Targeting Androgen Receptor Activation Function-1 with EPI to Overcome Resistance Mechanisms in Castration-Resistant Prostate Cancer

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Abstract

Purpose: Persistent androgen receptor (AR) transcriptional activity is clinically evident in castration-resistant prostate cancer (CRPC). Therefore, AR remains as a viable therapeutic target for CRPC. All current hormonal therapies target the C-terminus ligand-binding domain (LBD) of AR. By using EPI to target AR activation function-1 (AF-1), in the N-terminal domain that is essential for AR transactivation, we evaluate the ability of EPI to overcome several clinically relevant AR-related mechanisms of resistance.

Experimental Design: To study the effect of EPI on AR transcriptional activity against overexpressed coactivators, such as SRC1-3 and p300, luciferase reporter assays were performed using LNCaP cells. AR-negative COS-1 cells were employed for reporter assays to examine whether the length of polyglutamine tract affects inhibition by EPI. The effect of EPI on constitutively active AR splice variants was studied in LNCaP95 cells, which express AR-V7 variant. To evaluate the effect of EPI on the proliferation of LNCaP95 cells, we performed in vitro BrdUrd incorporation assay and in vivo studies using xenografts in mice.

Results: EPI effectively overcame several molecular alterations underlying aberrant AR activity, including overexpressed coactivators, AR gain-of-function mutations, and constitutively active AR-V7. EPI inhibited AR transcriptional activity regardless of the length of polyglutamine tract. Importantly, EPI significantly inhibited the in vitro and in vivo proliferation of LNCaP95 prostate cancer cells, which are androgen independent and enzalutamide resistant.

Conclusions: These findings support EPI as a promising therapeutic agent to treat CRPC, particularly against tumors driven by constitutively active AR splice variants that are resistant to LBD-targeting drugs. Clin Cancer Res; 22(17): 4466–77. ©2016 AACR.

See related commentary by Sharp et al., p. 4280

Introduction

Most prostate cancers require androgen receptor (AR) transcriptional activity for survival and growth, and therefore, inhibiting androgen synthesis and AR transcriptional activity are the therapeutic approaches to treat advanced prostate cancer. These hormone therapies consist of androgen deprivation therapy (ADT) and application of antiandrogens. Unfortunately, within 12 to 33 months, most patients treated with hormone therapies relapse with a more aggressive and lethal form of the disease referred to as metastatic castration-resistant prostate cancer (mCRPC; ref. 1). mCRPC is associated with poor prognosis and a mean survival time of 18 to 36 months (2).

Despite ADT achieving castrate levels of serum testosterone, CRPC is associated with aberrantly restored AR transcriptional activity. The clinical onset of CRPC is characterized by a rise in serum levels of PSA, which is a gene regulated by AR. There are several possible mechanisms underlying continued transcriptional activation of AR, including amplification of AR (3), gain-of-function mutations within the ligand-binding domain (LBD) of AR that enables activation by nonandrogenic steroids or antiandrogens (4, 5), ligand-independent activation of the AR N-terminal domain (NTD; refs. 6–9), overexpression of AR coactivators (10–14), intratumoral de novo synthesis of androgens (15), and expression of constitutively active AR splice variants with truncated LBD (16, 17). AR mechanisms of resistance to abiraterone and enzalutamide also include expression of constitutively active AR splice variants, elevated intratumoral androgen, and AR LBD gain-of-function point mutations (18, 19). Thus, novel approaches beyond AR LBD inhibition are required to block AR transcriptional activity for the treatment of mCRPC. One such approach involves targeting AR NTD, which would theoretically block all of the above AR mechanisms of resistance.

