

Proliferating cell nuclear antigen directly interacts with androgen receptor and enhances androgen receptor-mediated signaling

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Abstract. Androgen receptor (AR) and/or its constitutively active splicing variants (AR-Vs), such as AR-V7 and ARv567es, is required for prostate cancer cell growth and survival, and cancer progression. Proliferating cell nuclear antigen (PCNA) is preferentially overexpressed in all cancers and executes its functions through interaction with numerous partner proteins. The aim of the present study was to investigate the potential role of PCNA in the regulation of AR activity. An identical consensus sequence of the PCNA-interacting protein-box (PIP-box) was identified at the N-terminus of human, mouse and rat AR proteins. It was found that PCNA complexes with the full-length AR (AR-FL) and AR-V7, which can be attenuated by the small molecule PIP-box inhibitor, T2AA. PCNA also complexes with ARv567es and recombinant AR protein. The PCNA inhibitors, PCNA-IIS and T2AA, inhibited AR transcriptional activity and the expression of AR target genes in LNCaP-AI and 22Rv1 cells, but not in AR-negative PC-3 cells. The knockdown of PCNA expression reduced dihydrotestosterone-stimulated AR transcriptional activity and abolished the inhibitory effect of PCNA-IIS on AR activity. The PCNA inhibitor, PCNA-II, exerted additive growth inhibitory effects with androgen deprivation and enzalutamide in cells expressing AR-FL or AR-FL/AR-V7, but not in AR-negative PC-3 cells. Finally, R9-AR-PIP, a

small peptide mimicking AR PIP-box, was found to bind to GFP-PCNA at K_d of $2.73 \mu\text{M}$ and inhibit the expression of AR target genes, AR transcriptional activity and the growth of AR-expressing cells. On the whole, these data strongly suggest that AR is a PCNA partner protein and interacts with PCNA via the PIP-box and that targeting the PCNA-AR interaction may represent an innovative and selective therapeutic strategy against prostate cancer, particularly castration-resistant prostate cancers overexpressing constitutively active AR-Vs.

Introduction

Androgen receptor (AR) is a ligand-dependent transcription factor and regulates diverse aspects of development, cell growth and homeostasis (1-3). The full-length AR (AR-FL) functional domains mainly include a DNA binding domain (DBD) flanking with the N-terminal domain (NTD) and C-terminal ligand binding domain (LBD). The constitutively active AR splicing variants (AR-Vs) originate from aberrant splicing of AR pre-mRNA via various mechanisms, including the incorporation of cryptic exons containing a premature stop codon and exon-skipping events (4-7). The two predominant AR-Vs, AR-V7 and ARv567es, have lost all or part of the LBD, have become constitutively active, and enable androgenic signaling during androgen deprivation therapy (ADT) to drive the development of the castration-resistant prostate cancers (CRPCs). They function as AR-FL in binding the androgen response element (ARE) and interacting with coregulators (8). They can homodimerize and also heterodimerize with AR-FL in an androgen-independent manner to drive AR signaling during ADT (9). AR-Vs not only upregulate canonical AR target genes mainly related to metabolism, secretion and differentiation, such as PSA and FKBP5, but also AR-Vs-specific genes *UBE2C* and *CCNA2* associated with cell cycle progression (10,11). AR-V7 expression is very low in normal prostate, whereas it is increased in tumors and is further elevated in CRPC (6,12,13). Nuclear AR-V7 emerges with CRPC in 75% of cases following ADT in comparison with those in <1% prior to therapy (13,14). AR-V7, particularly in the circulating tumor cells, is a biomarker for predicting CRPC response to ADT (8,13,15,16). Nuclear ARv567es is also significantly elevated in CRPC and metastasis (17). The constitutively active AR-Vs escape challenges by the current repertoire of agents targeting the ligand binding domain

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Abbreviations: AR, androgen receptor; AR-FL, full-length AR; AR-Vs, constitutively active AR splicing variants; CRPC, castration-resistant prostate cancer; DHT, dihydrotestosterone; DBD, DNA binding domain; ENZ, enzalutamide; IDCL, interdomain connector loop; LBD, ligand binding domain; PCNA, proliferating cell nuclear antigen; PIP-box, PCNA-interacting protein-box; PCNA-Is, PCNA inhibitors; PSA, prostate-specific antigen

Key words: PCNA, androgen receptor, PCNA inhibitors, gene regulation, AR splicing variants

of AR. Immense efforts have been made to develop agents targeting AR-Vs in recent years (18-20). However, very few of these have been approved for clinical trials to date, such as EPI-506 that binds to the TAU-5 in N-terminal domain (19) and the repurposed anti-helminthic drug, niclosamide, that promotes the degradation of AR-V7 protein (20).

