

Regression of Castrate-Recurrent Prostate Cancer by a Small-Molecule Inhibitor of the Amino-Terminus Domain of the Androgen Receptor

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SUMMARY

Castration-recurrent prostate cancer (CRPC) is suspected to depend on androgen receptor (AR). The AF-1 region in the amino-terminal domain (NTD) of AR contains most, if not all, of the transcriptional activity. Here we identify EPI-001, a small molecule that blocked transactivation of the NTD and was specific for inhibition of AR without attenuating transcriptional activities of related steroid receptors. EPI-001 interacted with the AF-1 region, inhibited protein-protein interactions with AR, and reduced AR interaction with androgen-response elements on target genes. Importantly, EPI-001 blocked androgen-induced proliferation and caused cyoreduction of CRPC in xenografts dependent on AR for growth and survival without causing toxicity.

INTRODUCTION

Androgen ablation therapy causes a temporary reduction in prostate cancer tumor burden concomitant with a decrease in serum prostate-specific antigen (PSA). Unfortunately, prostate cancer will begin to grow again in the absence of androgens to form castrate-recurrent disease (CRPC), which is characterized before the onset of symptoms by a rising titer of serum PSA. Most patients succumb within 2 years of rising PSA. The current treatment for CRPC is docetaxel combined with prednisone, which increases survival by 2 months.

Androgen receptor (AR) is suspected to play a role in CRPC. The AR has distinct functional domains that include the ligand-binding domain (LBD), a DNA-binding domain (DBD), and an amino-terminal domain (NTD). AR NTD contains the AF-1 that contributes most of the activity to the ligand-bound AR (Jenster

et al., 1991; Rundlett et al., 1990; Simental et al., 1991), rather than AF-2 in the LBD, making the AR unique from other steroid receptors. The AR NTD is activated by alternative pathways in the absence of androgen (Sadar 1999; Ueda et al., 2002a, 2002b; Quayle et al., 2007). Binding of androgen to the LBD of the AR results in its activation such that the receptor can effectively bind to its specific DNA consensus site, termed androgen response element (ARE), on the promoter and enhancer regions of androgen regulated genes, such as PSA, to initiate transcription. Evidence supporting the AR in CRPC includes that many of the same genes that are increased by androgens in androgen-dependent prostate cancer xenografts become elevated in CRPC (Gregory et al., 1998). This suggests that the AR can be activated in the absence of testicular androgens in CRPC, which is consistent with the finding that nuclear AR protein is present in secondary prostate cancer tumors (Kim

Significance

Androgen receptor (AR) is a transcription factor that plays an important role in prostate cancer. We identified EPI-001 as a small molecule that inhibits transactivation of the AR amino-terminal domain (NTD). The advantages of EPI-001 are: (1) its mechanism is unique from antiandrogens that target the C-terminal ligand-binding domain (LBD) and fail presumably due to gain-of-function mutations in the LBD, or expression of constitutively active splice variants; (2) it does not appear to be toxic in the therapeutic dose range in vivo; and (3) currently there are no curative treatment options for castration-recurrent prostate cancer (CRPC). These findings suggest that the AR NTD is a promising target to develop therapeutics for the treatment of CRPC.

et al., 2002; van der Kwast et al., 1991). Other data supporting the role of AR in CRPC include amplification of the AR gene (Moul et al., 1995; Visakorpi et al., 1995), delayed onset of CRPC by altering the timing and sequence of use of antiandrogens (Bruchovsky et al., 2001), and the necessity of AR for proliferation and tumor growth (Zegarra-Moro et al., 2002; Chen et al., 2004). Importantly, in cell culture the AR can be activated in the absence of androgen (and absence of serum) by stimulation of the cAMP-dependent protein kinase (PKA) pathway, interleukin-6 (IL-6), growth factors (Culig et al., 1994; Nazareth and Weigel, 1996; Sadar, 1999; Ueda et al., 2002a, 2002b), and by bone-derived factors (Blaszczuk et al., 2004). The mechanism of transactivation of the AR in the absence of androgens by alternative pathways such as cAMP/PKA, IL-6, and epidermal growth factor involves the AR NTD (Sadar, 1999; Ueda et al., 2002a, 2002b; Blaszczuk et al., 2004; Quayle et al., 2007; Gregory et al., 2004) and CRPC is independent of the AF-2 region in the LBD (Dehm and Tindall, 2006). Thus, in vitro in the absence of androgen, or in vivo in the absence of testicular androgens, the AR NTD can be activated and is implicated in the molecular mechanism of CRPC. Recently, naturally occurring splice variants of the AR that lack the LBD have been reported in prostate cancer cell lines (LNCaP and 22Rv1), and also in CRPC (Dehm et al., 2008; Guo et al., 2009; Hu et al., 2009). These mutants are constitutively active and would not be inhibited by current therapies that target the AR LBD such as antiandrogens and androgen ablation therapy. Targeting the AR NTD blocks in vivo prostate cancer tumor growth in the presence (androgen sensitive) and absence of androgens (CRPC) (Quayle et al., 2007).

AR inhibitors used clinically all target the LBD. These antiandrogens predominantly fail presumably due to poor affinity and gain-of-function mutations in the LBD that lead to activation of the AR by these same antiandrogens (Taplin et al., 1999). Although the crystal structure has been resolved for the AR C-terminal LBD, this has not been the case for the NTD due to its high flexibility and intrinsic disorder in solution (Reid et al., 2002), thereby hampering virtual docking drug-discovery approaches. Here, our goal was to identify an antagonist to the AR NTD that: (1) interacts with the NTD; (2) inhibits protein-protein interactions with the NTD; and (3) potentially reduces CRPC tumor burden.

RESULTS

Transactivation of the AR NTD

Deletion experiments have shown that the NTD is essential for transcriptional activity of the AR in response to ligand as well as in the absence of ligand (Jenster et al., 1991). Thus, a small-molecule inhibitor of NTD function would block AR activity regardless of ligand. AR can be activated by androgen (ligand-dependent), and in the absence of both serum and androgen (ligand-independent) by forskolin (FSK), which stimulates PKA activity, or IL-6, by transactivation of the AR NTD. We screened a library of marine sponge extracts for inhibition of both ligand-dependent and ligand-independent activation of the AR by blocking transactivation of the AR NTD and identified small-molecule analogs designated EPI-001 and 185-9-1. The structures of both the active (EPI-001) and inactive analog (185-9-1) are shown in Figure 1A. The ability of EPI-001 to inhibit transactivation of the AR NTD

was tested in LNCaP cells cotransfected with an expression vector for a chimeric protein encoding amino acids 1–558 of the human AR NTD fused to the Gal4DBD with a reporter gene containing the Gal4-binding site. Androgen and the antiandrogen bicalutamide have no effect on this chimera due to the lack of the AR LBD in the Gal4DBD-AR₁₋₅₅₈ chimera. 185-9-1 is an analog of EPI-001 that has no activity in the concentration range employed and was used as a control. EPI-001 reduced both FSK-induced and IL-6-induced transactivation of the AR NTD to baseline levels (Figure 1B). EPI-001 had no effect on FSK-induction of transactivation of the Gal4DBD-CREB fusion protein, which supports that the inhibitory effects of EPI-001 are localized to the AR NTD and do not involve the Gal4DBD. EPI-001 has an IC₅₀ of ~6 μM for inhibition of transactivation AR NTD (Figure 1C).