Activation function-1 (AF-1) in AR NTD is essential for AR transcriptional activity (20) and, thus, a viable therapeutic target for CRPC. An antagonist of AR AF-1, EPI-002 (2R, 20S) is a single
EPI Overcomes Resistance Mechanisms in CRPC

**Translational Relevance**

Androgen receptor (AR) N-terminal domain (NTD) contains activation function-1 (AF-1), which is a viable therapeutic target for castration-resistant prostate cancer (CRPC). AF-1 antagonists, such as EPI, have potential to overcome all currently proposed AR-related mechanisms of resistance. Compelling evidence indicates that gain-of-function mutations in AR ligand-binding domain (LBD) and constitutively active AR splice variant V7 (AR-V7) with truncated LBD are associated with resistance to abiraterone and enzalutamide. Here, we demonstrate that EPI overcomes these clinically relevant AR mechanisms of resistance that may drive CRPC that include overexpressed AR coactivators, gain-of-function AR mutations, and expression of constitutively active AR-V7. These results support the development of antagonists to AR-AF-1 for the clinical management of CRPC. EPI is the first inhibitor that binds to the NTD of any steroid hormone receptor to be tested in clinical trials and is initially aimed at CRPC patients that have failed abiraterone or enzalutamide (ClinicalTrials.gov identifier: NCT02606123).

**Materials and Methods**

**Cell lines, transfection, and proliferation assay**

LNCaP cells were from Dr. Leland Chung (Cedars-Sinai Medical Center, Los Angeles, CA). LNCaP95 cells were from S.R. Plymato. COS-1 cells were from Dr. Rob Kay (Terry Fox Laboratory, Vancouver, BC, Canada). LNCaP and COS-1 cells were not further authenticated in our laboratory but were regularly tested to ensure mycoplasma-free (VenorGeM Mycoplasma Detection Kit, Sigma-Aldrich). In September 2013, LNCaP95 cells were authenticated by short tandem repeat analysis and tested to ensure mycoplasma-free at DDC Medical. All cells used were passaged less than 3 months after resuscitation. Transfection for luciferase reporter assays and cell proliferation assay were described previously (22). Details of transfection, proliferation assay, and plasmids and reagents are in the Supplementary Materials and Methods.

**Western blot analysis**

SRC-1, SRC-2 (TIF2), and SRC-3 (AIB-1) were probed with purified mouse anti-SRC-1, anti-TIF2, and anti-AIB-1, respectively (BD Biosciences). p300 was probed using p300 antibody (C-20, Santa Cruz Biotechnology). AR was probed by antianidrogen receptor antibody N-20 (Santa Cruz Biotechnology). Membranes were probed for β-actin using monoclonal mouse anti-β-actin antibody (a8226 from Abcam).

**Protein–protein interaction studies**

Cell-free SRC-1-3 and AR interactions were analyzed on a Scinti-strip microtiter 96-well plate (PerkinElmer Life Sciences), utilizing both purified recombinant AR-AF1 and in vitro transcribed/translated 35S-labeled binding partners, SRC-1a-CTD (amino acids 977–1240), SRC2-CTD (amino acids 1120–1464), and SRC3-CTD (amino acids 1093–1412; ref. 26). Cell-based immunoprecipitation assay was also employed to study SRC and AR interactions with details in Supplementary Materials and Methods.

**Endogenous expression of genes regulated by AR**

Forty-eight hours after treatment, total RNA was extracted by using RNeasy Micro Kit (Qiagen) and reverse transcribed to cDNA by SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen). Diluted cDNA and gene-specific primers were combined with Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). Transcripts were measured by qRT-PCR ABI PRISM 7900 Sequence Detection System (ABI PRISM, Applied Biosystems by Life Technologies) in triplicates for each biologic sample. Gene expression levels were normalized to housekeeping gene RPL13A. Primers have been described previously (22, 27).

**Xenografts and animal study approval**

Male NOD/SCID mice at 6 to 8 weeks old were subcutaneously injected with LNCaP95 cells (106 cells/site) using Matrigel (Becton Dickinson). Mice were castrated when tumor volumes reached approximately 100 mm3. Drug treatment started one week after castration. Animals bearing LNCaP95 xenografts were randomized and administered with 100 mg/kg body weight of EPI-002 twice daily or 50 mg/kg body weight of enzalutamide once daily or vehicle control (CMC/DMSO) once daily by oral gavage. Tumors were excised 2 days after the last treatment. All animal studies conformed to the relevant regulatory and ethical standards. The University of British Columbia Animal Care Committee approved all experiments involving animals.

**Steroid levels in xenografts and IHC**

Tumors were removed and 30 to 50 mg flash frozen for intratumoral steroid measurements as reported previously (18) and in the Supplementary Materials and Methods.