Proliferating cell nuclear antigen (PCNA) is a non-oncogenic protein essential for cell growth and survival. PCNA encircles DNA and serves as platforms for partner proteins involved in DNA replication and DNA repair, as well as other cellular processes (21-23). The partner proteins bind to PCNA through their PCNA interaction protein (PIP)-box, AlkB homologue 2 PCNA-interacting motif (APIM), and/or other motifs (22,24,25). PCNA is overexpressed in all tumors (22). PCNA overexpression is associated with advanced disease and the metastasis of prostate cancer (26-28). Targeting PCNA as a novel strategy for cancer therapy was previously explored with small peptides mimicking the APIM or 'cancer-associated PCNA' (caPCNA) (29,30), and small molecules T2AA targeting the PIP-box binding cavity of PCNA (31) and AOH1160 targeting caPCNA (32). Previously, the authors developed small molecule PCNA inhibitors (PCNA-Is) that block PCNA relocalization and chromatin association (33-36). PCNA-IIS, identified in the structure-activity relationship (SAR) analysis of PCNA-Is, is a more potent PCNA inhibitor than PCNA-I1 (34). These peptides and small molecules were shown to be well-tolerated in animals and exerted therapeutic effects against various types of tumors, particularly when combined with DNA damage drugs (29,30,32,35).

A previous study demonstrated that PCNA interacts with AR and several proteins involved in DNA synthesis and cell cycle regulation in replicating tumor cells (37). The aim of the present study was to investigate the potential role of PCNA in the regulation of AR activity. A PIP-box consensus sequence was identified at the N-terminus of AR and it was found that PCNA directly complexes with AR-FL, AR-V7 and ARv567es, very likely via the PIP-box. PCNA-IIS inhibits AR-FL transcriptional activity and the expression of the AR target genes, PSA and p21/WAF, as well as AR-FL and AR-V7 autoregulated by AR signaling. T2AA, a small molecule PIP-box inhibitor, reduces the PCNA interaction with AR-FL and AR-V7, and inhibits AR transcriptional activity and the expression of AR target genes. PCNA-I1 exerts additive inhibitory effects with ADT on the growth of CRPC cells expressing both AR-FL and AR-V7, but not on those without AR expression. The present study developed an AR PIP-box mimicking peptide R9-AR-PIP, which binds to PCNA and inhibits AR transcriptional activity, the expression of AR target genes and the growth of AR-expressing cells.

Materials and methods

Reagents. Dihydrotestosterone (DHT, cat. no. D-073), T2AA (cat. no. SML0794), recombinant AR and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, cat. no. M5655), were purchased from Sigma-Aldrich; Merck KGaA. Antibodies against mouse PCNA (cat. no. 2586) and rabbit PCNA (cat. no. 13110) and p21/WAF (cat. no. 2946) were purchased from Cell Signaling Technology, Inc. Antibodies against AR (N20, cat. no. sc-816 and H-280,

cat. no. sc-13062), TMPRSS2 (cat. no. sc-515727) and GAPDH (cat. no. sc-47724), as well as PCNA siRNA (cat. no. sc-29440) and control siRNA (cat. no. sc-36869), were purchased from Santa Cruz Biotechnology, Inc. Enzalutamide (ENZ, cat. no. S1250) was purchased from Selleckchem. Antibody against prostate-specific antigen (PSA) (cat. no. A0562) was obtained from Dako, Agilent Technologies, Inc. The DC Protein assay kit I (cat. no. 5000111) was purchased from Bio-Rad Laboratories, Inc. The luciferase assay system (cat. no. E1500) was obtained from Promega Corporation. Lipofectamine 3000® (cat. no. L3000015) was obtained from Thermo Fisher Scientific, Inc. IRDye 680LT anti-mouse fluorescent secondary antibody (cat. no. 926-68022) and IRDye 680LT anti-rabbit fluorescent secondary antibody (cat. no. 926-68023) for western blot analysis were from LI-COR Biosciences. The GST-PCNA expression plasmid was generously provided by Dr Shaochun Wang (University of Cincinnati, Cincinnati, OH, USA). PCNA-I1 and PCNA-IIS (33,34,36) were purchased from ChemBridge Corporation. R9-AR-PIP was synthesized by the custom peptide service at Biomatik Corporation.

