

## Original Article

# A new compound targets the AF-1 of androgen receptor and decreases its activity and protein levels in prostate cancer cells

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**Abstract:** Increased expression levels of constitutively active androgen receptor splice variants (AR-Vs) cause alterations in AR signaling, resulting in drug resistance and failed hormone therapy among patients with advanced prostate cancers. Several available compounds targeting the androgen axis and AR signaling have not demonstrated efficacy in preventing prostate cancer recurrence. Here, we investigated whether a new agent, 6-[6-ethoxy-5-isopropoxy-3,4-dihydroisoquinolin-2(1H)-yl]-N-[6-methylpyridin-2-yl]nicotinamide (EIQPN), has the potential for treating advanced prostate cancer. EIQPN interacted with the AR-activation fragment-1 (AF-1) domain and blocked its androgen-independent activity, robustly decreased the protein levels of AR and variants in prostate cancer cells by inducing AR protein degradation, and inhibited the androgen-independent proliferation of various AR-positive prostate cancer cells. In xenograft mouse models, EIQPN blocked the tumor growth of androgen-independent prostate cancer cells. Overall, these findings indicate that EIQPN could serve as a novel therapeutic agent for advanced recurrent prostate cancers.

**Keywords:** Prostate cancer, androgen receptor, splice variant, androgen-independent activity, protein degradation

## Introduction

Prostate cancer is a predominant lethal malignancy among males [1] and the second most common cause of male cancer death in the USA [2]. Androgen-deprivation therapy (ADT) through either medical or surgical castration has been the standard treatment for prostate cancer. However, cancer usually recurs after a couple of years, and progresses to advanced diseases such as castration-resistant prostate cancer (CRPC) [3-5].

Androgen receptor (AR) and its signaling play important roles in the cell proliferation, metastasis, and progression of prostate cancers [6, 7]. The AR comprises functional NH<sub>2</sub>-terminal transactivation (NTD), DNA-binding (DBD), and ligand-binding (LBD) domains. The AR-NTD contains most of the phosphorylated amino acid residues that regulate AR protein stabilization, cellular localization, and transcriptional activity [8-10]. A major activating domain of AR (activation fragment-1, AF-1), which is located within the NTD, has ligand-independent activity, wh-

ereas activation fragment-2 (AF-2) located within the LBD is associated with ligand-dependent activity [8, 9, 11]. The AF-1 in AR-NTD is essential for AR transcriptional activity, both androgen-dependent and -independent activity [12]. Androgen receptor signaling is activated via the NH<sub>2</sub>/COOH terminal (N/C) interaction, subcellular localization, and recruitment to androgen-response elements (AREs) in target gene promoters [6, 13].

Changes in AR signaling lead to development of advanced CRPC. Several proposed mechanisms underlying CRPC are AR amplification and mutations that lead to increased AR activity, altered levels of AR coregulator expression, ligand-independent AR activation by outlaw pathways, and altered steroidogenesis [14, 15]. Another recently proposed mechanism of progression to CRPC after failed androgen deprivation might involve an association between some recurrent cancers and the increased expression levels of constitutively active AR splice variants (AR-Vs) that lack the LBD [16]. Among the various AR-Vs that have been identi-

## A new compound targets the AF-1 of androgen receptor

fied in prostate cancer cell lines and tumors, AR-V7 (AR3) with androgen-independent trans-activation is the most prevalent and studied variant [16-18].

Since androgens play critical roles in prostate cancers, compounds targeting the androgen axis, including casodex (bicalutamide, BIC), abiraterone (CYP17 inhibitor) and enzalutamide (MDV-3100), have been developed to treat prostate cancers [19-22]. However, even the next generation AR signaling inhibitors such as enzalutamide could not prevent the progression of prostate cancer to CRPC [23]. Among the proposed mechanisms responsible for this resistance, the expression of AR-Vs that have lost the LBD and are therefore constitutively active has received considerable focus [24, 25]. A few compounds such as EPI-001 and UT-155 that target the AR-NTD have recently been developed as candidates to treat advanced prostate cancers [26, 27]. Currently, however, no drugs that target the AR-NTD and therefore become effective against these AR-Vs are clinically available.

In previous structure-activity relationship studies to develop AR antagonists [28, 29], we found that 6-[6-ethoxy-5-isopropoxy-3,4-dihydroisoquinolin-2(1H)-yl]-N-[6-methylpyridin-2-yl] nicotinamide (EIQPN) did not bind to AR-LBD unlike conventional AR antagonists. Therefore, in the present study, we investigated the effects of EIQPN on AR function and expression, as well as on prostate cancer cell proliferation and tumor growth in a CWR22rv xenograft mouse model, aiming to develop an agent for treating advanced prostate cancer. We found that EIQPN inhibited AR-AF1 activity and markedly decreased protein levels of AR and AR-Vs in prostate cancer cells. Furthermore, EIQPN inhibited the androgen-independent proliferation of AR-positive prostate cancer cells *in vitro* and *in vivo*. These findings suggest that EIQPN has potential for treating recurrent prostate cancers by blocking and downregulating the signaling pathways in CRPC/androgen-independent prostate cancer (AIPC).

### Materials and methods

#### Reagents

We purchased the following reagents from the described suppliers: radiolabeled dihydrotes-

tosterone ( $[^3\text{H}]5\alpha\text{-DHT}$ ; PerkinElmer Life and Analytical Sciences Inc., Waltham, MA, USA), forskolin (FSK) and chloroquine diphosphate salt (CQ; Sigma-Aldrich Corp., St. Louis, MO, USA), carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132; A.G. Scientific Inc., San Diego, CA, USA), human IL-6 (R&D Systems Inc., Minneapolis, MN, USA),  $5\alpha\text{-dihydrotestosterone}$  (DHT; Sigma-Aldrich), enzalutamide and bicalutamide (MDV-3100 and BIC; Sequoia Research Products Ltd., Newbury, England, UK). 6-(6-ethoxy-5-isopropoxy-3,4-dihydro-1H-isoquinolin-2-yl)-N-(6-methyl-pyridin-2-yl)-nicotinamide (EIQPN) was synthesized and provided by Medigene Co. (Daejeon, Republic of Korea).

#### Plasmids

The plasmids pARE2-TATA-Luc, GAL4.AR-LBD-658-919, VP16.AR1-660, 5xGAL4-luc3, pcR3.1 SRC-1, pcDNA3.AR, and pEGFP-AR have been previously described [28, 30]. For androgen-independent activity studies, the LBDs were removed from pcDNA3.AR and pEGFP-AR to generate pcDNA3.AR-NTD and GFP-AR-NTD, respectively. We constructed pB3.AR-AF1 to overexpress and purify AR-AF1 protein for *in vitro* protein-substrate interaction assay. The AR-AF1 domain (amino acids 110~485) was amplified from a human cDNA library and inserted into pB3 at a *Sma*I site to generate pB3.AR-AF1. **Table 1** lists the primers for cloning of AR-AF1.

#### Cell lines

The LNCaP (CRL-1740) and HEK 293T (CRL-11268) cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The C4-2 and CWR22rv cell lines were kindly provided by Dr. C. Jung (Chonnam National University Medical School), and PPC1 and DU145 cells were a kind gift from Dr. S. Back (Seoul National University). LNCaP, C4-2, and CWR22rv cells were maintained in RPMI-1640 media (HyClone Laboratories Inc., South Logan, UT, USA) with 5% Gibco fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA). The PPC1 and HEK 293T cells were cultured in Dulbecco's Modified Eagle's Media (DMEM; HyClone) with 10% FBS. Media contained 5% charcoal-stripped serum (cFBS) for starvation and steroid studies. All cell lines were maintained in media containing

## A new compound targets the AF-1 of androgen receptor

**Table 1.** Primers

Cloning	AR-AF1 LIC Forward: GGCGGTGGTGGCGGCATGGATGAGGAACAGCAACCT AR-AF1 LIC Reverse: TCTTCTCCTTTGCGCCCTACCGAGTGTAGCCGTAGGG
ChIP	ARE-2 Forward: ACAATCTCCTGAGTGCTGGTGT ARE-2 Reverse: GCAGAGGAGACATGCCAG ARE-3 Forward: TGAGAAACCTGAGATTAGGA ARE-3 Reverse: GTTCCTCCAGAGTAGGTCTGTTTTT $\beta$ -actin Forward: TCCTCCTCTTCTCAATCTCG $\beta$ -actin Reverse: AAGGCAACTTTCGGAACGG
RT-PCR	Gapdh Forward: ATCACCATCTTCCAGGAGCGAG Gapdh Reverse: GAGATGATGACCCTTTTGCTCC PSA Forward: GGCCAGGTATTTTCAGGTCAG PSA Reverse: TCGTGGCTGGAGTCATCAC TMPRSS2 Forward: CGCCAGAGCATTGTG TMPRSS2 Reverse: GCGGCTGTCACGATCC

100 units/mL penicillin and 100 mg/mL streptomycin (P/S; Thermo Fisher) under a 5% CO<sub>2</sub> atmosphere at 37°C.