**Statistical analysis**

Comparisons between two groups were performed with unpaired two-tailed Student t test, and comparisons between three or more groups were performed using one-way or two-way ANOVA (GraphPad Software, V6). P < 0.05 was considered statistically significant.

**Results**

EPI maintains effective inhibition of AR transcriptional activity despite elevated levels of coactivators

Elevated levels of AR coactivators, such as the p160 steroid receptor coactivator (SRC) family proteins (SRC-1, SRC-2, and SRC-3) and CBP/p300 are associated with prostate cancer
progression and poor prognosis (10–14). SRC and CBP/p300 directly interact with AR NTD (8, 26, 28, 29). EPI blocks interaction of AR with CBP (22), but it is unknown whether EPI also blocks interaction of AR with SRC. To test this, two approaches were employed. The first approach measured interaction in a cell-free assay with recombinant AR AF1 and SRC1,2,3-CTD proteins. This approach showed that EPI does not inhibit interaction between AF-1 and any of the SRC family members (Fig. 1A). The second approach was to immunoprecipitate endogenous protein complexes from LNCaP cells with and without EPI treatment. These studies also showed no effect of EPI on this interaction (Fig. 1B). Thus, EPI does not block interaction of AR with the SRC family of coactivators. To test whether EPI could maintain inhibition of AR transcriptional activity when SRC1-3 proteins were overexpressed, AR-driven PSA luciferase reporter assays were performed in LNCaP cells transiently transfected with increasing amounts of SRC expression vectors. Consistent with previous studies (8), overexpression of each of the SRC family proteins resulted in a dose-dependent increase of androgen-induced AR transcriptional activity (Fig. 1C, left). Western blot analysis confirmed the overexpression of each of the coactivators when compared with endogenous levels (Fig. 1C, right). Despite the increase of androgen-induced AR transcriptional activity as a result of overexpressed SRC family proteins, enzalutamide and EPI both maintained effective and consistent inhibition of AR activity, as indicated by the unchanged percentage of inhibition (Fig. 1C, middle). Similarly, overexpression of p300 led to a dose-dependent increase of androgen-induced AR transcriptional activity in LNCaP cells (Fig. 1D, left). Western blot analysis confirmed increased expression of p300 when compared with endogenous levels (Fig. 1D, right). Both EPI and enzalutamide effectively inhibited AR transcriptional activity in spite of elevated levels of p300 (Fig. 1D, middle).

Inhibition of AR transcriptional activity by EPI is not affected by polymorphic lengths of polyglutamine tract

The human AR gene contains a polymorphic CAG triplet repeat within exon 1 that encodes for a polyglutamine tract in its NTD. The number of CAG repeats range from 6 to 36 in the human population (30). As EPI binds AF-1 in the NTD, we tested whether variable lengths of polyglutamine tract would affect the efficacy of EPI to inhibit AR transcriptional activity. AR-negative COS-1 cells were cotransfected with plasmids containing AR-driven probasin (PB)-LUC reporter and expression vectors encoding human full-length AR with polyglutamine tract containing 0, 12, 20, 40, or 49 contiguous glutamines. Regardless of the length of polyglutamine tract, EPI demonstrated consistent inhibition of androgen-induced AR transcriptional activity (Fig. 2A and B). Consistent with previous published studies (30), these data confirmed the inverse correlation of the length of polyglutamine tract and AR transcriptional activity (Fig. 2C). Western blot analysis revealed that levels of ectopic AR expressed in COS-1 cells were within physiologic levels comparable with the endogenous AR level in LNCaP cells (Fig. 2D). However, expression of AR with 49 repeats of glutamine (CAG49) was reduced compared with the other ARs with shorter tracts. Enzalutamide reduced the total protein levels of AR, consistent with previous reports (31). These results support that variable lengths of polyglutamine tract within the NTD did not diminish inhibition of AR transcriptional activity by EPI.