EPI-001 Inhibits AR Activity Regardless of Ligand

An inhibitor of AR NTD function would block the activity of the full-length AR when activated by both ligand-dependent and ligand-independent mechanisms. To test this, we transfected LNCaP cells with the PSA(6.1 kb)-luciferase reporter that contains several AREs and is dependent upon AR for induction. LNCaP cells express endogenous AR that is activated by the synthetic androgen, R1881, and in the absence of both serum and androgen, by FSK or IL-6. EPI-001 blocked the induction of PSA-luciferase activity in response to androgen (R1881) and alternative pathways in the absence of androgen (FSK and IL-6) (Figure 1D). EPI-001 also blocked PSA-luciferase activity in 22RV1 cells that express both full-length AR and constitutively active splice variant of the AR that lacks LBD (Figure 1E). EPI-001 inhibited androgen-induced ARR3-luciferase reporter activity, that contains three repeats of the probasin ARE1 and ARE2 region, indicating that its effect was not specific to the PSA reporter (Figure 1F). To directly test if EPI-001 could inhibit a constitutively active deletion mutant, AR1-653, that contains the NTD, DBD and hinge region, but not the LBD (Jenster et al., 1991), was transfected into Cos1 cells which do not express endogenous AR. EPI-001 inhibited this constitutively active deletion mutant (Figure 1G). As expected, R1881 had no effect on AR1-653 because it lacks the LBD. EPI-001 also inhibited wild-type AR transfected into PC3 prostate cancer cells and exposed to R1881 as well as the constitutive activity of AR1-653 (Figure 1H). Levels of wild-type AR and AR1-653 protein levels from transfected cells are shown in Figure S1 (available online).

Endogenous Gene Expression

Induction of PSA and TMPRSS2 mRNAs in LNCaP cells is dependent upon AR. To test whether EPI-001 had an effect on endogenous gene expression, the levels of transcripts for numerous well-characterized androgen-regulated genes were measured in LNCaP cells. EPI-001 blocked androgen induction of endogenous PSA mRNA (Figure 2A), TMPRSS2 mRNA (Figure 2B), as well as 16 other well-known androgen-regulated genes (Figure S2A). However, EPI-001 did not inhibit androgen induction of all genes (an example is *BLVRB*).

AR Interaction with AREs on the DNA

Chromatin immunoprecipitation (ChIP) was used to assess if EPI-001 prevented AR binding to endogenous AREs in the regulatory regions of the *PSA* or *TMPRSS2* genes in the physiological

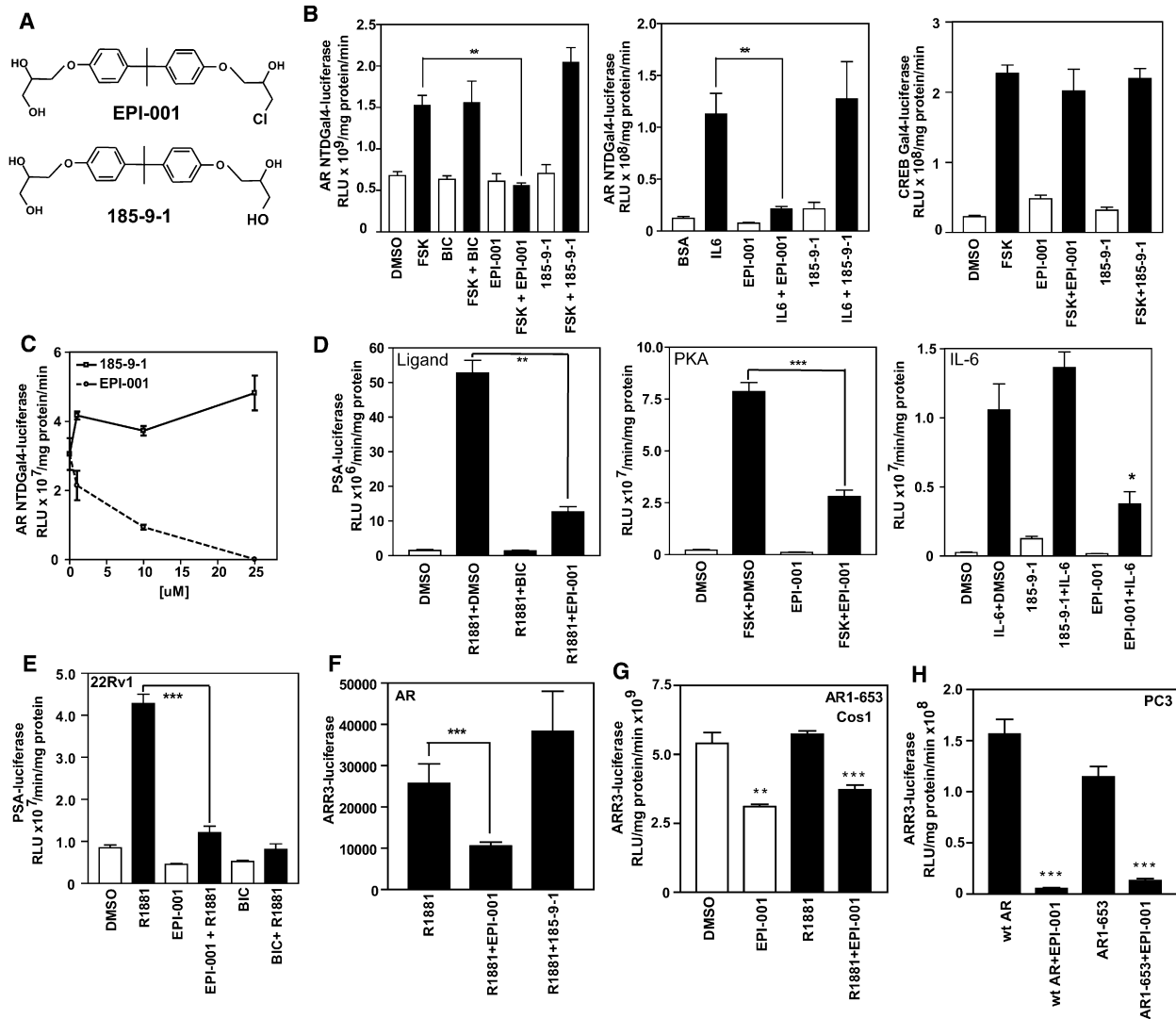


Figure 1. EPI-001 Blocks AR Transcriptional Activity

(A) Chemical structures of EPI-001 (BADGE.H₂O.HCl) or the inactive analog 185-9-1 (BADGE.2H₂O).
 (B) Transactivation assays of the AR NTD were performed in LNCaP cells cotransfected with p5 × Gal4UAS-TATA-luciferase and either AR-(1-558)-Gal4 DBD, Gal4DBD-CREB prior to incubation with FSK or IL-6 or vehicle for 24 hr. Bicalutamide (BIC), EPI-001, or 185-9-1 were added 1h before the addition of FSK or IL-6.
 (C) IC₅₀s of EPI-001 versus 185-9-1 on AR NTD transactivation were examined in LNCaP cells with varying concentrations of EPI-001 and 185-9-1.
 (D) EPI-001 inhibits both ligand-dependent and ligand-independent activation of the AR. LNCaP cells transfected with the PSA(6.1kb)-luciferase reporter and treated with R1881, FSK, or IL-6 under serum-free conditions for 24 hr.
 (E) 22Rv1 cells transfected with the PSA(6.1kb)-luciferase reporter, pretreated for 1 hr with BIC or EPI-001, prior to addition of R1881 for an additional 24 hr.
 (F) LNCaP cells stably transfected with ARR3-luciferase reporter were pretreated for 1 hr with EPI-001 (5 μg/ml) or 185-9-1 (5 μg/ml) before the addition of R1881 for an additional 24 hr. Bars represent the mean and standard deviation (± SD).
 (G) Cos-1 cells transfected with ARR3-luciferase reporter and an expression vector for AR1-653 were treated with R1881 or EPI-001 for 24 hr.
 (H) PC3 cells transfected with ARR3-luciferase reporter and an expression vector for wild-type AR or AR1-653 were treated with R1881 or EPI-001. Concentrations: R1881 (1 nM), FSK (50 μM), IL-6 (50 ng/ml), bicalutamide (BIC, 10 μM), EPI-001 (10 μg/ml), or 185-9-1 (10 μg/ml) unless stated otherwise. Error bars represent the mean and standard error of the mean (± SEM) in (B)–(E), (G), and (H). Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S1.

context of chromatin structure. Both *PSA* and *TMPRSS2* are androgen-regulated genes with well-characterized AREs. R1881 treatment significantly increased AR binding to AREs in promoter (*PSA-PR-ARE*) and enhancer (*PSA-EN-ARE*) of *PSA* (Figure 2C) and *TMPRSS2-ARE* (Figure 2D), which was significantly inhibited by EPI-001. CBP was also recruited to *PSA-EN-ARE*, *PSA-PR-ARE*, and *TMPRSS2-ARE* in response to

androgen (p < 0.01). Consistent with a decrease in AR binding to AREs, recruitment of CBP was also significantly decreased by EPI-001 (p < 0.05). The decrease in androgen-induced AR interaction with AREs by EPI-001 was not due to decreased levels of AR protein, general prevention of serine phosphorylation of AR, or prevention of AR nuclear translocation (Figures S2B–S2E).