### Cell transfection and reporter assays

Cells were transfected with the expression constructs, luciferase reporter, and pRSV-LacZ (Takara Bio USA Inc., Mountain View, CA, USA) using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA), starved, and then stimulated with androgen, growth factors, or AR-antagonists for 24 h. Cells were lysed in luciferase lysis buffer (0.2 M Tris-Cl (pH 8.0), 0.2% Triton X100, and 1% NP-40) at 25°C for 15 min. Luciferase activity was analyzed using Beetle Luciferin (Promega Inc., Madison WI, USA) and a Centro XS3 LB960 Luminometer (Berthold Technologies U.S.A. LLC., Oakridge, TN, USA). Results were normalized to  $\beta$ -galactosidase activity measured at a wavelength of 405 nm using a Versa Max microplate reader (Molecular Devices LLC., San Diego, CA, USA).

### Subcellular localization

HEK 293T cells were transiently transfected with GFP-AR-FL or GFP-AR-NTD, starved for 12 h, then incubated with 10  $\mu$ M EIQPN, MDV-3100, or BIC with or without 10 nM DHT for 2 h. The cells were fixed with 3.7% paraformaldehyde in PBS, stained with TOPRO-3. Subcellular localization of ARs was analyzed using a Nikon A1 laser-scanning Leica TCS SPE confocal microscope equipped with an ACS APO  $\times$  63/1.30 numerical aperture immersion objective.

Images were analyzed using the ImageJ software (<http://imagej.nih.gov/ij>, ImageJ 1.46r, Wayne Rasband, National Institutes of Health, USA).

### Chromatin immunoprecipitation (ChIP)

To examine the effect of EIQPN on a recruitment of AR to AREs, we conducted ChIP assays as described with modifications [31]. After starvation for 48 h, LNCaP cells were incubated with

vehicle or 10  $\mu$ M EIQPN and 10 nM DHT for 4 h, crosslinked with 1% formaldehyde (Sigma-Aldrich) for 10 min, sonicated to shear DNA, and then processed for ChIP assays. **Table 1** lists the primers used for ChIP.

### Competitive radioligand binding assays

To determine whether EIQPN interacts with the LBD of AR, we performed a competitive radioligand binding assay as described [32]. HEK 293T cells were transiently transfected with pcDNA3.AR, starved for 12 h, and then incubated with 5 nM [<sup>3</sup>H]5 $\alpha$ -DHT (~0.667  $\mu$ Ci) and various concentrations of competing unlabeled ligands (DHT and EIQPN) for 2 h. The cells were washed and harvested in lysis buffer (2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8), then radioactivity was measured using QuantaSmart™ -1.31 (Serial #: 428238, PerkinElmer Life and Analytical Sciences Inc.).

### AR-AF1 protein purification

We induced AR-AF1 protein with 0.2 mM IPTG for 4 h in BL21 *E. coli*. Cell pellets were sonicated in 50 mM Tris-Cl pH 7.5 containing protein inhibitors, and the supernatant was collected. The AR-AF1 protein was purified via immobilized metal affinity (IMAC) and anion exchange (Q-Sepharose) chromatography. Fractions containing AR-AF1 protein were identified by western blotting, and protein concentrations were determined by measuring  $\lambda$  at 280 nm. The purified AR-AF1 proteins were then used for *in vitro* protein-substrate interaction.

## A new compound targets the AF-1 of androgen receptor

**Table 2.** Antibodies and sources

Antibodies	Source
Anti-AR (PG-21)	Millipore
Anti-AR (C-19), Anti-PSA (C-19), Anti-HA (F-7), Anti-GAPDH, Rabbit-anti-goat	Santa Cruz
Anti-cleaved caspase 3	Cell Signaling
Goat anti-rabbit, Goat anti-mouse	Thermo Fisher

### *In vitro protein-substrate interaction*

To determine whether EIQPN interacts with AR-AF1 protein, the interaction reactions were performed using fluorescence spectroscopy as described with modifications [33, 34]. The intrinsic fluorescence was measured at excitation ( $\lambda_{Ex}$ ) and emission ( $\lambda_{Em}$ ) wavelengths of 278 and 300-400 nm, respectively, using a Shimadzu RF-5301 spectrofluorimeter (Shimadzu Corporation, Kyoto, Japan). EIQPN (5  $\mu$ M) was added to the AR-AF1 binding reaction. Conformational changes in the protein were evaluated as changes in quantum yield and/or peak shifts compared with those of native AR-AF1. Changes in the intrinsic fluorescence spectrum of AR-AF1 denatured with 8 M urea was also monitored.

### *3D computational modeling*

We analyzed 3D interactions between AR-AF1 and EIQPN and EPI-001. A putative 3D structure of the docking region within the TAU-5 domain of AF-1 was determined using the SWISS-MODEL online server (<https://swissmodel.expasy.org/>). Both EIQPN and EPI-001 were directly drawn and converted to 3D via the 1-Click docking online server by mcule (<https://mcule.com/apps/1-click-docking/>); then, each 3D-modeled docking region generated by SWISS-MODEL was docked with a specific docking center. More negative scores suggest a higher affinity. We analyzed interaction scores of the compounds at the same amino acid residue.

### *RNA isolation and RT-PCR*

Total RNA was isolated from cells using the TRI Reagent<sup>®</sup> (Molecular Research Center Inc., Cincinnati, OH, USA), and reverse transcribed using Oligo d(T)<sub>15</sub> (ELPiS, Daejeon, Korea) and M-MLV Reverse transcriptase kits (Promega Inc.) according to the manufacturer's instructions. Levels of mRNA were analyzed by RT-PCR

using *Taq* polymerase. **Table 1** lists the primers for RT-PCR.

### *Western blotting*

Cells were sonicated in 25 mM Tris-Cl (pH 8.0) containing protease inhibitors. The protein levels were analyzed by western blotting. Proteins were separated by SDS-PAGE, and transblotted onto Portran<sup>™</sup> Premium 0.2  $\mu$ M nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). Nonspecific binding was blocked, then the membranes were incubated with primary antibodies (**Table 2**) overnight at 4°C, washed, incubated with HRP-conjugated secondary antibodies for 1 h, and then visualized on X-ray films using the ECL<sup>™</sup> Western Blotting Analysis System (GE Healthcare).

### *Cell proliferation assays*

The LNCaP, C4-2 and CWR22rv cells were seeded onto 96-well plates and starved for 12 h, then incubated with 10  $\mu$ M of EIQPN, MDV-3100, or BIC with or without 1 nM DHT for 5 days. The PPC1 and DU145 cells were incubated with 10  $\mu$ M EIQPN, MDV-3100, or BIC for 5 days. For non-androgen-stimulated cell growth, LNCaP cells were starved for 12 h, and incubated with 10  $\mu$ M EIQPN, MDV-3100, or BIC, with or without IL-6 (50 ng/mL) for 5 days. Cell growth (%) and numbers were determined using MTS and trypan-blue staining, respectively.

### *Xenograft animal model*

We generated CWR22rv xenograft mouse models to examine the *in vivo* effect of EIQPN on androgen-independent prostate tumorigenesis. Healthy 4-week-old male NOD.CB17-Prkdc-SCID/J mice (mean weight [ $\pm$  SD], 20 g; KRIBB, Daejeon, Korea) were gently injected with 100  $\mu$ L of anesthetic (Zoletil 50:Rompun:saline buffer [20:10:270]) and warmed using a veterinary system. CWR22rv cells ( $4 \times 10^6$ /site [intact] or  $6 \times 10^6$ /site [castrated] mixed 1:1 with Matrigel) were subcutaneously implanted into the shoul-

## A new compound targets the AF-1 of androgen receptor

ders of the mice. After 1 week, the animals were injected i.p. with 50  $\mu$ L of vehicle or 25 mg/kg EIQPN in DMSO:PEG300 (2:8) three times per week for 7 weeks. The intact ( $n = 9$  per group) and castrated ( $n = 7$  per group) mice were euthanized with CO<sub>2</sub> for tumor dissection. All animals were maintained inside a cleaned bench in an animal room with a 12/12 h light/dark cycle and controlled temperature at 25°C. The cages, water, food, and bedding were sterilized and replaced weekly. The mice were injected with ultra-fine II short needles (U-100 INSULIN 30 gauge 5/16", 8 mm) to minimize pain and lesions. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Chonnam National University (permit number: 2012-44).