EPI inhibits transcriptional activities of AR with clinically relevant mutations

To assess the effectiveness of EPI against the transcriptional activities of several clinically relevant AR gain-of-function mutations, including mutations found in both the NTD and LBD, we performed AR-driven reporter gene assays using AR-negative COS-1 cells transiently cotransfected with various AR mutants and treated the cells with different inhibitors. First, we examined two AR NTD gain-of-function mutations derived from patients treated with antiandrogens. AR E255K interacts with an E3 ubiquitin ligase and enhances protein stability and androgen-independent nuclear localization (32). AR W435L increases interaction between the AR NTD and LBD (32). EPI significantly inhibited androgen-dependent transcriptional activities of these two AR NTD mutations, and the level of inhibition achieved was similar to that of the wild-type (WT) AR (Fig. 3A). This suggests that these NTD gain-of-function mutations did not impair the effectiveness of EPI to block transcriptional activity of AR. Next, we tested several AR LBD gain-of-function mutations. In the absence of androgen, EPI had no agonist activity, which was contrary to hydroxyflutamide and bicalutamide that stimulated substantial AR transcriptional activities for WT AR and the mutants tested (Fig. 3A). Enzalutamide also did not stimulate AR activity in the absence of androgen with any of these mutants. In the presence of androgen, EPI significantly blocked the transcriptional activities of WT, V715M, R761G, H874Y, and T877A (Fig. 3A). Hydroxyflutamide failed to significantly block androgen-dependent transcriptional activities of AR H874Y and T877A, which is consistent with previous reports that these two mutants confer resistance to flutamide (4, 33). No statistical significance was measured for the percentage of inhibition of androgen-dependent AR transcriptional activities between enzalutamide and EPI, with the exception of WT AR and AR T877A, in which enzalutamide displayed stronger inhibition (Fig. 3B). Relative levels of ectopic expression of each of the AR mutants are shown in Fig. 3C.

EPI blocks the noncanonical transcriptional program regulated by constitutively active AR splice variant

Expression of LBD-truncated and constitutively active AR splice variants AR-V7 or AR-V567es is associated with poor prognosis.
EPI inhibits the activities of AR-V567es in the presence and absence of full-length AR, whereas antiandrogens, including enzalutamide, have no effect (21). AR-V7 is reported to regulate a distinct transcriptional program compared with full-length AR (35–37). Any AR LBD-targeting therapies, including enzalutamide, may not inhibit AR-V7's transcriptional activity and thus may have no effect on expression of AR-V7's transcriptome. However, an inhibitor of AR AF-1, such as EPI, should block AR-V7 transcriptional activity and expression of its downstream target genes. To test this, the expression of genes regulated by full-length AR and AR-V7 was evaluated in LNCaP95 cells treated with antiandrogens or EPI. LNCaP95 cells express functional full-length AR and AR-V7 (16, 35). A PSA-luciferase assay confirmed that these cells do have a functional full-length
Figure 3.
EPI blocks the transcriptional activity of ARs with clinically relevant gain-of-function mutations. COS-1 cells were cotransfected with PB-luciferase reporter and expression vectors containing wild-type (WT) AR and mutant AR. Cells were pretreated with vehicle, 10 μmol/L enzalutamide (ENZ), 10 μmol/L bicalutamide (BIC), or 10 μmol/L hydroxyflutamide (FLU), or 25 μmol/L EPI (EPI-002) for 1 hour prior to treatment of 1 nmol/L R1881 or ethanol for 24 hours. A, luciferase activities were measured, and values were plotted after normalized to vehicle–ethanol treatment. B, percentage of inhibition by treatments was calculated on the basis of vehicle–R1881 treatment and plotted. Bar graphs are mean ± SEM, with n ≥ 3 independent experiments. One-way ANOVA compared treatment groups with vehicle controls in A and unpaired Student t test was used to compare EPI with ENZA in B. *, P < 0.05; **, P < 0.01; ***, P < 0.001. C, protein levels of ectopic expression of WT AR and AR mutants in COS-1 cells compared with endogenous levels of AR in LNCaP cells were shown in a representative Western blot using anti-AR N-20 antibody. β-Actin was used as a loading control.
AR that was responsive to androgen (Fig. 4A). Androgen-induced PSA luciferase reporter activities were inhibited by antiandrogens (bicalutamide and enzalutamide) and EPI. Consistent with LNCaP95 cells expressing functional full-length AR as shown with the PSA-reporter gene construct, endogenous expression levels of PSA and FKBP5 were induced by androgen (Fig. 4B). Both enzalutamide and EPI decreased androgen-induced levels of PSA and FKBP5 transcript (Fig. 4B). Next, we measured levels of mRNA of genes that are distinctively regulated by AR-V7, such as UBE2C, CDC20, and AKT1 (35). Firstly, expression of these genes was not induced by androgens, thereby supporting that they were not regulated by full-length AR. Consistent with this interpretation, enzalutamide, which blocks full-length AR, had no effect on the levels of expression of these genes (Fig. 4C). Contrary to antiandrogens, EPI significantly reduced the levels of expression of UBE2C, CDC20, and AKT1 (Fig. 4C). Levels of mRNA for full-length AR and AR-V7 were not significantly affected by enzalutamide or EPI (Fig. 4D), nor were protein levels of full-length AR or AR-V7 decreased by any of the treatments compared with relevant controls and androgen treatments (Fig. 4E). These data support the ability of EPI to inhibit the transcriptional activities of full-length AR and constitutively active AR-V7 to block expression of their target genes.

