. 2A). To confirm this change was through inhibition of mTOR: LNCaP95 cells were treated with everolimus (10 nmol/L) which yielded a similar increase in PSA-luciferase activity (Fig. 2A, right). Importantly, in LNCaP cells, BEZ235 did not enhance the activity of FL-AR in response to androgen (Fig. 2B). To address whether BEZ235 affected AR-Vs transcriptional activities, Cos-1 cells that do not express endogenous AR were cotransfected with PB-luciferase and expression vectors for AR-V567es or AR-V7. BEZ235 had no effect on the transcriptional activities of either AR-V567es or AR-V7 (Fig. 2C). Protein levels of AR-V7 and AR-567es were comparable with endogenous levels in LNCaP cells (Fig. 2C, lower). To determine whether BEZ235 directly enhanced AR transactivation, we employed the AR-NTD transactivation assay using both LNCaP and LNCaP95 cells. The AR-NTD is essential for full transcriptional activities (37). In LNCaP cells, transactivation of the AR-NTD can be induced with IL6 or by stimulation.
Figure 2.
Effect of inhibition of mTOR on AR transcriptional activity. LNCaP95 (A) and LNCaP (B) cells transfected with PSA-, ARR3-, or PB-luciferase reporters were treated with DMSO, EPI-002 (EPI), enzalutamide (ENZ), BEZ235 (BEZ), or combination for 1 hour prior to the addition of R1881 for 48 hours. LNCaP95 cells transfected with PSA-luciferase reporter were also treated with everolimus (EVE; 10 nmol/L) or combination with enzalutamide or EPI-002 (A, right). C, Cos-1 cells transfected with PB-luciferase and expression vectors for AR-V567es or AR-V7 were treated with DMSO, EPI-002, BEZ235, or combination of EPI-002 and BEZ (left). Ectopic protein levels of AR-V7 and AR-V567es in Cos-1 cells are shown relative to endogenous levels of FL-AR in LNCaP cells (right). D, transactivation assays of the AR-NTD were performed in LNCaP (left and middle) and LNCaP95 cells (right) cotransfected with p5xGal4UAS-TATA-luciferase and AR-NTD-Gal4DBD. (Continued on the following page.)
Cotargeting AR-Vs and mTOR in CRPC

EPI-002 and BEZ235 effects on endogenous genes regulated by FL-AR and AR-V7. LNCaP95 cells were treated with DMSO, EPI-002 (EPI; 35 μmol/L), enzalutamide (ENZ), BEZ235 (BEZ), or combination of enzalutamide and BEZ235 or EPI-002 and BEZ235 for 1 hour prior to the addition of R1881 or EtOH for 48 hours. A, transcript levels of FL-AR-regulated genes KLK3, FKBP5, and TMPRSS2. B, transcript levels of AR-V7-regulated genes UBE2C, CDC20, and AKT1. C, transcript levels of FL-AR and AR-V7. All transcripts were normalized to levels of RPL13A. Error bars, mean ± SEM from three independent experiments. One-way ANOVA, post hoc Turkey multiple comparisons test. *, versus DMSO control; †, versus EPI-002 treatment group; ‡, versus BEZ235 treatment group. n.s., not statistically significant; *, P < 0.05; **, P < 0.01; ††, P < 0.001; †††, P < 0.0001; †‡, P < 0.01; ‡‡, P < 0.05; ‡‡‡, P < 0.001; ***‡‡‡, P < 0.0001.

(Continued.) EPI-002, BEZ235, or combination of EPI-002 and BEZ were added 1 hour before addition of IL6 (50 ng/mL) or FSK (50 μmol/L) to LNCP cells and harvested after 24 hours. LNCaP95 cells were harvested 24 hours after the treatment of indicated compound (right). Luciferase activities are shown as percentage of vehicle control. Data are presented as the mean ± SEM from three independent experiments. One-way ANOVA, post hoc Turkey multiple comparisons test. *, versus DMSO control; †, versus EPI-002 treatment group; ‡, versus BEZ235 treatment group. n.s., not statistically significant; *, P < 0.05; **, P < 0.01; ††, P < 0.001; †††, P < 0.0001; †‡, P < 0.01; ‡‡, P < 0.05; ‡‡‡, P < 0.001; ***‡‡‡, P < 0.0001.
Figure 4.
Combination therapy with EPI-002 (EPI) and BEZ235 (BEZ) significantly decreased tumor growth both in vitro and in vivo compared with vehicle and each monotherapy-treated group. A, LNCaP95 cells were treated with DMSO, EPI-002, enzalutamide (ENZ), BEZ235, or combination of EPI-002 and BEZ235 for 1 hour prior to the addition of R1881 (0.1 nmol/L) for 48 hours. Proliferation was measured by BrdUrd incorporation. B, LNCaP95 cells were also treated with everolimus (EVE, 10 nmol/L) instead of BEZ235. Proliferation was assessed by BrdUrd incorporation. (Continued on the following page.)
on the intrinsic activity of AR-NTD in LNCaP95. Taken together, BEZ235 has differential effects on AR transcriptional activities that possibly involve cell-specific differences in signal transduction pathways.