### Statistical analysis

All graphs were created using GraphPad Prims (GraphPad software Inc., Version 5.0, La Jolla, CA, USA). Data are presented as means  $\pm$  SEM of more than three independent experiments. Statistical significance was calculated using one-way ANOVA with Tukey post hoc tests and two-tailed t-tests. Values with  $P < 0.05$  were considered statistically significant.

## Results

### *Novel EIQPN inhibits androgen-independent AR transactivation in prostate cancer cells*

While developing new antagonists of AR, we identified the new compound EIQPN (**Figure 1A**) that inhibited androgen-independent AR activity in addition to androgen-dependent activity. In hypothesis-testing mechanistic studies, EIQPN inhibited the transactivation of endogenous AR activated by the androgen DHT and the non-androgen forskolin (FSK) and IL-6 in LNCaP cells (**Figure 1B-D**). Moreover, EIQPN blocked endogenous AR transactivation in AIPC cell line CWR22rv that expresses full-length AR (AR-FL) and AR-Vs (**Figure 1E**).

We further analyzed the effects of EIQPN on androgen-independent AR transactivation by comparing its effects on AR-NTD that lacks the LBD, with those on AR-FL in PPC1 cells. We found that EIQPN inhibited the transactivation of exogenous AR-FL to an extent comparable to that of the same concentration of the established AR antagonists MDV-3100 and BIC in an

androgen-dependent manner (**Figure 1F**). Notably, EIQPN blocked the androgen-independent transactivation of AR-NTD with an IC<sub>50</sub> of 0.7865  $\mu$ M, whereas MDV-3100 and BIC did not (**Figure 1G, 1H**). These results suggest that unlike conventional AR antagonists, EIQPN targets AR-NTD and inhibits androgen-independent AR transactivation as well as androgen-dependent transactivation.

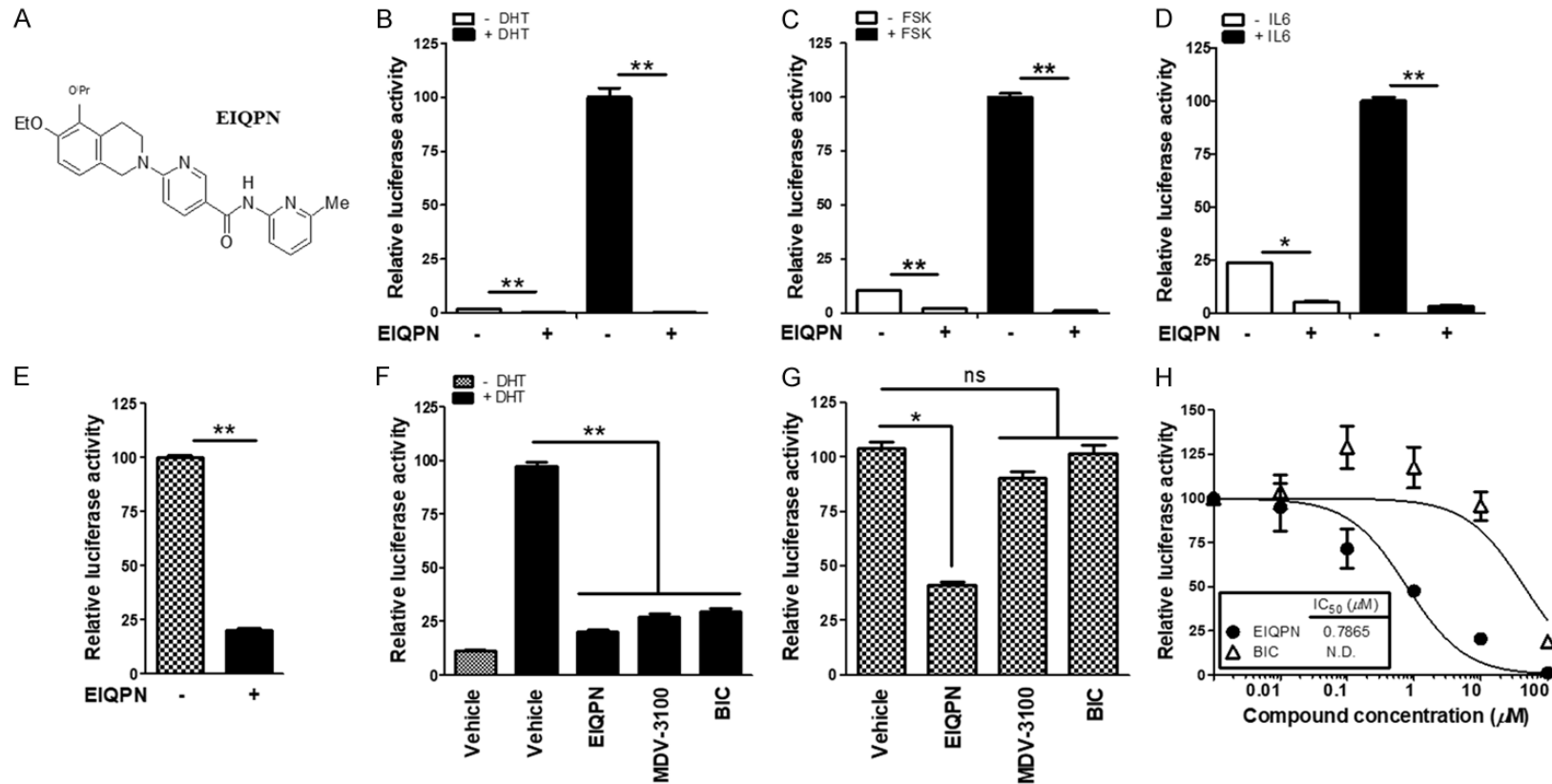
### *Activation of AR at various stages is blocked by EIQPN*

To elucidate the mechanism through which EIQPN inhibits AR transactivation, we investigated the effects of EIQPN on the AR activation steps, comprising N/C interaction, subcellular localization, and AR recruitment to AREs. In N/C interaction assays using AR NH<sub>2</sub>-terminal (VP16.AR1-660) and COOH-terminal (GAL4.AR658-919) constructs, EIQPN blocked the N/C interaction of AR under induction by DHT or FSK more than MDV-3100 and BIC (**Figure 2A, 2B**).

We then investigated whether EIQPN androgen-dependently and -independently alters the subcellular localization of AR by overexpressing GFP-AR-FL and GFP-AR-NTD in HEK 293T cells. We found that AR-FL, which is located in the cytoplasm in the absence of DHT, was completely translocated to the nucleus by stimulation with DHT. However, EIQPN blocked the DHT-induced nuclear translocation of AR-FL, promoting AR protein aggregation in the cytoplasm (**Figure 2C**). The localization of AR-NTD, which was distributed in the cytoplasm and nucleus under starvation, was barely affected by EIQPN, whereas the antiandrogens, MDV-3100 and BIC, induced the nuclear import of AR-NTD (**Figure 2D**).