Cells and cell culture. The prostate cancer cell lines, LNCaP (cat. no. CRL-1740), PC-3 (cat. no. CRL-1435) and 22Rv1 (cat. no. CRL-2505), were obtained from ATCC. Androgen-independent LNCaP-AI cells were generated from androgen-dependent LNCaP cells in a previous study by the authors (38). The cells were expanded and kept in cryogenic storage for long-term safekeeping. The cells were authenticated genetically with PCR identifying the short tandem repeat (STR) and cell-specific profiling against the ATCC database at the University of Arizona Genetics Core. The CWR-R1-D567 (R1-D567, cat. no. EMN028-FP) cells (39) were obtained from Kerafast. The LNCaP, PC-3, R1-D567 and 22Rv1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. The LNCaP-AI cells were cultured in stripped medium containing RPMI-1640 medium supplemented with 10% charcoal-dextran treated FBS (sFBS). Cells in exponential growth phase were harvested by treatment for 2-5 min with a 0.25% Trypsin-0.02% EDTA solution and resuspended in medium. The suspensions of single cells with viability >95% (ascertained by Trypan blue exclusion; data not shown) were used in the present study.

Knockdown of PCNA expression. Lipofectamine 3000® was used for the transfection of siRNA according to the protocol provided by the manufacturer. PC-3 cells (2x10⁵/well) in six-well plates were transfected with 5 µM siRNA with 10 µl Lipofectamine 3000 in 100 µl of Opti-MEM medium (Thermo Fisher Scientific, Inc.) for 3 days. Subsequently, the cell extracts were prepared and quantitated for western blot analysis.

Co-immunoprecipitation assay. Co-immunoprecipitation was performed in a modified radio-immunoprecipitation assay (RIPA) buffer [PBS, 0.1% NP-40, 0.1% sodium deoxycholate, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1X protease inhibitor cocktail]. Mouse PCNA antibody was first incubated with 1 mg of cell extract at 4°C on a rocker platform for 1-2 h.

A total of 50 μ l of Protein G plus/protein A agarose beads (Calbiochem, Thermo Fisher Scientific) was then added, and the samples were incubated at 4°C on a rocker platform overnight. The beads were washed with the modified RIPA lysis buffer at 2,500 x g for 5 min each for four times at room temperature and boiled in SDS loading buffer. The protein samples were subjected to 12% SDS-PAGE and western blot analysis.

GST-PCNA pull-down assay. GST-PCNA vector was transformed into BL21 bacteria. The transformed bacteria were cultured in L-broth with the addition of 100 μ M of IPTG to induce GST-fusion protein expression. The bacteria were then harvested, sonicated and subjected to GST fusion protein purification by using Glutathione Sepharose 4B (#17-0756-01, GE Healthcare Bio-sciences AB). For the pull-down reaction, 5-10 μ g of GST-PCNA were incubated with 1 mg of cell extract or 0.25-0.5 μ g of recombinant AR in the modified RIPA buffer. The samples were incubated at 4°C on a rocker platform overnight. The beads were washed with the modified RIPA buffer for four times and boiled in SDS loading buffer. The protein samples were subjected to 12% SDS-PAGE and western blot analysis.

Western blot analysis. Western blot analysis was performed as previously described (40). Briefly, aliquots of samples with the same amount of protein, determined using the DC Protein assay kit, were mixed with loading buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 100 mM dithiothreitol and 0.005% bromophenol blue), boiled, fractionated in an 12% SDS-PAGE, and transferred onto 0.45- μ m nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked with 2% fat-free milk in PBS for 1 h, and probed with primary antibody in PBS containing 0.01% Tween-20 (PBST) and 1% fat-free milk. The membranes were incubated overnight at 4°C with primary antibodies (1:200 dilution for antibodies from Santa Cruz Biotechnology, Inc. and 1:400 dilution for all other antibodies). The membranes were then washed four times in PBS and incubated with IRDye 680LT secondary antibodies with at a 1:1,000 dilution in PBST containing 0.01% Tween 20 (PBST) and 1% fat-free milk for 1 h at room temperature. After washing four times in PBS, the membranes were visualized using Odyssey imaging system (Li-COR).