EPI-002 inhibited FL-AR- and AR-V7-regulated genes and BEZ235 inhibited AR-V7-regulated genes

LNCaP95 cells were used to examine the effects of BEZ235 and combination therapies on endogenous gene expression regulated by FL-AR and AR-Vs. EPI-002 and enzalutamide inhibited expression of KLK3, TMPRSS2, and FKBP5, which are genes regulated by FL-AR in response to androgen. BEZ235 significantly increased androgen-induced levels of PSA transcripts compared with levels induced by androgen alone (Fig. 3A, compare column 4 to column 1). In the absence of androgen, BEZ235 also induced levels of PSA transcript which could be blocked by EPI-002 but not enzalutamide. No similar effects were observed for TMPRSS2 or FKBP5 in response to BEZ235.

AR-V7 regulates a subset of genes that are unique from FL-AR. Enzalutamide increased levels of UBE2C transcripts in cells treated with androgen, while monotherapy with EPI-002 or BEZ235 attenuated UBE2C levels regardless of androgen (Fig. 3B). In the absence of androgen, enzalutamide had no effect on transcript levels of any of the AR-V7 target genes, contrary to monotherapies with EPI-002 or BEZ235 that consistently reduced the levels of expression of these AR-V7 target genes. Combination of EPI-002 and BEZ235 were significantly more effective than monotherapies. BEZ235 did not increase levels of FL-AR transcript (Fig. 3C, left). Surprising was the >2-fold increase in transcript levels of AR-V7 induced with BEZ235 in the absence of androgen which was blocked by EPI-002 (Fig. 3C).

Combination therapy with EPI-002 and BEZ235 significantly reduced CRPC tumor growth

Proliferation of LNCaP95 cells is androgen-independent and driven by AR-V7 (32, 38). As expected, enzalutamide had no effect on proliferation of these cells (Fig. 4A). Thus, LNCaP95 cells are enzalutamide-resistant. EPI-002 or BEZ235 monotherapies inhibited proliferation with the combination being significantly better than each monotherapy. Everolimus also inhibited the proliferation of LNCaP95 cells, indicating that this additional inhibition was by blocking mTOR (Fig. 4B). Everolimus in combination with EPI-002 was significantly better than the monotherapies.

A small pilot in vivo study was completed to determine the nontoxic oral dose of BEZ235 that could be administered daily. Doses of BEZ235 at 45 mg/kg body weight resulted in the mortality of 66% of the animals (data not shown). BEZ235 orally administered daily at 5 mg/kg body weight was nontoxic and sufficient to block mTOR but not pAkt in tumors. Therefore, a dose of BEZ235 at 5 mg/kg body weight was used in the following in vivo studies. Castrated mice were daily treated orally either with vehicle (NMPIPEG400, 1:9, v/v), a half-dose of EPI-002 (100 mg/kg), BEZ235 (5 mg/kg), or a combination (EPI-002 100 mg/kg + BEZ235 5 mg/kg). Final tumor volume in the EPI-002 + BEZ235 combination group was significantly reduced compared with those in vehicle (DMSO), EPI-002, and BEZ235 groups (Fig. 4C). There was no significant difference in body weight among the treatment groups (Fig. 4D). Protein levels of FL-AR and AR-V7 in response to BEZ235 were reduced (Fig. 4E) contrary to in vitro results which may be the result of differences in time course, exposure levels, and contributions from other signaling pathways in the tumor microenvironment which are not present in vitro. Consistent with in vitro data, protein levels of UBE2C and pS6 were reduced in harvested tumors treated with EPI-002, BEZ235, and combination treatment. No significant change observed in levels of pAkt and p4EBP1 when normalized to total Akt or 4EBP1, respectively (Fig. 4E, bottom). In vivo, EPI-002 and BEZ235 monotherapies reduced protein levels of FKBP5, an FL-AR target gene, and UBE2C, an AR-V7 target gene thereby supporting that the transcriptional activities of FL-AR and AR-Vs were blocked. Immunohistochemical analysis of these same harvested xenografts revealed that EPI-002 and BEZ235 reduced levels of UBE2C and pS6 staining (Fig. 5A), which were consistent with Western blot data. EPI-002 significantly decreased proliferation (Fig. 5B) and increased apoptosis (Fig. 5C) as indicated by staining of Ki67 and TUNEL, respectively.