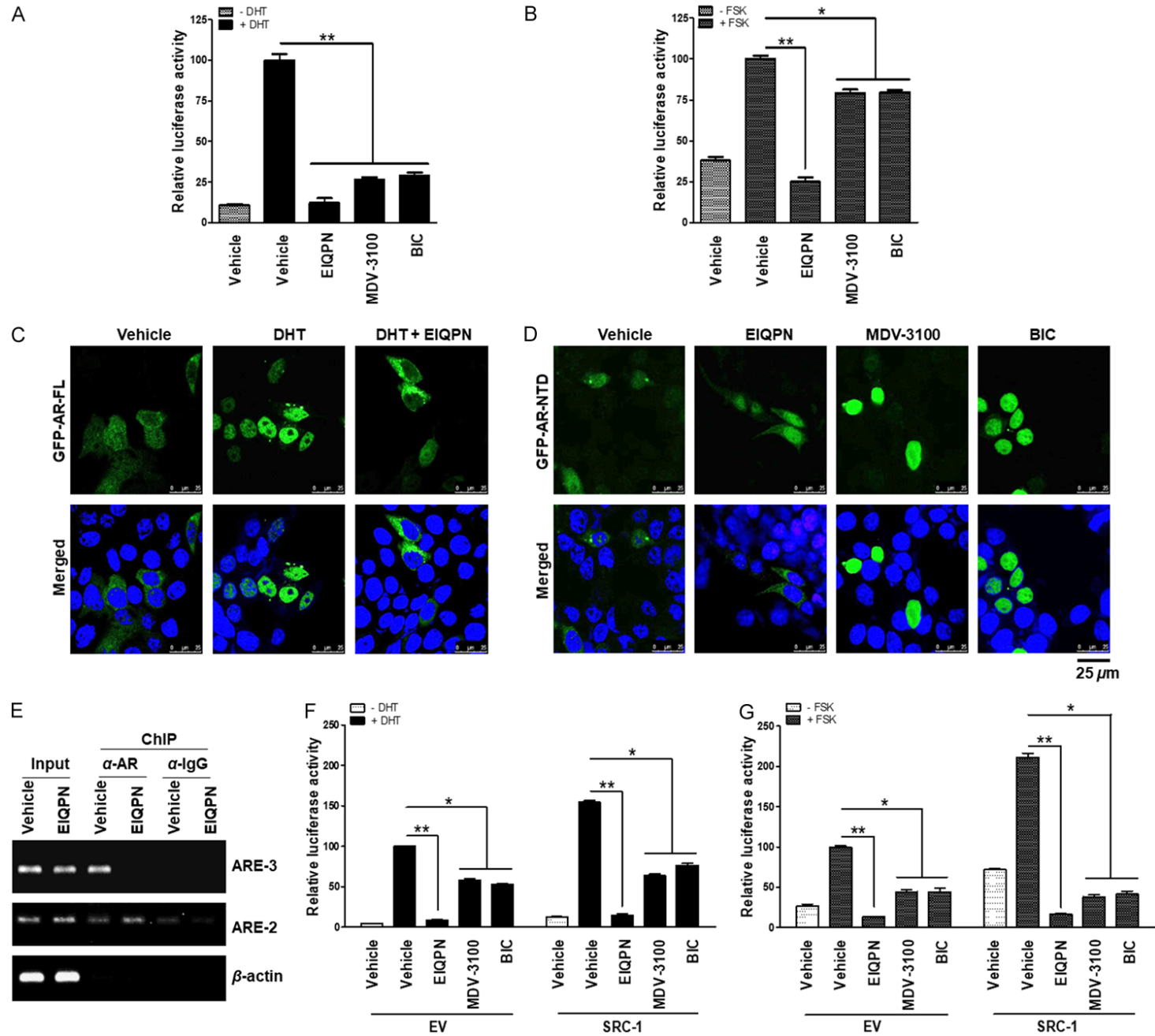
We also assessed whether EIQPN alters the recruitment of AR to AREs. The DHT-induced recruitment of AR to the ARE-3, which is located within the distal major enhancer ~4 kb upstream of the transcription start site of the AR target PSA gene in LNCaP cells, was prevented by EIQPN (**Figure 2E**). This major enhancer's activity is more sensitive to androgen stimulation than that of the proximal enhancer containing ARE-2 [6, 13]. In addition, overexpressed SRC-1 enhanced AR transactivation under starvation, and DHT or FSK induction, whereas EIQPN blocked this AR co-activation, suggest-

A new compound targets the AF-1 of androgen receptor



**Figure 1.** EIQPN inhibits androgen-independent AR transactivation as well as androgen-dependent. (A) Chemical structure of (6-[6-ethoxy-5-isopropoxy-3,4-dihydroisoquinolin-2(1H)-yl]-N-[6-methylpyridin-2-yl]nicotinamide (EIQPN). (B-E) EIQPN inhibits endogenous AR transactivation in prostate cancer cells. LNCaP (B-D) and CWR22rv (E) cells were transiently transfected with pARE2-TATA-luc reporter and incubated with 10  $\mu$ M EIQPN and with or without 10 nM DHT (B), 50 ng/mL IL-6 (C), or 50  $\mu$ M FSK (D). (F, G) EIQPN inhibits exogenous AR transactivation in PPC1 cells. Cells were transiently transfected with full-length AR (AR-FL) (F) or AR N-terminal domain (AR-NTD) (G) expression construct along with pARE2-TATA-luc, and incubated with 10  $\mu$ M EIQPN, MDV-3100, or BIC, and with and without 10 nM DHT. Luciferase activity was normalized to  $\beta$ -galactosidase activity. Data are shown as means  $\pm$  SEM of at least three independent experiments. \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ ; one-way ANOVA with Tukey post hoc tests. ns, not significant. (H) EIQPN dose-dependently inhibits AR-NTD transactivation. HEK 293T cells overexpressing AR-NTD were incubated with various doses of EIQPN or BIC. Inhibitory concentration ( $IC_{50}$ ) was obtained by nonlinear regression analysis.

A new compound targets the AF-1 of androgen receptor



## A new compound targets the AF-1 of androgen receptor

**Figure 2.** EIQPN blocks AR activation at various stages. (A, B) EIQPN inhibits the N/C interaction of AR. PPC1 cells co-transfected with AR N-terminal (VP16/AR1-660) and C-terminal (GAL4/AR-LBD658-919) domain constructs along with 5xGAL4-luc3 reporter, were incubated with 10  $\mu$ M EIQPN, MDV-3100, or BIC, and 10 nM DHT (A) or 50  $\mu$ M FSK (B). (C, D) EIQPN blocks AR nuclear translocation. HEK 293T cells overexpressing GFP-AR-FL (C) and GFP-AR-NTD (D) were incubated with 10  $\mu$ M EIQPN, MDV-3100, or BIC, and with or without 10 nM DHT for 2 h. Subcellular localization of ARs was detected as green fluorescent protein (GFP) signals. Nuclei were stained blue with TOPRO-3. Images were acquired using a confocal microscope. Scale bars, 25  $\mu$ m. (E) EIQPN prevents AR recruitment to ARE. Recruitment of AR protein to ARE-2 and ARE-3 within PSA promoter was determined by CHIP assays using anti-AR (C-19) antibody. LNCaP cells were incubated with 10  $\mu$ M EIQPN and 10 nM DHT for 2 h. Changes in AR enrichment at ARE was examined by PCR. The loading control was  $\beta$ -actin. (F, G) EIQPN inhibits coactivator recruitment to AR. PPC1 cells co-transfected with AR expression construct and SRC-1 or empty vector (EV) along with pARE2-TATA-luc were incubated with 10  $\mu$ M EIQPN, MDV-3100, or BIC, and with or without 10 nM DHT (F) or 50  $\mu$ M FSK (G). Data represent means  $\pm$  SEM of at least three independent experiments. \*,  $P < 0.01$ , and \*\*,  $P < 0.001$ ; one-way ANOVA with Tukey post hoc tests.

ing the inhibition of coactivator recruitment to AR (**Figure 2F, 2G**). Notably, coactivator recruitment was inhibited more by EIQPN than by MDV-3100 and BIC (**Figure 2F, 2G**). These results suggest that EIQPN blocks AR activation at various stages.

*EIQPN interacts with the AF-1 domain of AR, but not with the LBD*

We aimed to elucidate the molecular mechanism through which EIQPN inhibited AR activity. We first determined whether EIQPN interacts with the LBD and/or NTD domain of AR using competitive radioligand binding assays and protein-substrate interaction assays *in vitro* based on fluorescence spectroscopy. In competitive radioligand binding assays, 0.1 to 100  $\mu$ M EIQPN did not bind to the AR-LBD, as binding signals were undetectable ( $< 15\%$ ). In contrast, DHT, a high affinity AR ligand, bound to the AR-LBD with a  $K_d$  of 0.001  $\mu$ M (**Figure 3A**). This finding appeared to contradict the inhibition of androgen-dependent AR activity by EIQPN (**Figure 1B, 1F**), because binding or interaction with the LBD is important for androgen-dependent AR transactivation. Therefore, we speculated that EIQPN binds to or interacts with the NTD, preferably the AF-1 domain of AR, to inhibit the transactivation of AR-FL and AR-NTD.