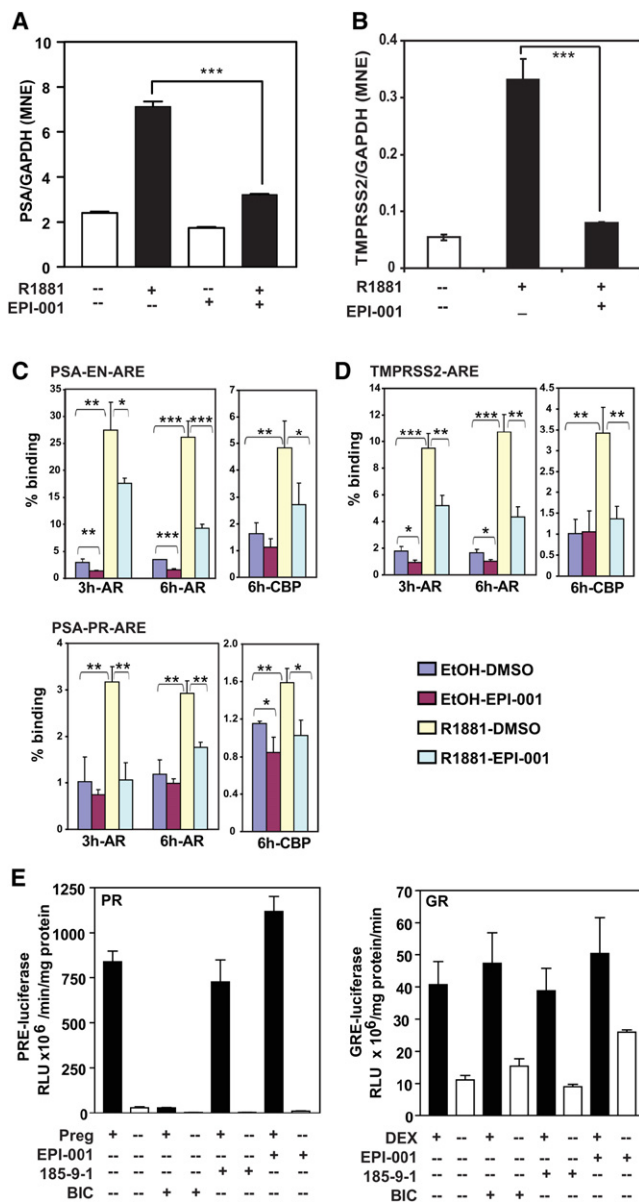


Figure 2. EPI-001 Inhibits Endogenous Expression of Androgen-Regulated Genes

(A and B) EPI-001 inhibits endogenous expression of PSA (A) and TMPRSS2 (B). LNCaP cells were pretreated with EPI-001 prior to incubation for 16 or 24 hr with R1881 and harvesting total RNA. Levels of PSA and TMPRSS2 mRNAs were measured by qRT-PCR and normalized to GAPDH mRNA. Bars represent the mean \pm SD.

(C) ChIP assay used LNCaP cells that were pretreated with EPI-001 (or DMSO as the vehicle control for 1 hr) prior to the addition of R1881 for 3 and 6 hr. Rabbit immunoglobulin G (IgG) (no antibody negative control) or anti-AR antibody or anti-CBP antibody was used in ChIP assay. Eluted DNA fragments were purified and used for qPCR with primers designed to amplify the PSA-ARE site in the enhancer (PSA-EN-ARE) and promoter (PSA-PR-ARE) of PSA locus. Error bars represent the mean \pm SD.

(D) DNA from cells was also used with primers to the ARE in *TMPRSS2* locus. The percentage input of each sample was averaged from triplicates. Bars represent the mean \pm SD.

(E) EPI-001 does not inhibit other steroid hormone receptors in LNCaP cells in response to ligand. Preg: 4-pregnene-3,20 dione (10 nM); DEX: dexametha-

Steroid Receptor Specificity

Sequence similarities of amino acids in the AR with related human steroid receptors progesterone receptor (PR) and glucocorticoid receptor (GR) are significant in some domains such as the DBD. The AR NTD shares less than 15% homology with the NTDs of PR and GR, but these receptors interact with some of the same proteins such as CBP. Therefore, reporter gene assays were used to determine if EPI-001 would inhibit GR or PR transcriptional activities. Cells were cotransfected with expression plasmids for full-length human GR, PR β , and the relative reporter, and then treated with ethanol vehicle, dexamethasone (GR), or 4-pregnene-3,20 dione (progesterone) (PR). Importantly, EPI-001 did not inhibit either PRE-luciferase or GRE-luciferase activities in response to ligand (Figure 2E). These data provide evidence that EPI-001 does not have general effects on transcription or translation because it did not inhibit induction of PRE- or GRE-luciferase reporters in response to their cognate ligands. Thus, EPI-001 appears specific to the AR without inhibiting the transcriptional activities of other steroid receptors.

EPI-001 Blocks N/C Interaction

Ligand-dependent activity of the AR requires N/C interaction for antiparallel dimer formation (He et al., 1999). Antiandrogens such as bicalutamide, flutamide, and cyproterone acetate do not stimulate this interaction on their own, and each inhibits N/C interaction induced by androgen (Wong et al., 1993; Langley et al., 1995; Kempainen et al., 1999; Masiello et al., 2002). We applied the mammalian two-hybrid system to measure N/C interaction in CV1 cells that do not express endogenous AR. Cells were cotransfected with expression vectors for a chimera protein of amino acids 1–565 of the AR NTD fused to VP16 (VP16-ARTAD, the N terminus), the expression vector for the Gal4DBD fused to the LBD of the AR (amino acids 628–919; Gal4-ARLBD; the C terminus), and the Gal4-luciferase reporter. As expected, there was no detectable N/C interaction in the absence of androgen (Figure 3A, compare columns 1 and 2). Androgen stimulated this interaction as measured by increased luciferase activity which was blocked by bicalutamide (columns 2 to 8). Importantly, EPI-001 inhibited androgen-stimulated N/C interaction (columns 4 and 2), whereas the inactive analog 185-9-1 had no effect (columns 6 and 2).

EPI-001 Does Not Prevent Ligand Binding

Deletion of AF-1 in the NTD kills transcriptional activity but does not prevent ligand binding (Jenster et al., 1991). To test whether EPI-001 affects ligand binding, we employed fluorescence polarization to measure competition of binding of a fluoromone to recombinant AR-LBD. Both R1881 and bicalutamide bound to the AR-LBD as expected (Figure 3B). EPI-001 in the concentration range of 0.1 nM to 100 μ M did not prevent ligand-binding as shown by not competing with binding of fluoromone. These data suggest that EPI-001 does not interact with the LBD or reduce ligand-binding.

son (10 nM). Error bars represent the mean \pm SEM. Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S2.