Reporter assay. Cells (10⁵/well) were seeded in 12-well tissue culture plates. The following day, the cells were transfected with reporter plasmids (200 ng) without or with AR expression vector (200 ng) with Lipofectamine 3000[®] reagent for 4 h. The cells were then treated with various concentrations of PCNA-IIS, T2AA, R9-AR-PIP and/or DHT as indicated in the figures or figure legends for 24 h, respectively. Subsequently, the cell extracts were prepared and protein concentration from each sample was determined using the DC Protein assay kit. Each sample was normalized by protein concentration relative to the control sample. Luciferase activity was assessed in a Berthold Detection System (Titertek-Berthold) using the luciferase assay kit following the manufacturer's instructions. For each assay, cell extract (20 μ l) was used and the reaction was started by injection of 50 μ l of luciferase substrate (Promega Corporation). Each reaction was measured for 10 sec in a Sirius

luminometer (Berthold Detection Systems). The luciferase activity was defined as light units/mg protein.

Reverse transcription-quantitative PCR (RT-qPCR). RNA from the control and treated 22Rv1 cells was isolated using the RNeasy Plus Universal Mini kit (Qiagen GmbH). RNA samples were then treated with the DNase using DNA-free TM kit (Thermo Fisher Scientific) and subjected to reverse transcription reactions using High Capacity cDNA Reverse Transcription kits (Applied Biosystems; Thermo Fisher Scientific). The expression of AR and PSA was analyzed by RT-PCR using the Fast SYBR-Green Master Mix (Thermo Fisher Scientific) in a 7300 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific) with primer pairs as follows: AR, 5'-GGTGAGCAGAGTGCCTATC-3'/5'-GGCAGTCTCCAAACGCATGTC-3' and PSA, 5'-GACCACCTGCTACGCCTCA-3'/5'-AACTTGCGCACACACGTCATT-3', as previously described (41,42). Following an initial step at 95°C for 10 min, PCR was performed at 95°C for 3 sec and 60°C for 30 sec for 40 cycles according to the manufacture instruction. The cycle threshold values were used to calculate the normalized expression of target genes against β -actin using Q-Gene software (43).

MTT assay. Cells were plated in 96-well plates and treated as indicated with enzalutamide (ENZ, 5 μ M), R9-AR-PIP (up to 20 μ M), and/or PCNA-II (150 nM) in normal and/or stripped medium for 96 h. Viable cells in the control and treated cultures were stained with MTT as described in a previous study by the authors (44). Growth inhibition (%) in the treated cell cultures in comparison with that in the untreated controls was calculated using the following formula: (1-A₅₇₀ of treated wells/A₅₇₀ of control wells) x100, which reflects the effects of the treatments on cell growth inhibition. The IC₅₀ was defined as the concentration of a drug that inhibited cell growth by 50% as compared with untreated control.

PCNA binding assay. The binding and affinity of R9-AR-PIP to PCNA was measured using microscale thermophoresis (MST) following a previously described protocol (45). PC-3 cells in 100-mm plates were transfected with a mammalian expression vector pGFP-PCNA (34,46) or pGFP (Promega Corporation) encoding GFP-tagged PCNA or GFP (control) using Lipofectamine 3000[®], respectively. After two days, the expression levels of GFP-PCNA and GFP in ~80% cells were confirmed under an Olympus IX51 inverted fluorescent microscope. The cells were washed with PBS and scrapped in 10 ml pre-chilled PBS. Following centrifugation at 250 x g for 5 min at 4°C, the cells were resuspended in 250 μ l of 25 mM Tris-HCl (pH 8.0) containing 1X protease inhibitor mixture and 1 mM PMSF, and sonicated on ice for 3x10 sec at 30% amplitude using a Sonicator Ultrasonic Processor (Misonix). The soluble fraction of the cell lysate was collected following centrifugation at 12,000 x g for 10 min at room temperature. R9-AR-PIP at increasing concentrations was incubated with the lysate (1.5 μ g in 30 μ l) for 1 h at room temperature in binding buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 10 mM MgCl₂, 0.01% Tween-20). The alterations in the thermophoresis of GFP-PCNA or GFP protein were measured using Monolith NT.115 (NanoTemper Technologies). The