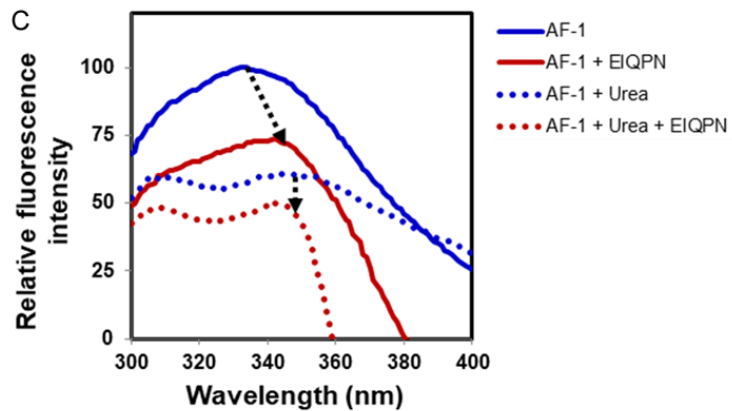
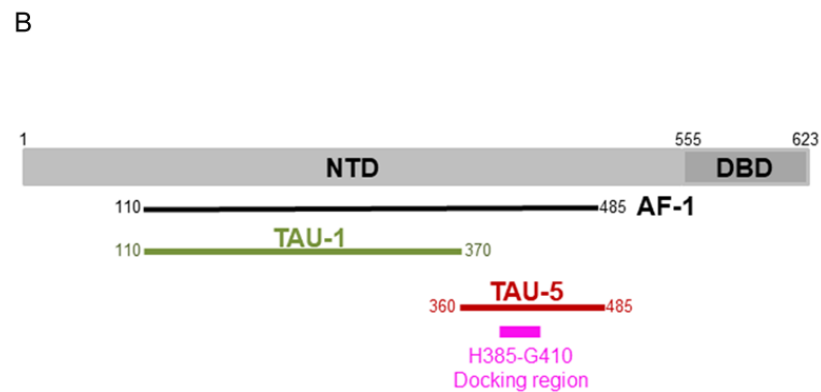
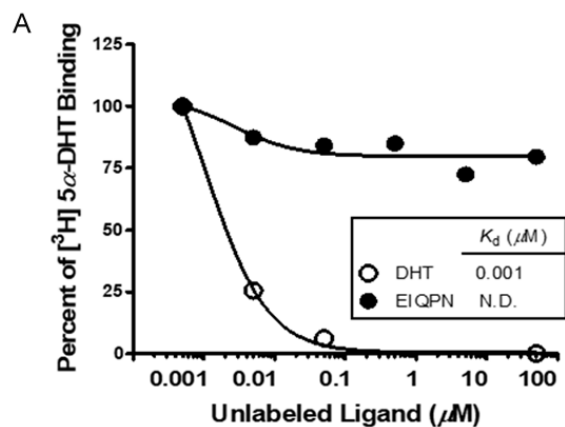
Interactions between protein and substrates often cause protein conformational changes that can be measured by fluorescence spectroscopy [26, 27, 35]. We investigated whether EIQPN interacts with AR-AF1. We prepared AR-AF1 domain proteins (amino acids 110~485) containing TAU-1 and TAU-5 domains but not the glutamine-rich region (**Figure 3B**), and assayed protein-substrate interactions *in vitro* (**Figure 3C**). The steady-state intrinsic fluores-

cence spectrum for native AF-1 protein was characterized by an emission maximum (peak) at 333 nm due to tryptophan residues and a tyrosine shoulder at  $\sim 309$  nm. EIQPN caused quantum yield decrease and tryptophan peak shift from 333 nm to 343-345 nm (**Figure 3C**). These results indicate that the tryptophan residues became losing energy and its signal was quenched due to the interaction with EIQPN. Meanwhile, the steady-state fluorescence spectrum of AF-1 protein denatured with urea showed two distinct peaks with decreased intensity. Unlike native AF-1 protein, adding EIQPN to denatured AF-1 protein did not cause a peak shift. These results suggest that the binding of EIQPN to the AR-AF1 domain requires some AF-1 secondary structures.

We also computationally modeled an interaction site within the TAU-5 domain of AR-AF1 (docking region) (**Figure 3B**), based on the results of the present protein-substrate, and previous EPI-001-TAU-5 interaction analyses [36]. EPI-001 binds to the TAU5 domain of AR-AF1 and inhibits AR-NTD activity [27]. The modeling results showed a lower affinity score for EIQPN (-6.0) than EPI-001 (-5.6) at the docking region (H385-G410 TAU-5) (**Figure 3D-F**), suggesting that EIQPN has higher interaction affinity than EPI-001. The modeled image shows the 3D structure of the TAU-5 domain (orange), and EIQPN and EPI-001 interacting with the TAU-5 domain at a K389/E391/P393/W400 pocket, which might result in a reduction of quantum yield and a red-shift of tryptophan peak (**Figure 3C, 3E, 3F**). These results suggest that EIQPN interacts with the AR-AF1 domain and induces conformational changes that eventually result in the loss of AR transactivation in both androgen-independent and -dependent manners.

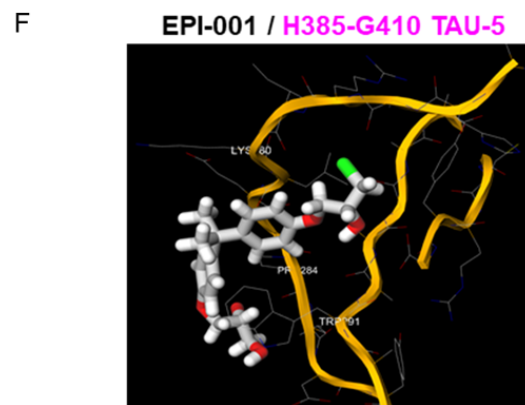
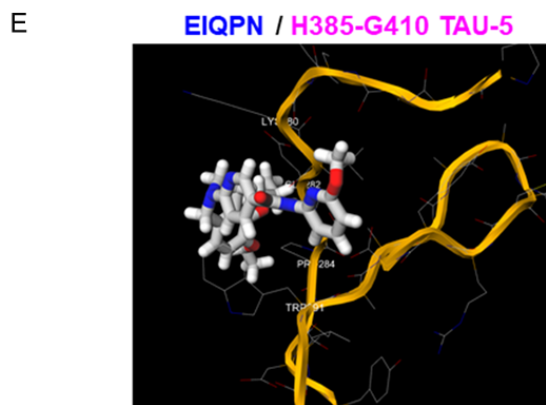


A new compound targets the AF-1 of androgen receptor



**D**

<b>Docking region: H385-G410 TAU-5</b>	
<b>Docking center L390</b>	
Compound	Docking score
EIQPN	-6.0
EPI-001	-5.6



## A new compound targets the AF-1 of androgen receptor

**Figure 3.** EIQPN interacts with AR-AF1 domain, but not with LBD. (A) EIQPN does not bind AR-LBD. HEK 293T cells overexpressing AR-FL were incubated with 5 nM [<sup>3</sup>H]5 $\alpha$ -DHT and various concentrations of unlabeled ligand (DHT or EIQPN).  $K_d$  values were obtained by nonlinear regression analysis. (B) Schema of cloned AR-AF1 and docking region within TAU-5 domain. AR-AF1 domain (amino acids 110-485) was cloned for protein-substrate interaction assays *in vitro*. Docking region (H385-G410) contains 26 amino acids of TAU-5 domain. (C) Conformational change in AR-AF1 protein caused by interaction with EIQPN. Changes in intrinsic fluorescence spectrum of native or urea-denatured AR-AF1 protein caused by EIQPN were monitored using fluorescence spectroscopy. (D-F) Computational modeling of EIQPN interaction with AF-1 domain. Molecular PDB model of docking region of AR-AF1 was obtained using SWISS-MODEL. Novel interaction between EIQPN and docking region was determined by 1-Click docking. Summary of docking interaction scores (D). Images show 3D structure of H385-G410 TAU-5 docking region (orange) and EIQPN interacting in a K389/E391/P393/W400 pocket (E). EPI-001 was the positive control (F).

### *EIQPN decreases AR protein levels*

We found that EIQPN robustly decreased the protein levels of endogenous ARs in various AR-positive prostate cancer cells. In androgen-dependent (LNCaP) and -independent (CWR-22rv) prostate cancer cells, 10  $\mu$ M EIQPN reduced the protein levels of AR and AR-Vs > 90% (**Figure 4A, 4B**). The same concentration of the control compounds, MDV-3100 and BIC, showed a little effect on AR protein levels in LNCaP cells but significantly enhanced those in CWR22rv cells as previously reported in several other cell lines [17, 37, 38]. In addition, EIQPN reduced the protein levels of both endogenous and exogenous AR-FL and AR-NTD overexpressed in LNCaP, and DU145 but not in PPC1 and HEK 293T cells (**Figure 4C-F**).