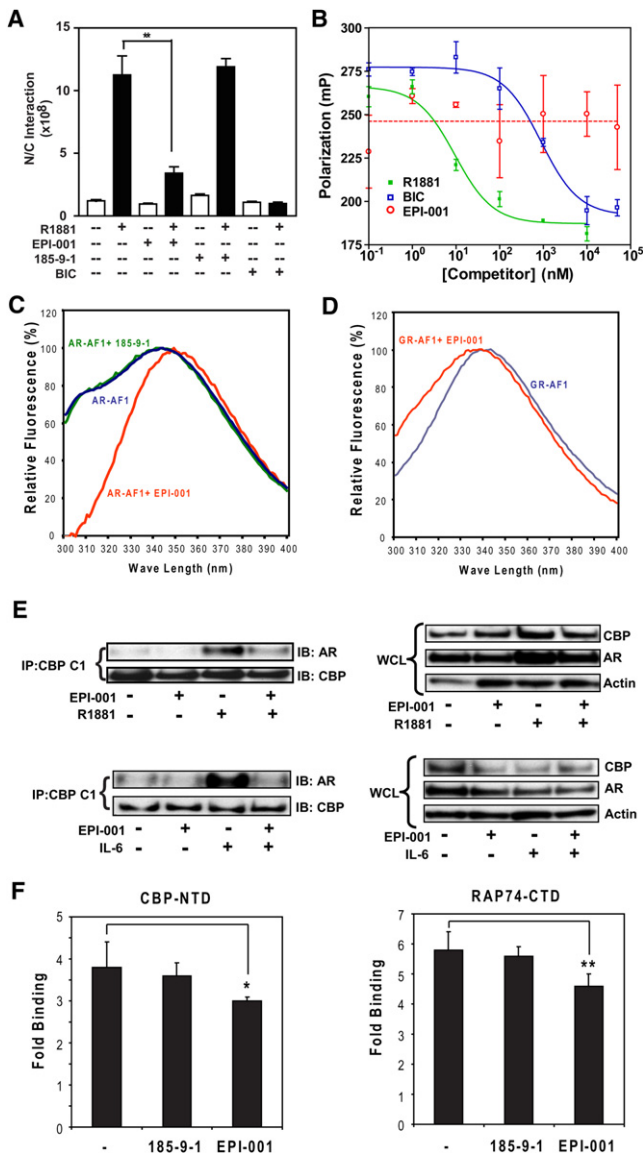


Figure 3. EPI-001 Interacts with AF-1 in the NTD to Prevent Protein-Protein Interactions

(A) EPI-001 blocks N/C interaction induced by androgen. CV1 cells transfected with GAL4-AR DBD and/or VP16-ARTAD and Gal4-luciferase reporter were pretreated for 1 hr with bicalutamide, EPI-001, or 185-9-1 before addition of R1881 for 24 hr. Error bars represent the mean \pm SEM, ** $p < 0.01$.

(B) A representative competition binding curve showing inhibition of ligand (2 nM) binding to recombinant AR-LBD (20 nM) by the antiandrogen bicalutamide and agonist R1881. EPI-001 did not inhibit ligand-binding. Mixtures were incubated for 5 hr before measurement of fluorescent polarization. Data represent the mean \pm SEM, $n = 3$.

(C) Steady-state fluorescence emission spectra for AR-AF1 polypeptides in buffer, buffer plus EPI-001 (2.9 μ M), or buffer plus 185-9-1 (5.3 μ M). In buffer, or buffer plus 185-9-1, the steady state spectra for AR-AF1 has a distinctive shoulder at 305 nm and a λ_{max} for tryptophan at 343 nm.

(D) Steady-state fluorescence emission spectra for GR-AF1 in buffer, or buffer plus EPI-001 (5.3 μ M). λ_{max} for tryptophan was 341 nm in buffer and 338 nm in the presence of EPI-001. Spectra have been corrected for buffer effects and normalized to the λ_{max} for tryptophan (100%).

(E) EPI-001 prevents physical interaction between endogenous AR and CBP. LNCaP cells were serum-starved and then exposed to R1881, IL-6, or vehicle

Interaction of EPI-001 with the AR-AF1 Region in the NTD

AR-AF1 is characterized by the presence of natural disordered structure, which adopts a more folded conformation in the presence of binding partners or the natural osmolyte TMAO. The conformational changes in this domain can be studied by measuring the steady-state fluorescence spectra for endogenous aromatic amino acids (tyrosine and tryptophan) (Reid et al., 2002). In buffer, the steady-state spectrum is characterized by a λ_{max} for tryptophan at 343 nm and shoulder of tyrosine emission at 305 nm (Figure 3C). In the presence of EPI-001 there is a dramatic loss of the tyrosine shoulder and a red-shift of the tryptophan λ_{max} to 349 nm. These changes are consistent with a change in the local environment surrounding the tryptophan residues and a quenching of the tyrosine signal due to an interaction of EPI-001 with AR-AF1. These changes were not observed in the presence of the inactive compound 185-9-1. Importantly, the steady state spectrum for GR-AF1 was not affected by EPI-001 (Figure 3D). Taken together, the data suggest that interaction of EPI-001 with the AR-AF1 domain alters the steady-state fluorescence emissions for tyrosine and tryptophan through direct binding and/or a conformational change in the polypeptide.

EPI-001 Blocks Protein-Protein Interactions with AR

CBP and its homolog p300 are bridging factors and CBP interacts with the AR NTD (Frønsdal et al., 1998) to potentially stabilize both N/C interaction as well as AR binding to AREs. CBP/p300 increases AR transactivation of the AR in response to androgen or IL-6 (Frønsdal et al., 1998; Debes et al., 2002). Ligand-dependent and ligand-independent interactions between CBP and AR were examined using endogenous complexes isolated from LNCaP cells that were pretreated with EPI-001. AR was immunoprecipitated with CBP as expected in response to both R1881 and by IL-6 and this interaction was reduced by EPI-001 (Figure 3E). Reprobing the blot for CBP protein revealed similar levels of CBP were immunoprecipitated in each lane thereby supporting that EPI-001 decreased interaction of CBP with AR in response to both R1881 and IL-6. Consistent with these data, EPI-001 also inhibited interaction between AF-1 (AR NTD) and CBP-CTD recombinant proteins (Figure 3F) as well as AF-1 interaction with the large subunit of general transcription factor TFIIF (RAP74), a protein previously shown to interact with AF-1 in the AR NTD (Reid et al., 2002). In the presence of EPI-001, binding of CBP-CTD and RAP74-CTD were each impaired by 21%. In contrast there was little or no effect on binding in the presence of 185-9-1. Therefore, EPI-001 is capable of disrupting at least a subset of interactions with the AR-AF1 region in the NTD. Because EPI-001 did not block PR or GR transcriptional activity (Figure 2E) that require CBP interaction, these data support the potential specificity of EPI-001 for the AR NTD.

for 6 hr. Whole cell lysates were precleared with mouse IgG, immunoprecipitated with anti-CBP antibodies (IP), and then analyzed by immunoblotting (IB). (F) In vitro protein-protein interactions. Immobilized AR-AF1 was incubated with radiolabeled CBP-NTD or with RAP74-CTD polypeptides in the absence (-) or presence of EPI-001 (2.54 μ M) or 185-9-1 (2.66 μ M). Fold-binding is plotted relative to a BSA control (= 1) and represents the mean \pm SD for at least $n = 3$ independent measurements. Student's t test: * $p = 0.05$ and ** $p = 0.02$.