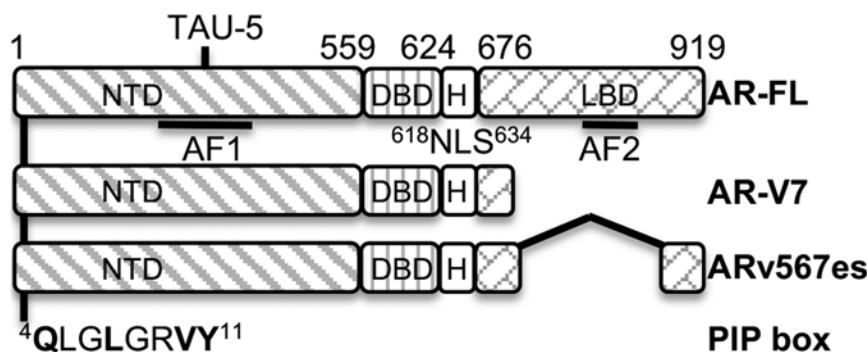


Figure 1. AR-FL and AR-V functional domains. NTD, N-terminal domain containing the activation function 1 (AF1); DBD, DNA binding domain; LBD, ligand binding domain containing the activation function 2 (AF2); PIP-box (QLGLGRVY), PCNA-interaction protein box is located at the N-terminus of AR; NLS, nuclear localization signal; TAU-5, transcription activation unit 5.

apparent dissociation constant (K_d) of the binding was calculated by non-linear regression analysis using MO Affinity Analysis v2.1.3 (NanoTemper Technologies).

Statistical analysis. Data from each assay are expressed as the mean $\pm$ SD. Statistically significant differences between two groups were determined using a Student's t-test. All statistical analyses were carried out using Prism 9 software (GraphPad). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Interaction between PCNA and AR. The interdomain connector loop (IDCL) on the outer surface of PCNA trimers interacts with proteins containing the PIP-box, defined by the consensus sequence Q1-x2-x3-h4-x5-x6-a7-a8, where 'h' and 'a' represent hydrophobic (ILMV) and aromatic (FYH) amino acids, respectively (47). The canonical Q1 docks into a 'Q pocket' and h4, a7 and a8 bind in a large hydrophobic pocket formed by the IDCL. A previous study demonstrated that PCNA interacts with AR (37). The present study performed a sequence analysis of human, mouse and rat AR proteins, and found an identical consensus sequence of the PIP-box (QLGLGRVY) located at the amino acid 4 position consisting of Q, L and Y for Q1, h4 and a8, respectively (Fig. 1).

To determine whether PCNA complexes with AR via the potential N-terminal PIP-box, the cell extracts from LNCaP (AR-FL⁺) and 22Rv1 (AR-FL⁺/AR-V7⁺) cells (48) were subjected to co-immunoprecipitation assay. It was found that PCNA complexed with both AR-FL (Fig. 2A-C) and AR-V7 (Fig. 2B and C), and this was attenuated by the PIP-box inhibitor, T2AA, following treatment in either the cell culture or by addition to the 22Rv1 cell lysate during co-IP (Fig. 2C). Furthermore, GST pull-down assay was performed using the cell extracts from 22Rv1 cells, as well as R1-D567 cells that only express ARv567es (39). The cell extract from AR-negative PC-3 cells was used as a control. It was found that GST-PCNA complexed with AR-FL, AR-V7 and ARv567es (Fig. 2D). More importantly, GST-PCNA also bound to recombinant AR (Fig. 2E). Taken together, the results from these experiments demonstrated that PCNA bound directly to the N-terminal domain of AR, very likely via the PIP-box at the N-terminus of AR.

Inhibitory effects of PCNA-IIS and T2AA on the expression of endogenous canonical AR target genes. The present study further investigated the effects of PCNA-IIS on the expression of the endogenous canonical AR target genes, PSA and p21/WAF (49), as well as AR that is autoregulated by androgens (50), due to the presence of the ARE in the AR gene (50,51). As shown in Fig. 3A, PCNA-IIS reduced the expression of p21/WAF in 22Rv1 cells, but not in AR-negative PC-3 cells (49). PCNA-IIS also downregulated the expression of both AR-FL and AR-V7 in 22Rv1 cells (Fig. 3A). Moreover, PCNA-IIS inhibited the expression of AR-FL and PSA in a time-dependent manner in the CRPC LNCaP-AI (AR-FL⁺) cells (38) (Fig. 3B). Similarly, the expression of AR-FL and AR-V7 in the 22Rv1 cells was also attenuated by the PIP-box inhibitor, T2AA, in a concentration-dependent manner (Fig. 3C). Furthermore, treatment of the CRPC 22Rv1 cells with PCNA-IIS reduced the mRNA levels of AR and PSA genes by 46 and 31%, respectively (Fig. 3D).