We then explored the mechanism through which EIQPN decreased AR protein levels. The ubiquitin-proteasome pathway mainly regulates AR protein degradation in prostate cancers, although the lysosomal pathway is also involved in AR protein degradation [8]. Blocking the ubiquitin-proteasome pathway with MG-132, but not the lysosomal pathway with chloroquine (CQ), fully recovered AR protein levels that were decreased by EIQPN in CWR22rv and C4-2 cells (**Figure 4G, 4H**). These results suggest that protein degradation of AR in cells incubated with EIQPN occurs mainly through the ubiquitin-proteasome pathway.

We investigated EIQPN-induced changes in ubiquitinated AR (ubi-AR) levels in CWR22rv cells transiently transfected with an HA-tagged E3 ubiquitin ligase Mdm2 expression construct to understand more about AR degradation promoted by EIQPN. Levels of ubi-AR were significantly enhanced in cells incubated with, than without 10  $\mu$ M EIQPN for 2 h (**Figure 4I**), confirming that EIQPN mediates AR protein degradation via the ubiquitin-proteasome pathway.

### *Proliferation of prostate cancer cells is inhibited by EIQPN*

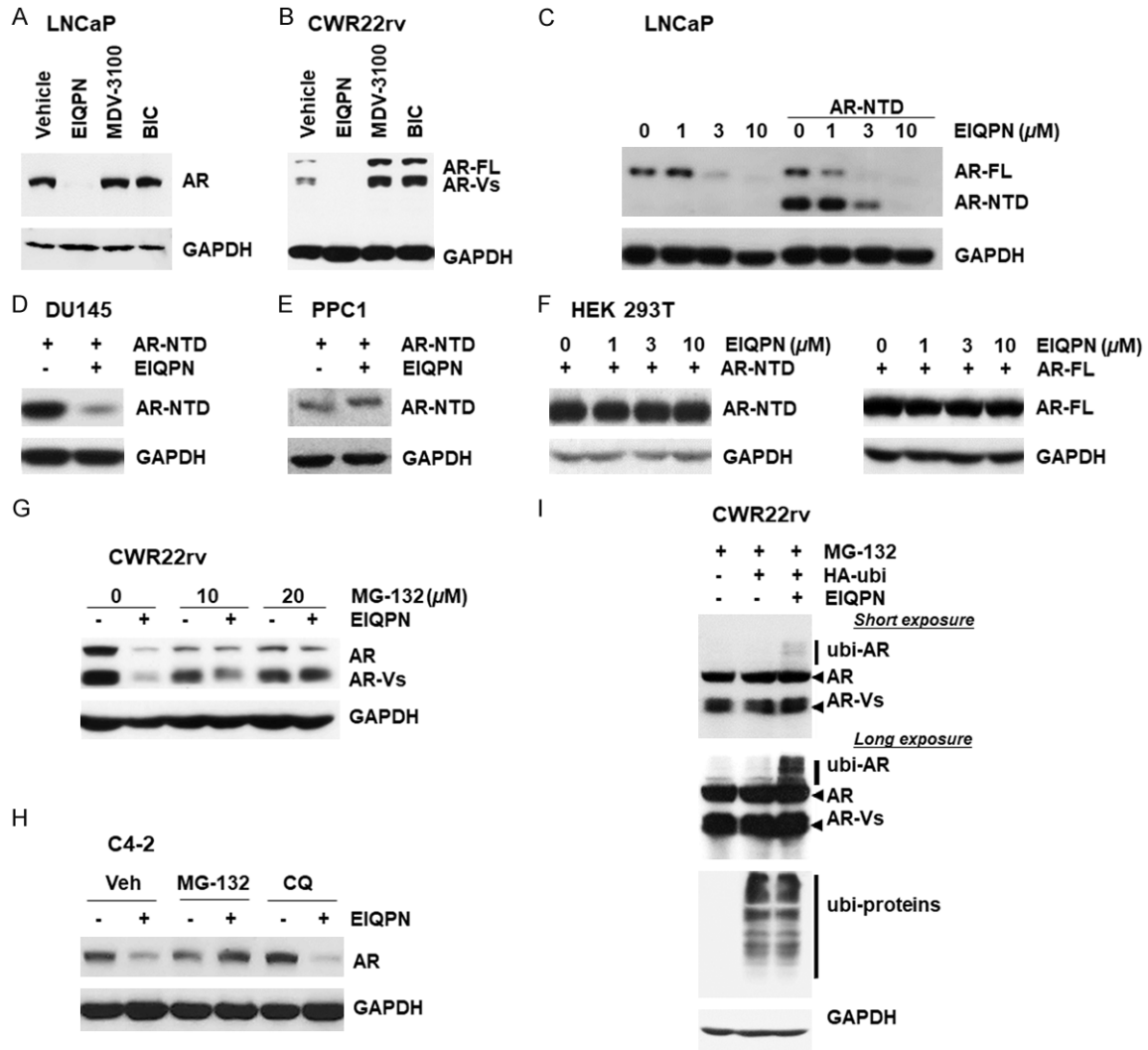
Because EIQPN negatively affected AR signaling in prostate cancer cells, we explored the effects of EIQPN on the survival and proliferation of various prostate cancer cell lines. The proliferation of LNCaP cells under androgen-independent and -dependent stimulation with IL6 and DHT, respectively, was more effectively inhibited by EIQPN than by MDV-3100 and BIC (**Figure 5A, 5B**). Furthermore, EIQPN also inhibited the androgen-independent proliferation of castration-resistant (C4-2) and androgen-independent, advanced prostate cancer cells (CWR22rv), with  $IC_{50}$  values of 1.100 and 1.514  $\mu$ M, respectively (**Figure 5C-F**). In contrast, MDV-3100 exerted some, whereas BIC exerted no significant effects on the androgen-independent proliferation of both cell lines. Meanwhile, EIQPN, like MDV-3100 and BIC, did not affect the proliferation of AR-negative DU145 prostate cancer cells (**Figure 5G**). Taken together, these results suggest that EIQPN inhibits the androgen-independent and-dependent proliferation of prostate cancer cells.

### *Formation and growth of CWR22rv xenograft tumors in vivo are inhibited by EIQPN*

The effects of EIQPN on prostate tumorigenesis *in vivo* were examined using CWR22rv xenograft mouse models. Compared with control mice, EIQPN (~10 mg/kg/day, i.p.) caused an approximate 3-week delay in tumor formation and inhibited tumor growth by ~65% and ~90% in intact and castrated mice, respectively (**Figure 6A-D**). The absence of significant changes in the body and testis weight of intact and castrated mouse models suggest minimal toxicity (**Figure 6A, 6B**).

Molecular analysis of tumors dissected from intact and castrated mice showed that EIQPN

## A new compound targets the AF-1 of androgen receptor



**Figure 4.** EIQPN reduces the AR protein levels. (A, B) Protein levels of AR and AR-Vs are decreased by EIQPN. LNCaP (A) and CWR22rv (B) cells were incubated with 10  $\mu$ M EIQPN, MDV-3100, or BIC. Amounts of AR proteins were analyzed by western blotting, with GAPDH as loading control. (C) Western blots show decreased protein levels of endogenous AR and exogenous AR-NTD in LNCaP cells incubated with various doses of EIQPN. (D) Western blots show decreased protein levels of exogenous AR-NTD in DU145 cells incubated with 10  $\mu$ M EIQPN. (E, F) Western blots show no effects of 10  $\mu$ M EIQPN on protein levels of exogenous AR-NTD in PPC1 cells (E) and of different doses of EIQPN on exogenous AR-NTD (left) and AR-FL (right) in HEK 293T cells (F). (G, H) Ubiquitin-proteasome inhibitor MG-132 fully recovered AR protein levels decreased by EIQPN. CWR22rv (G) and C4-2 (H) cells cultured with or without 10  $\mu$ M EIQPN for 16 h were incubated with MG-132 or chloroquine (CQ) for 8 h. (I) EIQPN significantly enhances ubiquitinated AR (ubi-AR) levels. CWR22rv cells were transfected with HA-tagged E3 ubiquitin ligase Mdm2 (HA-ubi) expression construct and incubated with 10  $\mu$ M EIQPN for 2 h in the presence of 10  $\mu$ M MG-132.