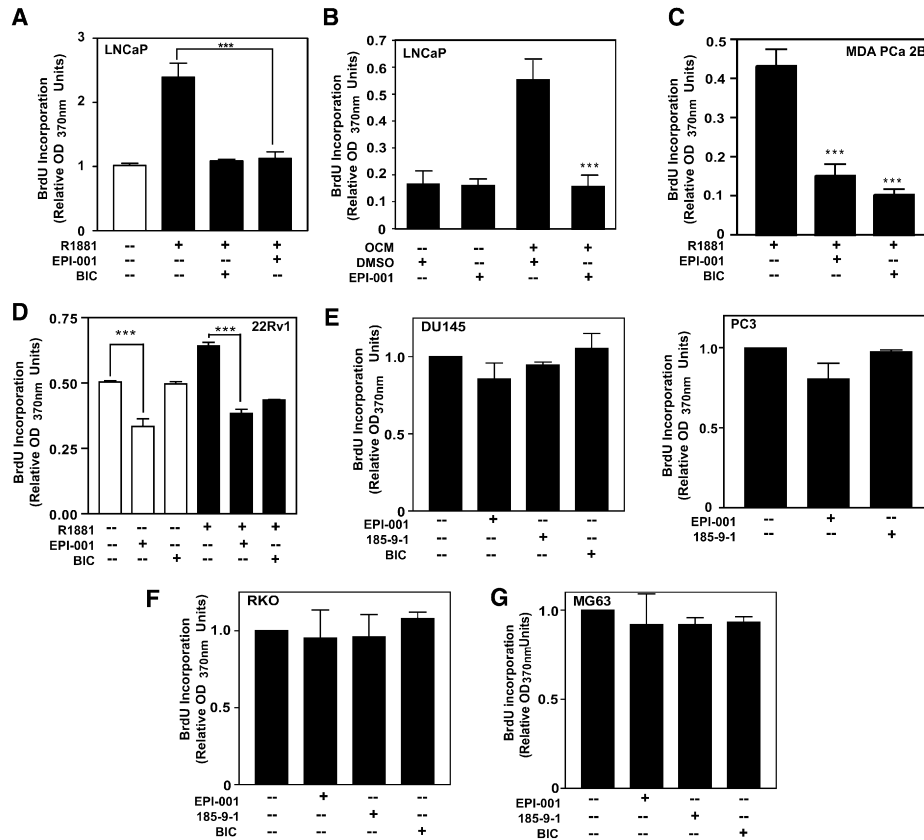


Figure 4. EPI-001 Blocked AR-Dependent Proliferation

(A) LNCaP cells treated with bicalutamide (BIC) or EPI-001 for 1 hr before the addition of R1881 (0.1 nM) for 4 days. Error bars represent the mean \pm SEM. (B) LNCaP cells treated with OCM (50%) with, or without, EPI-001 for 3 days (serum-free, androgen-free, phenol red-free conditions) Error bars represent the mean \pm SD. (C) MDA PCa 2B cells in BRFF-HPC1 (contains 0.1 nM DHT) were treated with R1881 and EPI-001 for 5 days. Error bars represent the mean \pm SEM. (D) 22Rv1 cells treated for 3 days with, or without androgen, and EPI-001 or BIC. Bars represent the mean \pm SEM. (E) EPI-001 does not block proliferation of PC3 or DU145 cells treated with vehicle or EPI-001 for 3 days. Error bars represent the mean \pm SD. (F) EPI-001 does not prevent proliferation of RKO colon cancer cells treated for 3 days. Error bars represent the mean \pm SD. (G) EPI-001 does not alter proliferation of MG63 osteosarcoma cells treated for 3 days. Error bars represent the mean \pm SD, t test, $p > 0.05$ for all cells except LNCaP, MDA PCa 2B, and 22Rv1 ($p < 0.001$). Proliferation was assessed by BrdU incorporation.

EPI-001 Blocks AR-Dependent Proliferation

EPI-001 was next tested to examine if it had effects on proliferation that were specific to prostate cancer cells that express AR. LNCaP cells increase proliferation in response to androgen (0.1 nM) and in the absence of androgen (serum-free and phenol red-free) by osteoblast-conditioned media (OCM) by a mechanism dependent on AR (Blaszczuk et al., 2004; Wang et al., 2009). EPI-001 inhibited both androgen-dependent (Figure 4A) and androgen-independent (OCM-induced) (Figure 4B) proliferation of LNCaP cells. EPI-001 decreased proliferation of MDA PCa2B cells (Figure 4C) and 22Rv1 cells (Figure 4D), which both express AR. EPI-001 did not block proliferation of PC3 or DU145 human prostate cancer cells (Figure 4E, $p > 0.05$ t test) that do not express functional AR and do not rely on the AR for growth and survival. EPI-001 did not inhibit proliferation of RKO human colon cancer cells (Figure 4F) or MG63 osteosarcoma cells (Figure 4G). These data support the specificity of EPI-001 for targeting the AR to block AR-dependent proliferation.

In Vivo, EPI-001 Blocks the Androgen-Axis and Inhibits Androgen-Dependent Tumor Growth

Prostate is an androgen-dependent tissue that involutes with androgen ablation or administration of antiandrogens. In vivo testing of inhibitors of the androgen axis can therefore be assessed by measuring changes in the weight of the prostate. Intravenous (i.v.) injection of EPI-001 (50 mg/kg body weight) significantly reduced the weight of prostates from intact mice compared with control-treated animals (Figure 5A). These data are consistent with EPI-001 inhibiting the androgen axis in mice. Similarly, LNCaP subcutaneous (s.c.) xenografts from intact male mice treated with EPI-001 by i.v. were significantly reduced in volume (Figure 5B). Staining sections of harvested xenografts revealed that EPI-001 significantly reduced proliferation (Ki67 staining) and increased apoptosis (TUNEL staining) (Figure 5C), which was consistent with reduced tumor growth.

To further expand our finding, we include a newly established patient-derived prostate cancer xenograft model.

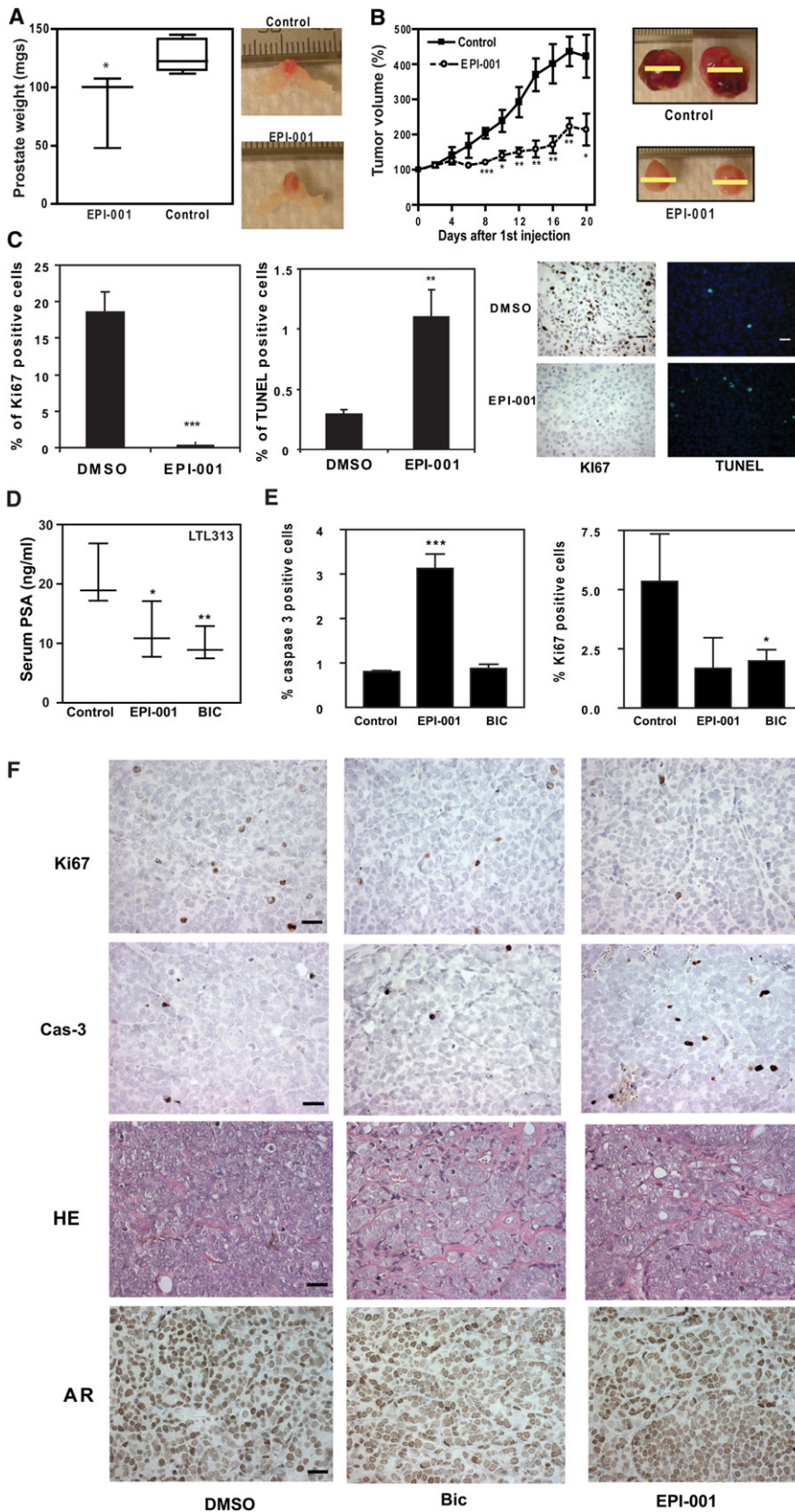


Figure 5. EPI-001 Inhibits Androgen-Dependent Growth In Vivo

(A) Weight of prostates from intact mice treated with EPI-001 (i.v. 50 mg/kg body weight) every other day for a total of 10 doses. Prostates were harvested 2 days after the last injection. EPI-001, n = 5; DMSO, n = 6.