Discussion

AR-Vs are a potential mechanism of resistance to abiraterone and enzalutamide in CRPC (14, 39, 40). AR-NTD targeting drugs have benefits over drugs targeting the AR-LBD because the NTD is essential for the transcriptional activities of both FL-AR and AR-Vs. Antagonists of AR-NTD, such as EPI-002, could therefore provide therapeutic responses for CRPC patients with malignancies that express constitutively active AR-Vs and are resistant to abiraterone or antiandrogens. In addition to AR, the PI3K/Akt/mTOR pathway is implicated as a potential driver of CRPC (21, 26, 41). Previous reports have shown therapeutic benefits for the treatment of CRPC by a combination of antiandrogen with an inhibitor of PI3K/Akt/mTOR (23, 26, 29). However, those studies focused on cross-talk with FL-AR and PI3K/Akt/mTOR pathways. As CRPC that is resistant to antiandrogens and abiraterone has been shown to be correlated to the expression of constitutively active AR-Vs, it is of interest to investigate therapeutic effects and mechanisms by combination treatments using an inhibitor of both FL-AR and AR-Vs, such as EPI-002, with an inhibitor of PI3K/Akt/mTOR. Here, such studies showed the following: (i) a low, nontoxic concentration of BEZ235 (15 nmol/L) that did not inhibit pAkt was a potent inhibitor of mTOR; (ii) inhibition of mTOR caused an increase in levels of FL-AR (protein) and its target gene PSA (protein and transcript); (iii) inhibition of mTOR also increased levels of AR-Vs, but decreased endogenous expression of its target

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genes such as UBE2C, CDC20, and Akt1; (iv) inhibition of mTOR decreased the proliferation of enzalutamide-resistant human prostate cancer cells which are considered to be driven by AR-V7. Combination therapy to block mTOR and the AR-NTD provided significantly better suppression of proliferation than individual monotherapies; and (v) cotargeting PI3K/Akt/mTOR and AR-NTD in vivo was superior to monotherapies and sufficient to suppress FL-AR and AR-Vs transcriptional activities, and decrease the growth of enzalutamide-resistant CRPC xenografts. Together, these findings support the rationale for cotargeting mTOR and AR-NTD (blocks both FL-AR and AR-Vs) signaling pathways for the treatment of CRPC.

Previous work has implicated that inhibition of FL-AR activates Akt through reducing levels of PHLPP (23). Here, enzalutamide and EPI-002 both decreased mTOR-regulated pS6 while androgen increased pS6. These data suggest that FL-AR regulates mTOR activity, which is consistent with recent studies (42, 43). Importantly, here the levels of AR were increased by both BEZ235 and everolimus without decreasing pAkt. This suggests that mTOR plays an important role in regulating AR protein levels and that Akt was not directly involved. PI3K/Akt inhibitors can have various effects on AR protein levels in prostate cancer cell lines through Akt-independent mechanisms (25). Also surgical and chemical castration is reported to have no effect on activation of Akt and mTOR (26). Here, BEZ increased protein levels of FL-AR and AR-V7 without concomitant increases in levels of their respective transcripts. Thus, inhibition of mTOR may regulate AR protein levels through posttranslational modifications (44). Taken together, our results suggest the possibility of an alternative mechanism of cross-talk between these pathways apart from a

Figure 5.
EPI-002 (EPI) decreased proliferation, UBE2C, and pS6 immunostaining, and increased apoptosis in CRPC LNCaP95 xenografts. A, IHC of representative xenografts stained for hematoxylin and eosin (H&E), UBE2C, pS6, K67, and TUNEL. % K67 (B) and % TUNEL-positive cells (C) were counted in sections from xenografts for each treatment. At least 3,000 cells per xenograft were counted. Cells that were positive for K67 or TUNEL staining were counted in sections from three xenografts per treatment. The total number of cells counted was as follows: 4,712 (vehicle, K67), 4,833 (EPI-002, K67), 5,167 (BEZ235, K67), 4,123 (combination, K67), 4,502 (vehicle, TUNEL), 3,733 (EPI-002, TUNEL), 4,109 (BEZ, TUNEL), and 3,715 (combination, TUNEL). Error bar, mean ± SEM. One-way ANOVA post hoc Bonferroni multiple comparison test; *, P < 0.05; ***, P < 0.001; ****, P < 0.0001.
UBE2C decreased expression of AR-V7 target genes, such as levels of AR-V7 protein by inhibition of mTOR. A signaling pathway is shown in Fig. 6.