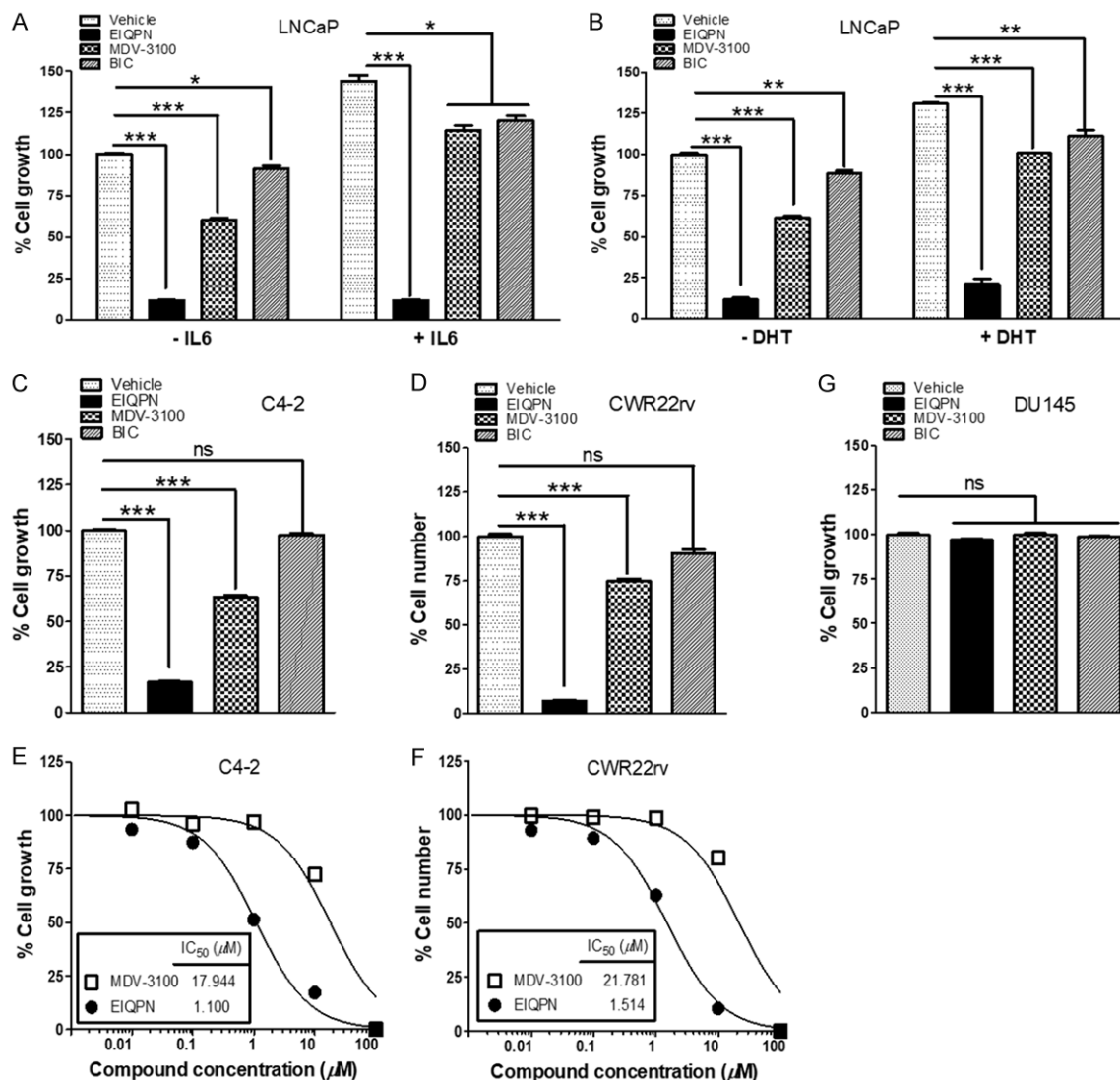
significantly increased and reduced protein levels of the apoptosis marker, cleaved caspase 3, and AR, respectively (Figure 6E, 6F). Furthermore, EIQPN also tended to downregulate expression of the AR target genes, PSA and TMPRSS2, especially in tumors from castrated mice (Figure 6G, 6H). These results were consistent with the negative effects of EIQPN on AR signaling in prostate cancer cells, which

resulted in the inhibition of cell survival and proliferation. These results suggest that EIQPN inhibits the formation and growth of advanced, androgen-independent prostate tumors.

### Discussion

Failed castration and antiandrogen therapy are associated with the increased expression of

## A new compound targets the AF-1 of androgen receptor

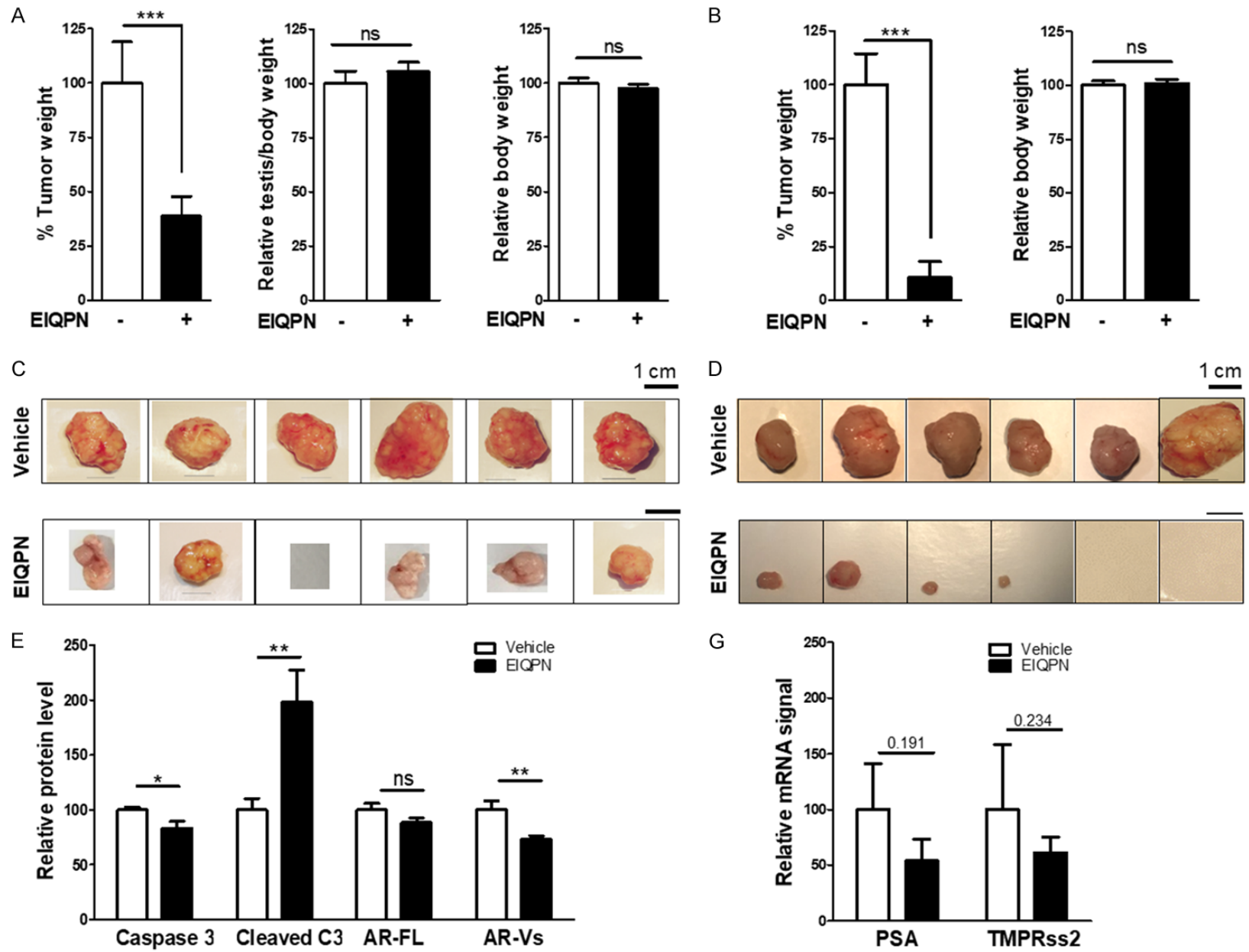


**Figure 5.** EIQPN inhibits androgen-dependent and -independent growth of AR-positive prostate cancer cells. (A-F) Proliferation of AR-positive prostate cancer cells is inhibited by EIQPN. LNCaP (A, B), C4-2 (C) and CWR22rv (D) cells were incubated for 5 days with 10 μM EIQPN, MDV-3100, or BIC, and with or without 50 ng/mL IL-6 (A) or 1 nM DHT (B). C4-2 (E) and CWR22rv (F) cells were incubated with different doses of EIQPN or MDV-3100. Half maximal inhibitory concentrations (IC<sub>50</sub>) were determined by nonlinear regression analyses. Cell growth was assessed by MTS assays (A-C and E) or counting cells stained with trypan blue (D and F). (G) Proliferation of AR-negative prostate cancer cells is not affected by EIQPN. DU145 cells were incubated with 10 μM EIQPN, MDV-3100, or BIC, then growth was assessed using MTS assays. Data represent means ± SEM of at least three independent experiments. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; two-tailed t-test analysis. ns, not significant.