EPI attenuates the growth of enzalutamide-resistant LNCaP95 xenografts

Proliferation of LNCaP95 cells is androgen independent and driven by AR-V7 transcriptional activity (16, 35). Therefore, if EPI blocks the transcriptional activity of AR-V7 as suggested here, then EPI should also impact the proliferation of these cells. Consistent with previous reports, proliferation of LNCaP95 cells was androgen independent and resistant to antiandrogens, such as bicalutamide and enzalutamide (Fig. 5A). Consistent with EPI blocking...
AR-V7 transcriptional activity, EPI significantly reduced the number of proliferating LNCaP95 cells. Together these data suggest LNCaP95 cells are enzalutamide resistant yet sensitive to EPI by a mechanism involving inhibition of the transcriptional activity of AR-V7. To test in vivo effects of EPI on potentially enzalutamide-resistant tumors, LNCaP95 cells were grown as xenografts in male SCID mice that were castrated prior to treatments. EPI significantly attenuated the growth of LNCaP95 xenografts, whereas enzalutamide had no significant effect (Fig. 5B). By the end of the study, animals treated with EPI had xenografts that were significantly smaller than those treated with vehicle or enzalutamide. EPI reduced the tumor size by more than 50% compared with vehicle control (Fig. 5C and D) but did not result in tumor regression of LNCaP95 xenografts contrary to regression achieved with EPI in LNCaP xenografts (21, 22). These studies demonstrate the in vivo efficacy of EPI on the growth of castration-resistant and enzalutamide-resistant LNCaP95 tumors and support previous data showing the superior efficacy of EPI compared with anti-androgens on VCaP xenografts maintained in castrated hosts (21). Together, these data are consistent with the notion that LNCaP95 tumors are driven by constitutively active AR-V7. Support for this can be provided by analysis of levels of expression of genes regulated by full-length AR and AR-V7 in the harvested xenografts. QPCR of RNA isolated from harvested tumors revealed that enzalutamide substantially increased levels of full-length AR and AR-V7 transcripts (Fig. 5E), consistent with in vitro data (38), whereas EPI did not alter transcript levels of full-length AR or AR-V7. EPI treatment decreased levels of expression of genes...
regulated by full-length AR and AR-V7, including PSA, FKBP5, UBE2C, and UGT2B17 (Fig. 5E), consistent with the in vitro findings and gene expression data from VCaP xenografts maintained in castrated hosts treated with EPI (21). Measurement of androgen levels in these same xenografts confirms that LNCaP95 xenografts were not driven by androgen in castrated hosts (Fig. 5F).

Immunohistochemical analysis of harvested xenografts revealed similar levels of total AR protein with all treatments (Fig. 6A). EPI significantly decreased proliferation as indicated by the lowered number of Ki67-stained cells, while it significantly induced apoptosis as indicated by the increased number of TUNEL-positive cells (Fig. 6B). Consistent with TUNEL staining, cleaved caspase-3 staining of these xenografts also showed significantly increased level of cleaved caspase-3 in EPI treatment group (Supplementary Fig. S1). In contrast, enzalutamide had no effect on the proliferation and apoptosis in these xenografts. In accordance with previously published data that enzalutamide increases AR-V7 protein expression in LNCaP95 cells (35), we observed stronger nuclear AR-V7 staining with enzalutamide treatment, while full-length AR staining was similar between the treatment groups (Supplementary Fig. S2). Together, these data support the efficacy of EPI in blocking the growth of castration-resistant and enzalutamide-resistant tumors by a mechanism that is consistent with blocking the transcriptional activities of both full-length AR and constitutively active AR splice variants.