(B) LNCaP tumor volume decreased in intact mice administered with EPI-001 (same protocol as in A). Tumors were harvested 2 days after the last injection. Error bars represent the mean \pm SEM. Scale Bar = 10 mm.

(C) EPI-001 decreases proliferation (Ki67 staining) and increases apoptosis (TUNEL staining) in s.c. LNCaP tumors harvested from intact mice. % Ki67 and % TUNEL positive cells were counted in sections from three xenografts for each treatment. At least 1000 cells per xenograft were counted. Total number of cells counted: 4156 (DMSO, Ki67), 3108 (EPI-001, Ki67), 4048 (DMSO, TUNEL), and 3089 (EPI-001, TUNEL), n = 3 tumors. Representative slides with staining are shown.

(D) EPI-001 decreases serum PSA in intact mice bearing LTL313 xenografts implanted under the renal capsule. Mice were administered EPI-001 (i.v. 50 mg/kg body weight every other day) or BIC (gavage daily with 10 mg/kg body weight) for a duration of 20 days. n = 4 or 5.

(E) Caspase-3 staining was used for determining the apoptotic index, whereas Ki-67 staining was used for determining the proliferative index of LTL313 cancer cells. EPI-001 significantly increased apoptosis as compared with both the control and bicalutamide. The proliferative index was reduced by both EPI-001 and BIC.

(F) Immunohistochemistry of representative sections of LTL313 xenografts harvested at the duration of the experiment and stained for Ki67, caspase-3 (Cas-3), hematoxylin and eosin (H&E), and AR. Error bars represent the mean \pm SD (C and E). Box and whisker plots (A and D) represent the median, minimum, and maximum. Scale bars represent 20 μ m in all micrographs.

LTL313 was developed from needle biopsy specimen obtained from an 80-year-old man diagnosed with Gleason grade 8 prostate cancer and presenting serum PSA of 17 ng/ml (see details at <http://www.livingtumorlab.ca>). LTL313 is a slow-growing tumor with a doubling time of greater than 20 days in intact male SCID mice, making it difficult to measure short-term treatments on tumor volume. LTL313 xenografts express AR and serum PSA provides a sensitive measure for biochemical responses to treatments. After just 2 weeks of treatment, serum PSA from the EPI-001-treated group was significantly lower than that in the control group and comparable to levels achieved with bicalutamide (Figure 5D). EPI-001 treated xenografts had a higher apoptotic index compared to tumors from control-treated mice as determined by caspase-3 staining (Figure 5E). Proliferation was low in LTL313 as expected compared with LNCaP tumors. EPI-001 did show a trend to decrease proliferation that was borderline significant ($p = 0.056$) at this short treatment time that was limited because of frequent tail vein injections. Histology shows LTL313 cells express AR and levels of caspase-3 and Ki-67 in harvested tumors (Figure 5F).

In Vivo Efficacy of EPI-001 with No Apparent Toxicity

For testing whether EPI-001 would have an effect on CRPC tumor growth, male mice bearing LNCaP s.c. xenografts were castrated when the tumors were $\sim 100 \text{ mm}^3$. Within just 2 weeks, EPI-001 by i.v. reduced tumors from $100.3 \pm 1.72 \text{ mm}^3$ to $73.03 \pm 29.6 \text{ mm}^3$ ($n = 10$), whereas tumors in vehicle-treated animals continued to grow from $103.2 \pm 3.89 \text{ mm}^3$ to $148.2 \pm 19.39 \text{ mm}^3$ ($n = 7$) (compare the red line for EPI-001 in Figure 6A with the blue line for dimethyl sulfoxide [DMSO]). Thus, the animals treated with EPI-001 had tumors that were less than half the size of controls ($p = 0.0028$). Immunohistochemistry (IHC) using markers for apoptosis (TUNEL) and proliferation (Ki67) showed that i.v. delivery of EPI-001 increased apoptosis and reduced proliferation (Figure 6B). This was consistent with cytorreduction of the tumors as well as in vitro analysis of apoptosis showing that at 72 hr of treatment with R1881, that LNCaP cells had only $5.15 \pm 0.89\%$ apoptosis while EPI-001 increased this by almost 2-fold to $9.55\% \pm 2.27\%$ ($p = 0.01$). EPI-001 did not cause general toxicity to animals indicated by no change in animal behavior or body weight (Figure 6C). Pathology review of sections of lung, heart, liver, spleen, and kidney harvested from mice receiving EPI-001 by i.v. delivery showed no signs of toxicity (Figure 6D). Longer treatment periods of animals were feasible by intratumoral (i.t.) injections. Animals were castrated when tumors were $\sim 100 \text{ mm}^3$ (mean = $131.1 \pm 24.9 \text{ mm}^3$, $n = 19$). EPI-001 (20 mg/kg body weight every 5 days) significantly reduced the tumors, even after the first injection (compare the green line for EPI-001 with the black line for DMSO in Figure 6A). Note that the slope of the tumor volume for i.v. and i.t. experiments overlap. At the duration of the experiment, the EPI-001-treated tumors were reduced to $35.4 \pm 15.7 \text{ mm}^3$, whereas vehicle-treated tumors continued to grow and were $435.6 \pm 334.9 \text{ mm}^3$. Thus EPI-001 reduced the tumor volume and did not just slow the growth. This suggests EPI-001 may be potentially curative for CRPC. Harvested tumors from animals treated with EPI-001 also tended to be less bloody in appearance (Figure 6E). IHC and western blot analysis reveal

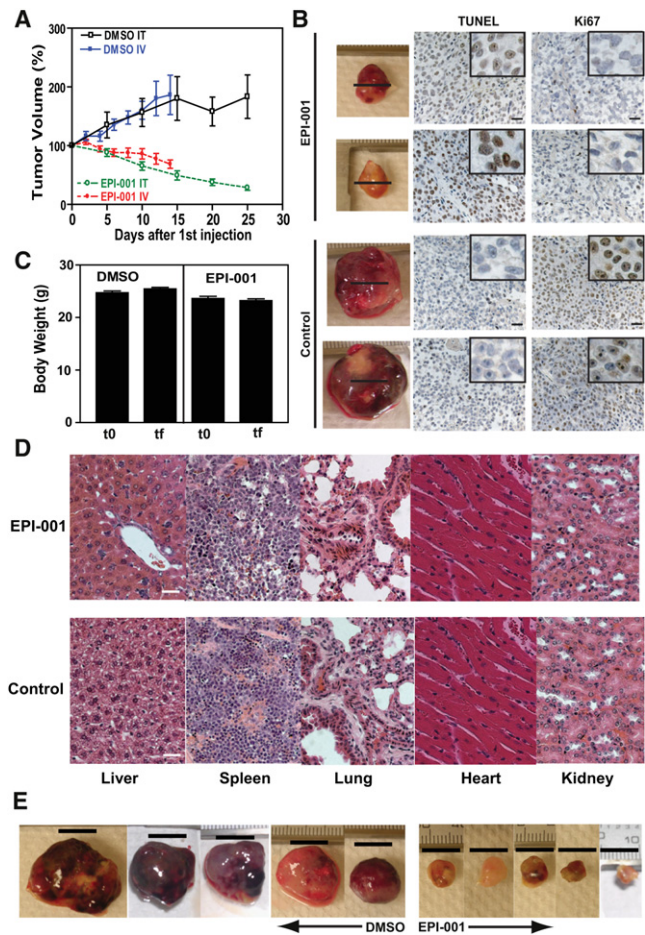


Figure 6. Systemic Delivery of EPI-001 Results in Cytorreduction of CRPC without Causing Toxicity