Here an important observation was that in spite of increased levels of AR-V7 protein by inhibition of mTOR there was decreased expression of AR-V7 target genes, such as UBE2C, respectively. EPI-002 reduces mTOR-regulated pS6. B, mTOR inhibitors such as everolimus (EVE) and low dose of BEZ235 (BEZ) blocks mTOR with concomitant increases in levels of FL-AR and AR-V. Increased levels of FL-AR lead to increased levels of expression of its target gene, PSA. Levels of AR-V7 target genes such as UBE2C are inhibited possibly by decreased transactivation of AR-NTD or other unidentified mechanisms. C, increased transcriptional activity of FL-AR due to increased levels in response to mTOR inhibition is blocked by AR-NTD antagonist. Combination of AR-NTD antagonist (EPI) and mTOR inhibitor blocks mTOR-regulated pS6 and transcriptional activity of AR-V7 to reduce levels of its target gene, UBE2C. Lines, effects on activity; thick arrows, changes in levels of expression.

Figure 6. Hypothetical model of cross-talk mechanisms between FL-AR, AR-V, and mTOR signaling pathways. A, EPI-002 (EPI) inhibits transcriptional activity of FL-AR and AR-Vs, which results in reduced expression of target genes such as PSA and UBE2C, respectively. EPI-002 reduces mTOR-regulated pS6. B, mTOR inhibitors such as everolimus (EVE) and low dose of BEZ235 (BEZ) blocks mTOR with concomitant increases in levels of FL-AR and AR-V. Increased levels of FL-AR lead to increased levels of expression of its target gene, PSA. Levels of AR-V7 target genes such as UBE2C are inhibited possibly by decreased transactivation of AR-NTD or other unidentified mechanisms. C, increased transcriptional activity of FL-AR due to increased levels in response to mTOR inhibition is blocked by AR-NTD antagonist. Combination of AR-NTD antagonist (EPI) and mTOR inhibitor blocks mTOR-regulated pS6 and transcriptional activity of AR-V7 to reduce levels of its target gene, UBE2C. Lines, effects on activity; thick arrows, changes in levels of expression.

Cotargeting AR-Vs and mTOR in CRPC

reciprocal feedback regulation of Akt and AR signaling. A model of possible cross-talk mechanisms among FL-AR, AR-V7, and mTOR signaling pathway is shown in Fig. 6.

Here an important observation was that in spite of increased levels of AR-V7 protein by inhibition of mTOR there was decreased expression of AR-V7 target genes, such as UBE2C, CDC20, and Akt1. Reduced levels of AR-V7 target genes by inhibition of mTOR appeared specific because there was no decrease in expression of genes regulated by FL-AR or other non-AR-V7 mechanisms (e.g., transcript levels of FL-AR or AR-V7). This suggests that inhibition of mTOR might attenuate transactivation of AR-V7 possibly by mechanisms involving post-translational modifications of AR-Vs or coregulators unique to AR-Vs, but this remains to be established. Paradoxically, increased expression of FL-AR target genes and enhanced reporter activities in response to BEZ235 seem likely due to induced levels of AR-V7 protein rather than increased transactivation because BEZ did not increase the transcriptional activities of ectopic AR-V567es or AR-V7 in Cos-1 cells or transactivation of AR-NTD. However, cell-specific responses to inhibition of mTOR would not be unexpected due to variation in signaling pathways in different cell lines.

Upregulation of the FL-AR pathway was, at least in part, blocked here by enzalutamide and EPI-002, which supports a rationale that cotargeting both PI3K/Akt/mTOR and FL-AR should achieve better efficacy. However, a clinical trial using a combination of everolimus and bicalutamide to block those pathways failed to achieve a better response when compared with bicalutamide monotherapy (43). A possible explanation for the lack of efficacy could be that the FL-AR signaling pathway was not directly related to the CRPC growth observed and perhaps those tumors were driven by AR-Vs, which would not be impacted by an inhibitor of the AR-LBD, which is consistent with the clinical evidence that neither enzalutamide nor abiraterone can reverse resistance mediated by AR-Vs (15). In accordance with this notion, enzalutamide did not inhibit the growth of LnCaP95 cells, despite that it effectively blocked FL-AR transcriptional activity. Most importantly, EPI-002 and BEZ, but not enzalutamide, reduced levels of UBE2C, an AR-V7 target gene.

In conclusion, our findings demonstrate that cotargeting mTOR and AR-NTD to block both FL-AR and AR-Vs showed maximum antitumor efficacy in PTEN-negative enzalutamide-resistant CRPC with acceptable tolerability. As AR-LBD-targeting drugs may have limited or no effect on AR-Vs, this novel approach may provide a therapeutic advantage for CRPC patients that are resistant to abiraterone or antiandrogens by a mechanism involving expression of AR-Vs.

Disclosure of Potential Conflicts of Interest

C.A. Banuelos and N.R. Mawji have ownership interest (including patents) in ESSA Pharma Inc. M.D. Sadar is a Director, Officer, and a consultant/advisory board member for ESSA Pharma Inc. No potential conflicts of interest were disclosed by the other authors.

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