Discussion

Restoration of AR transcriptional activity is believed to be the major driver of most lethal mCRPC (39). Discovery of new drugs that block persistent AR transcriptional activities is crucial for improving the clinical management of this disease. In contrast to

Figure 6.
EPI reduces proliferation and increases apoptosis in LNCaP95 xenografts. A, representative xenograft tumors stained for hematoxylin and eosin (HE), AR, Ki67, and TUNEL. Scale bars (red), 20 μm. B, percentages of Ki67 and TUNEL-positive cells were counted in xenograft sections of each treatment. Total number of cells counted: 2,361 (control, Ki67), 2,658 (EPI-002, Ki67), 2,409 (enzalutamide (ENZA), Ki67), 1,209 (control, TUNEL), 1,210 (EPI-002, TUNEL), and 1,137 (enzalutamide, TUNEL). Bar graphs are mean ± SEM, with n = 3 different xenograft sections. One-way ANOVA comparing treatment groups with control: *, P < 0.05; **, P < 0.01. NS, not significant.
all current hormone therapies that act through AR C-terminal LBD, including abiraterone and enzalutamide, an antagonist of AR AF-1 in the NTD has the potential to block all known AR mechanisms of resistance. The current study revealed that EPI, an AF-1 antagonist, was capable of blocking such AR mechanisms of resistance. AR belongs to the steroid hormone receptor family that includes glucocorticoid receptor, progesterone receptor, estrogen receptor, and mineralocorticoid receptor. These receptors share structural features, such as LBD, DNA-binding domain, and an intrinsically disordered NTD. There are no small molecules reported that bind to NTDs of any other steroid hormone receptor. Thus, EPI is precedent in the field of steroid hormone receptors as the first small molecule discovered that directly interacts with AR NTD (21, 22). AR transcriptional activity is dependent on a functional AF-1 region within its NTD, which is comprised of Tau-1 (amino acid residues 110–370) and Tau-5 (amino acid residues 360–528). Tau-1 and Tau-5 may act independently of one another (20). EPI binds AF-1 (21, 22) and recent NMR spectroscopy has mapped its binding specifically to Tau-5 (24).

AR interacts with p160 SRC coactivators through both its C-terminal LBD and AF-1 (40–42) and with CBP/p300 in its NTD (29). CBP/p300 are bridging factors that are necessary for AR transcriptional activity (29, 43). The expression levels of SRCs and CBP/p300 are increased in CRPC and these increases in expression are proposed as an AR mechanism of resistance to current hormone therapies (10–14). Here, we demonstrated the ability of EPI to maintain an effective and consistent inhibition of AR transcriptional activity in spite of elevated levels of these AR coactivators. EPI did not block interaction of AF-1 with p160 SRCs using recombinant peptides or with endogenous proteins coimmunoprecipitated from cells. This implies that p160 SRCs interaction with AF-1 cannot override or compensate for the loss of interaction with CBP and RAP74 caused by EPI to decrease AR transcriptional activity (22). P300 and RAP74 interact with Tau-5 of AR AF-1 (24, 44). RAP74 is a subunit of transcriptional factor IIIF (TFIIF) and an essential part of the basal transcriptional machinery to recruit RNA polymerase II that is necessary for transcription. Hence, SRC interaction is not sufficient on its own to drive AR transcriptional activity in the absence of RAP74 and CBP/p300.