(A) Tumor growth in SCID mice bearing s.c. tumors receiving i.v. injections of EPI-001 or vehicle control follows the same slope as that obtained by i.t. injection. Mice received i.v. delivery of EPI-001 (50 mg/kg body weight) every other day, or i.t. delivery (20 mg/kg body weight) every 5 days, that began 7 days after castration. Bars represent the mean \pm SEM. (B) Photographs of xenografts treated i.v. with EPI-001 with corresponding IHC for markers of proliferation (Ki67) and apoptosis (TUNEL). Sections of xenografts were immunostained (diaminobenzidine with hematoxylin counterstaining) for Ki67 and TUNEL. The box within each image is a cropped region from the image to enable a closer view of the expression patterns. (C) Body weight of animals administered EPI-001 by i.v. over the duration of the experiment. DMSO: $n = 7$, $p = 0.152$. EPI-001: $n = 10$, $p = 0.483$. t_0 = body weight the day of 1st injection. t_f = body weight 2 days after the last injection. Bars represent the mean \pm SEM. (D) HE staining of sections of liver, spleen, lung, heart and kidney harvested at the duration of the experiment from hosts receiving i.v. delivery of EPI-001 (top row) or vehicle control (bottom row). Tissues are from the same animals shown in (B). (E) Photographs of harvested tumors from animals that received EPI-001 by i.t. The black bar equals 10 mm. Scale bars represent 20 μm in all micrographs. See also Figure S3.

that EPI-001 did not decrease in vivo levels of AR protein compared with levels of AR protein in vehicle-treated xenografts (Figure S3).

In an attempt to more closely mimic human disease, we tested whether EPI-001 would be effective in castrated mice bearing orthotopic LNCaP xenografts. Serum PSA was used to provide an

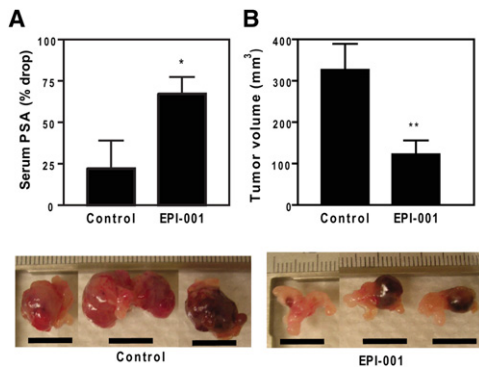


Figure 7. EPI-001 Inhibits Serum PSA and Growth of Orthotopic LNCaP Xenografts in Castrated Mice

(A) Mice were administered 50 mg/kg body weight EPI-001 by i.v. every other day for a total of 8 doses. Serum PSA was measured 2 days after the last dose when the prostates were harvested and tumor volume measured. Initial serum PSA: 69 ± 11 (control) and 60 ± 13 ng/ml (EPI-001), $p = 0.36$. Serum PSA is represented as the percentage drop from the start of the experiment. Error bars represent the mean \pm SEM.

(B) Tumor volumes and photographs of representative prostates with LNCaP tumors from mice administered DMSO or EPI-001. Bars represent the mean \pm SEM.

indication of tumor burden prior to castration and was measured again at the duration of the experiment. Initial serum PSA levels were similar in both treatment groups (69 ± 11 versus 60 ± 13 ng/ml, $p = 0.36$) prior to castration. Serum PSA was decreased by only $22.1\% \pm 29.4\%$ at the duration of the experiment in control mice compared to $67.2 \pm 24.8\%$ in EPI-001-treated mice (Figure 7A). These significant differences in serum PSA between the two groups correlated to differences in tumor volume (Figure 7B). Prostatic tumors harvested from hosts receiving EPI-001 were approximately 62% smaller than tumors from control mice (122.6 ± 104.9 mm³ versus 326.5 ± 125.6 mm³, respectively, $p = 0.009$).

Subcutaneous PC3 Xenograft Models

The LNCaP and LTL313 xenograft models reflect the majority of clinical CRPC that express AR and are dependent on endogenous AR for growth and survival. We employed PC3 human prostate cancer cells that are insensitive to androgen and do not express functional AR to provide an indication of specificity of EPI-001. In vitro, EPI-001 did not significantly affect proliferation of PC3 cells (Figure 4E). Male mice were castrated and randomized into two groups when PC3 s.c. tumors were approximately 50 mm³ (tumor volume = 56.5 ± 5.8 mm³, $n = 14$). Four days after castration, the animals were treated every other day with an i.v.

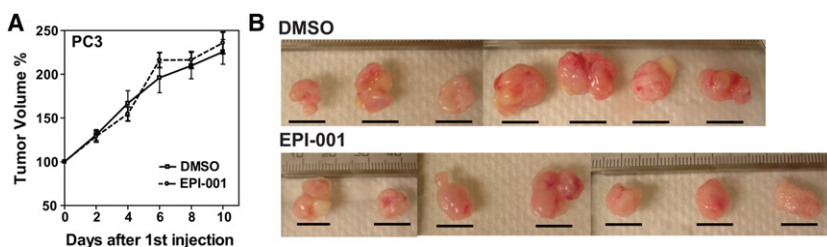


Figure 8. EPI-001 Does Not Cause Cytorreduction of PC3 Xenografts

(A) PC xenograft growth in SCID mice bearing s.c. tumors receiving i.v. injections of EPI-001 ($n = 7$) or vehicle control ($n = 7$). Four days after castration, animals received 50 mg/kg body weight by tail vein injection every other day for a total of five injections. Tumors were harvested 2 days after the last injection. Bars represent the mean \pm SEM.

(B) Photographs of harvested tumors. Scale bars represent 10 mm.

dose of 50 mg/kg body weight EPI-001 or matching volume of vehicle (control, DMSO). In contrast to LNCaP xenografts, EPI-001 did not reduce tumors (Figures 8A and 8B). These data support that EPI-001 is specific to the AR and does not affect cells that do not depend on functional AR for growth and survival.

DISCUSSION

Current therapies that target AR LBD provide transient responses but do not provide cures for patients with CRPC and presumably fail due to gain-of-function mutations in the LBD or to expression of constitutively active splice variants lacking the LBD. The AF-1 in the NTD contributes most of the transcriptional activity to the AR. AF-1 mutations in the NTD do not affect ligand-binding but obliterates transcriptional activity of the AR (Jenster et al., 1991). Drugs that target the AR NTD would block the activity of the AR regardless of ligand. Thus, while conventional therapy has concentrated on androgen-dependent activation of the AR through its C-terminal LBD, our concept of blocking transactivation of the NTD for therapy of CRPC has not been previously addressed. Here we identified a lead compound for developing drugs that can be used to delay or potentially cure CRPC. We show that EPI-001: (1) inhibited transactivation of the AR NTD; (2) was specific for inhibition of the AR without inhibiting PR or GR transcriptional activities; (3) blocked induction of androgen-regulated genes; (4) interacted with the AF-1 region in the NTD; (5) reduced protein-protein interactions with the NTD; (6) inhibited constitutively active AR1-653 that lacks the LBD; (7) reduced AR interaction with well-characterized AREs on target genes; (8) blocked androgen-induced proliferation; and (9) caused cytorreduction of CRPC in xenografts dependent on AR for growth and survival without causing toxicity.