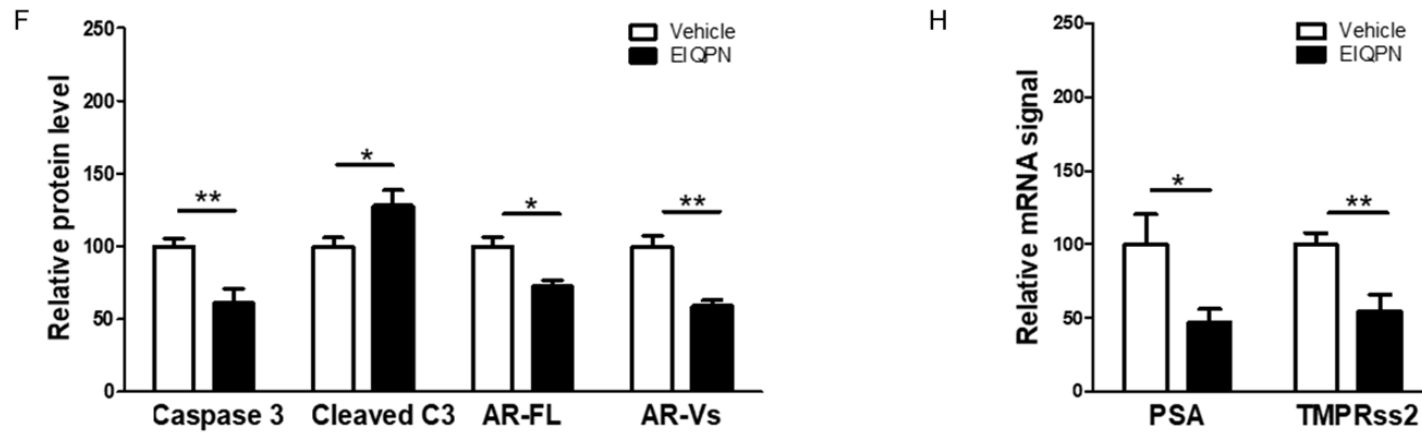
constitutively active AR-Vs without the LBD [16, 24, 25]; hence, AR-NTD is an alternative target for the development of compounds to treat advanced prostate cancers. EPI-001 interacts with the TAU-5 domain in the AF-1 region of the AR-NTD [36]. It blocks the androgen-independent function of TAU-5 and prevents 433-WHTLF437/AF2 interaction, which attenuates androgen-dependent AR transactivation [26, 27]. The present study showed that EIQPN

interacted with the AR-AF1 domain in the same manner as EPI-001 and caused conformational changes. Such interaction likely prevented AR N/C interaction and/or the recruitment of AR and coactivator SRC-1 to AREs, resulting in the loss of androgen-dependent and -independent AR activity. Additionally, the interaction between EIQPN and the AF-1 domain blocked the nuclear translocation of both androgen-induced AR-FL and androgen-independent AR-NTD.

A new compound targets the AF-1 of androgen receptor



A new compound targets the AF-1 of androgen receptor



**Figure 6.** CWR22rv xenograft tumor growth *in vivo* is inhibited by EIQPN. (A-D) Tumor growth in intact (A, C) and castrated (B, D) mouse xenograft models is prevented by EIQPN. CWR22rv cells were injected into shoulders of intact and castrated 4-week-old, male NOD.CB17-PrkdcSCID/J mice. After 1 week, mice were injected i.p. with 25 mg/kg EIQPN in DMSO:PEG300 (2:8) or vehicle three times per week for 7 weeks. Two days after the final injection, tumor (left), testis (middle) and body (right) weight of intact (A) and castrated (B) mice were assessed. Intact mice, n = 9; castrated mice, n = 7. (C, D) Representative tumors dissected from intact (C) and castrated (D) mice. Scale bars, 1 cm. (E, F) Levels of cleaved caspase 3 (cleaved C3) and AR are significantly increased and decreased, respectively, by EIQPN in tumors dissected from intact (E) and castrated (F) mice. Protein band signals were scanned by Image Studio Lite, and visualized by GraphPad Prism 5.0. (G, H) EIQPN downregulated expression of AR target genes, PSA and TMPRss2, in tumors from intact (G) and castrated (H) mice. Levels of mRNA were analyzed by RT-PCR. Data represent means  $\pm$  SEM of at least three independent experiments. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; two-tailed t-test analysis. ns, not significant.

## A new compound targets the AF-1 of androgen receptor

However, unlike EPI-001, EIQPN interaction with the AF-1 domain not only blocked AR activity, but also induced protein degradation and reduced AR protein levels in most prostate cancer cell lines. The ubiquitin-proteasome pathway is probably the main mechanism through which EIQPN promoted AR protein degradation. Androgen receptors are similarly degraded by EIQPN, galeterone and IRC117539, that is, via the ubiquitin-proteasome pathway [39]. However, > 10  $\mu$ M galeterone is needed to induce AR degradation in androgen-dependent LNCaP and LAPC4 cells, and AR T878A mutation is necessary for proteasome-dependent degradation [40]. Meanwhile, IRC117539 exerted antiandrogenic effects on the prostate by inducing castration-like histopathological changes. Nevertheless, IRC117539 (25 mg/kg/day for 50 days) reduced LNCaP-derived tumor volumes only by 42% in intact Balb/c nude mice, whereas EIQPN (3  $\times$  25 mg/kg/week ( $\sim$ 10 mg/kg/day) for 49 days reduced CWR22rv xenograft tumor weight by  $\sim$ 65% and  $\sim$ 90% in intact and castrated mice, respectively.

The selective and potent AR antagonist UT-155 also behaves like EIQPN. It targets the AR-NTD, promote AR protein degradation potentially through the ubiquitin-proteasome pathway and inhibiting CWR22rv cell growth with an  $IC_{50}$  of  $\sim$ 1  $\mu$ M [41]. However, UT-155 (100 mg/kg/day s.c.) for 20 days and EIQPN ( $\sim$ 10 mg/kg/day i.p.) for 49 days reduced  $\sim$ 25% [41] and  $\sim$ 90%, respectively, of CWR22rv xenograft tumor weight in castrated mice. These findings indicate that EIQPN is comparable with other agents that inhibit AR function and expression as well as prostate cancer cell proliferation.

In conclusion, EIQPN inhibited AR-AF1 activity and reduced AR protein levels, thereby inhibiting prostate cancer cell survival and proliferation. A functional study found that EIQPN delayed and inhibited advanced androgen-independent prostate tumorigenesis in intact and castrated xenograft mouse models *in vivo*. These findings suggest EIQPN could serve as a novel agent to treat and prevent the recurrence of advanced prostate cancers.

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### Disclosure of conflict of interest

None.

### Abbreviations

A (ALA), alanine; ADT, androgen-deprivation therapy; AF-1, activation fragment-1; AF-2, activation fragment-2; AR, androgen receptor; ARE, androgen-response element; AR-V, androgen receptor variant; CRPC, castration-resistant prostate cancer; DBD, DNA-binding domain; E (GLN), glutamate; EIQPN, 6-[6-ethoxy-5-isopropoxy-3,4-dihydroisoquinolin-2[1H]-yl]-N-[6-methylpyridin-2-yl]nicotinamide; G (GLY), glycine; H (HIS), histidine; K (LYS), lysine; L (LEU), leucine; LDB, ligand-binding domain; NTD,  $NH_2$ -terminal transactivation domain; P (PRO), proline; PSA, prostate-specific antigen; TAU, ligand-dependent transcriptional activation unit; TMPRSS2, transmembrane serine protease 2; W (TRP), tryptophan.

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