The length of polyglutamine tract influences interaction of AR with corepressors and AR transcriptional activity (45). The AR repressor, silencing mediator for retinoid acid and thyroid hormone receptor, binds Tau-5 and LBD, and its ability to repress AR activity is highly influenced by the length of AR polyglutamine tract, with only 17% inhibition of AR with 9 repeats and 56% of AR with 42 repeats (45). Inhibition of AR transactivation by EPI was not affected by the length of polyglutamine tract within the NTD. EPI achieved significant and similar levels of inhibition of transactivation of AR with variable lengths of polyglutamine tract in the range of 0 to 49 repeats. The ability of EPI to continue to inhibit AR transcriptional activity in spite of differences in polyglutamine tract length is relevant to the clinical application of EPI to treat prostate cancer patients and particularly those patients with shorter polyglutamine tracts that correlate with more aggressive disease (46).

AR LBD is a structurally ordered domain with an open conformation for ligand binding, making the binding pocket for anti-androgen readily accessible (47). Contrary to this, the NTD is intrinsically disordered and acts as a hub for interaction with many proteins. Such differences in protein structure between the LBD and NTD may explain the rapid efficacy of antiandrogens in short-term in vitro inhibition of AR transcriptional activity compared with EPI in reporter assays. It may take longer, perhaps one doubling time, for the binding site in the NTD to be accessible to EPI. However, in long term in vivo studies, the activities of antiandrogen and EPI were comparable in xenografts that express solely full-length AR in spite of the short half-life of EPI relative to antiandrogens (24). AR W435L (Tau-5) and AR E255K (Tau-1) are both cell-specific and reporter-specific gain-of-function AR NTD mutations that were originally detected using tissue from patients treated with antiandrogens (32). EPI effectively inhibited all the mutant gain-of-function ARs tested here, including E255K and W435L and several LBD mutations. The requirement of AF-1 for AR transcriptional activity supports a prediction that an antagonist to AF-1 should inhibit all LBD gain-of-function mutations.

EPI binds AF-1 (21, 22) and within this region specifically to Tau-5 (24), which is especially important for ligand-independent transcriptional activity of constitutively active truncated AR lacking LBD (20). Consistent with EPI binding to Tau-5, functional assays show that EPI blocks the activity of ectopic constitutively active AR splice variant and truncated AR1-653 deletion mutant (21, 22). LBD-truncated AR splice variants, such as AR-V7 and AR-V567es, are correlated with poor prognosis (34). Aberrant and persistent AR transcriptional activity caused by constitutively active AR splice variants is likely a main common mechanism of resistance to abiraterone and enzalutamide, and compelling evidence suggests that these variants provide resistance to current LBD-targeting therapies in vitro and in vivo (18, 38, 48). A recent clinical study revealed that the levels of AR-V7 in the circulating tumor cells from CRPC patients are correlated with resistance to abiraterone and enzalutamide (49). These findings indicate the potential of constitutively active AR variants to be a major mechanism of resistance to current AR LBD-targeting therapies, but more importantly, they highlight the need for inhibitors of AR AF-1. In LNCaP95 cells, that endogenously express both functional full-length AR and AR-V7, EPI reduced the expression of genes regulated by full-length AR and AR-V7, EPI reduced the expression of genes regulated by full-length AR, such as PSA and F K B P 5 , and most importantly genes distinctly regulated by AR-V7, such as UBE2C and CDC20, whereas, enzalutamide had no effect. Androgen-independent proliferation of LNCaP95 cells was significantly reduced by EPI, whereas antiandrogens had no effect. Consistent with these in vitro results, in vivo EPI caused significant reduction of the growth of castration-resistant and enzalutamide-resistant LNCaP95 xenografts that recapitulate CRPC tumors driven by constitutively active AR splice variants lacking the LBD. Evidence for in vivo, on-target activity was provided by analysis of gene expression of the harvested xenografts showing EPI decreased expression of genes regulated by full-length AR and AR-V7. These results support previous findings that EPI specifically decreases the growth of castration-resistant VCaP xenografts that express AR-V7 splice variant with concomitant evidence of on-target activity provided by gene expression analysis (21). Thus, antagonists of AF-1, such as EPI, represent a viable therapeutic approach to treat CRPC tumors that are driven by AR splice variants, which have emerged as a clinically significant mechanism underlying the progression and therapy resistance in CRPC. EPI-506, a prodrug of EPI-002, is in clinical trials for patients that have failed.
abicinone and enzalutamide, with expression of AR-V7 proposed to be measured in circulating tumor cells (50).

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

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