EPI-001-related compounds were isolated from the marine sponge *Geodia lindgreni* and have structural resemblance to BADGE (Bisphenol A Diglycidic Ether), which indicates that they may be of industrial origin. The collected sponge presumably bioaccumulated the BADGE derivatives from contaminated seawater. Approximately 20 BADGE analogs were tested in cell-based assays to obtain additional Structure Activity Relationship for the pharmacophore. The compound BADGE.HCl.H₂O, or EPI-001, had the most potent activity. EPI-001 and 185-9-1 (BADGE.2H₂O) are harmless metabolites of bisphenol A with no estrogenic or androgenic effects (Poole et al., 2004; Volkel et al., 2002; Tsai, 2006; Stroheker et al., 2004). Metabolic systems are unable to transform BADGE into bisphenol A (Poole et al., 2004). BADGE and its chlorohydrins were examined with conclusions that they are not carcinogenic or genotoxic at daily doses well in excess of those used here (EFSA, 2004). There are no

adverse effects with daily oral doses of 450 mg/kg body weight BADGE for 3 months (Poole et al., 2004). Consistent with these observations, here i.v. delivery of 50 mg/kg body weight EPI-001 had no effects on the histology of major internal organs, or the body weights and behavior of the animals. EPI-001 did not significantly reduce the proliferation of cells that do not require functional AR for growth and survival. Interestingly, doses of 250 and 1000 mg/kg body weight BADGE daily for 2 years had mild effects on the adrenal cortex and seminiferous tubules in males, and in females slight atrophy of the endometrium and myometrium of the ovary (Poole et al., 2004). These tissues express AR and tend to respond to androgens (Wilson and McPhaul, 1996), thereby supporting their antagonist effects on the androgen pathway. Consistent with those observations, here we show that EPI-001 reduced the weight of androgen-dependent prostate tissue.

BADGE.2HCl (AR-binding IC_{50} , 19 μ M) and BADGE.2H₂O/185-9-1 (AR-binding IC_{50} , 190 μ M) are suggested to bind to the full-length AR at high concentrations by measurement of liberated testosterone that competed with anti-testosterone antibody and peroxidase-labeled testosterone (Satoh et al., 2004). Those studies did not examine EPI-001 (BADGE.HCl.H₂O), nor did they examine dissociation kinetics. The LBD functions independent of other domains to bind ligand (Jenster et al., 1991). However, a compound that interferes with the NTD such as EPI-001 could affect the ligand-dissociation rate even though it does not bind to the LBD. Deletion of the NTD increases the dissociation rate of bound ligand (Zhou et al., 1995), and importantly N/C interaction results in slower dissociation of bound ligand (Langley et al., 1998). Although EPI-001 does not bind to the LBD, it may cause ligand to dissociate more quickly because it blocks N/C interaction.

AR NTD is essential for AR activity and is implicated in the underlying molecular mechanism of CRPC. In the absence of androgen, full-length AR is activated through its NTD by factors secreted from bone and by alternative pathways. Changes in protein-protein interactions of the AR NTD by stimulation of alternative pathways may mediate a transcriptionally active receptor in the absence of ligand. Precedence for this mechanism is drawn from estrogen related receptors (ERR) that are activated in the absence of ligand by interactions with steroid receptor coactivator (SRC) and PPAR- γ coactivator 1 (PGC-1) families (Xie et al., 1999; Huss et al., 2002; Schreiber et al., 2003; Hong et al., 1999). In the absence of ligand, crystal structure analyses of ERR α bound to a PGC-1 α peptide, and ERR γ bound to a SRC-1 or RIP-140 peptides, have shown both ERRs assume the conformation of ligand-activated nuclear receptors (Kallen et al., 2004; Wang et al., 2006; Greschik et al., 2002). This suggests that agonist ligand is not an essential requirement for the activation of receptors. The estrogen receptor (ER)- β and chick PR are also transactivated in the absence of ligand by the PKA pathway through enhanced protein-protein interactions with SRC-1 and CBP (Dutertre and Smith, 2003; Rowan et al., 2000; Tremblay et al., 1999). The AR NTD is flexible with a high degree of intrinsic disorder with characteristics of a collapsed disordered conformation (Lavery and McEwan, 2008). The limited structure of the AR NTD is thought to require interactions with other proteins to assume correct folding for further protein-protein interactions including interactions with bridging factors

such as CBP and the basal transcriptional machinery for active transcription (Reid et al., 2002). Thus an inhibitor that blocks essential protein-protein interactions with the NTD would block AR transcriptional activity regardless of ligand. Indeed, here we show that EPI-001 interacts with AF-1 and inhibits essential protein-protein interactions including CBP, RAP74, and N/C. EPI-001 inhibited both ligand-independent and ligand-dependent interaction with CBP interaction. CBP interacts with the AR NTD (Frønsdal et al., 1998), enhances AR transcriptional activity (Aarnisalo et al., 1998), and levels of CBP/p300 are associated with clinical progression (Debes et al., 2003; Comuzzi et al., 2004). CBP may stabilize N/C interactions and AR binding to AREs. PR and GR transcriptional activities require CBP interactions (Smith et al., 1996; Kamei et al., 1996) that were not attenuated by EPI-001, which was consistent with AR-specificity of EPI-001. Specificity of EPI-001 for AR was also supported by: (1) lack of effect of EPI-001 on the proliferation of cells that do not require functional AR; (2) in vivo data showing no effect of EPI-001 on the growth of PC3 xenografts; and (3) no effects on body weight, or histology of internal organs, of animals receiving i.v. delivery of EPI-001. The selectivity of EPI-001 to inhibit transactivation of the AR NTD and attenuate AR activity through blocking protein-protein interactions and reducing binding to AREs has significant therapeutic potential for the treatment of CRPC.

EXPERIMENTAL PROCEDURES

Cell Proliferation, Transfections, Luciferase Assays, and Gene Expression Analysis

LNCaP, 22RV1, MDA PCa2b, PC3, Du145, RKO, and MG63 cells were seeded in 96-well plates for 24 hr before pretreating for 1 hr with bicalutamide (10 μ M), EPI-001, or 185-9-1 prior to addition of 0.1 nM R1881 for LNCaP and 22RV1 cells. BrdU incorporation was measured after 4 days for LNCaP, 5 days for MDA PCa2b, and 3 days for 22RV1 PC3, Du145, RKO, and MG63 cells using BrdU ELISA kit (Roche Diagnostics). BRFF HPC1 media for MDA PCa2b cells contains 0.1 nM DHT, making androgen-free conditions not possible. Transfections, luciferase assays, and gene expression analysis were performed as described previously. Details are available in the [Supplemental Experimental Procedures](#).

Fluorescence Polarization

The Polar Screen Androgen Receptor Competitor Assay kit (Invitrogen) was employed according to the manufacturer's protocol with 20 nM AR-LBD and 2nM Fluoromone. The reactions were done in 40 μ l aliquots in triplicates in Greiner 384 black clear bottom plates and fluorescence polarization read using the Infinite M1000 (Tecan) with excitation at 530 nM and emission at 590 nM.

Coimmunoprecipitation, Chromatin Immunoprecipitation, and Immunohistochemistry

Coimmunoprecipitations, chromatin-immunoprecipitation assays, and immunohistochemistry were performed as previously described. Details are available in [Supplemental Experimental Procedures](#).

Animals

Six- to eight-week-old male NOD-SCID mice were maintained in the Animal Care Facility in the Research Center of the British Columbia Cancer Agency. All experiments involving animals conform to the relevant regulatory standards and the experiments were approved by the University of British Columbia Animal Care Committee.

Subcutaneous Tumors

Mice bearing subcutaneous (s.c.) tumors were castrated when tumors averaged 100 mm³ (LNCaP) or 50 mm³ (PC3) in size. Intact animals with s.c. tumors began treatment when tumors were approximately 50 mm³. Tumor volumes

were calculated by the formula length \times width \times height \times 0.5236. Tumors and major organs were excised 5 days after the last i.t. injection or 2 days after the last i.v. injection and prepared for western blot analyses and IHC.

Orthotopic LNCaP Xenograft

Subcutaneous LNCaP xenografts were grafted into the anterior prostates of male mice. When serum PSA was above 40 ng/ml, animals were randomized into two groups and animals were castrated.

LTL313 Renal Capsule Xenograft

The patient-derived prostate cancer tissue line LTL313 was maintained by serial transplantation of subrenal capsule xenografts into male NOD-SCID mice supplemented with testosterone (10 mg/mouse). The protocol was approved by the Clinical Research Ethics Board of the University of British Columbia/BC Cancer Agency. When the tumors reached an average volume of about 50 mm³, testosterone pellets were removed and mice were randomly distributed into three groups for treatment for control, EPI-001, and bicalutamide. At the duration of the experiment, serum PSA was measured, tumors were harvested and measured, and sections were prepared for immunohistochemistry.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.ccr.2010.04.027](https://doi.org/10.1016/j.ccr.2010.04.027).

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