Inhibitory effects of PCNA-IIS and T2AA on AR transcriptional activity. The effects of PCNA inhibitors on AR transcriptional activity were determined in reporter assays using two luciferase reporters PSA(4.3)-luc driven by PSA promoter containing ARE III region (-4,086/-3,981 bp) and ARE I region (-231/-143 bp) (52) and p21(-215)-luc driven by p21/WAF promoter containing an ARE at -200 bp position (49). It was found that PCNA-IIS inhibited both the basal and DHT-induced promoter activities of PSA and p21/WAF genes in the LNCaP-AI and 22Rv1 cells (Fig. 4A-C), and also the DHT-induced promoter activity of the PSA gene in AR-negative PC-3 cells co-transfected with an AR expression vector (Fig. 4D). The inhibitory effects on PSA promoter activity were also observed in the 22Rv1 cells treated with T2AA (Fig. 4E). These AR functional assays suggest that PCNA promotes AR transcriptional activity, likely through interaction with AR via the PIP-box.

Effects of knockdown of PCNA expression on AR transcriptional activity and the inhibitory effects of PCNA-IIS. To validate the role of PCNA in the regulation of AR transcriptional activity and the target specificity of PCNA-IIS, the effects of the knockdown of PCNA expression on AR activity and the inhibitory effects of PCNA-IIS were determined. As shown in Fig. 5A, the expression of PCNA was markedly

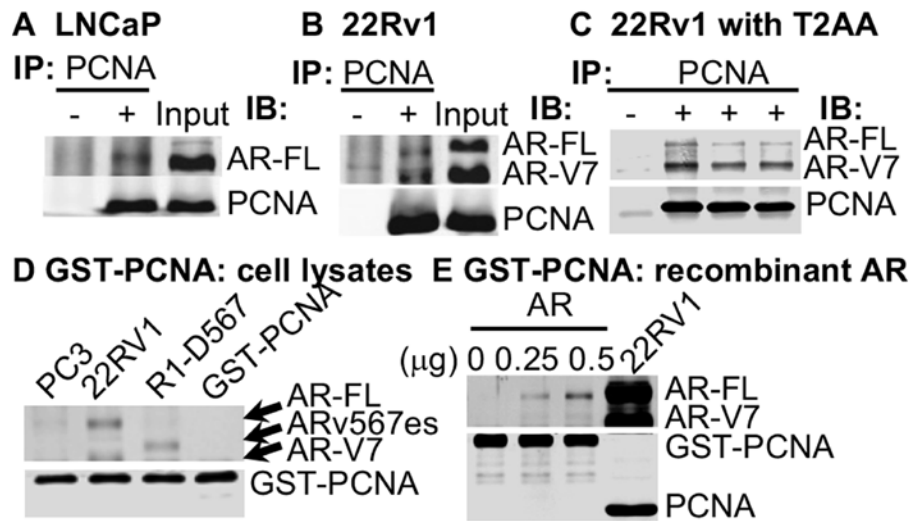


Figure 2. AR is a novel PCNA partner protein. (A) LNCaP and (B) 22Rv1 cell extracts were used for co-IP with a mouse antibody to PCNA for IP and with rabbit antibodies to AR (N20 binding to the NTD of AR and PCNA for western blot analysis). (C) Co-IP was performed with extracts from 22Rv1 cells incubated for 24 h in medium without (lanes 1 and 2) or with 5 μ M T2AA (lane 3). T2AA (30 μ M) (lane 4) was added to the lysates of untreated 22Rv1 cells during co-IP. (D) Cell extracts from PC-3, 22Rv1, and R1-D567 cells were subjected to GST pull-down reaction with GST-PCNA alone as a control. (E) GST-PCNA pull-down reaction was performed in the presence of 0, 0.25, or 0.5 μ g (lanes 1, 2 and 3) of recombinant AR protein, with cell lysate from 22Rv1 cells (lane 4) as a control, followed by western blot analysis using anti-AR and anti-PCNA antibodies. co-IP, co-immunoprecipitation; AR, androgen receptor; PCNA, proliferating cell nuclear antigen; NTD, N-terminal domain.

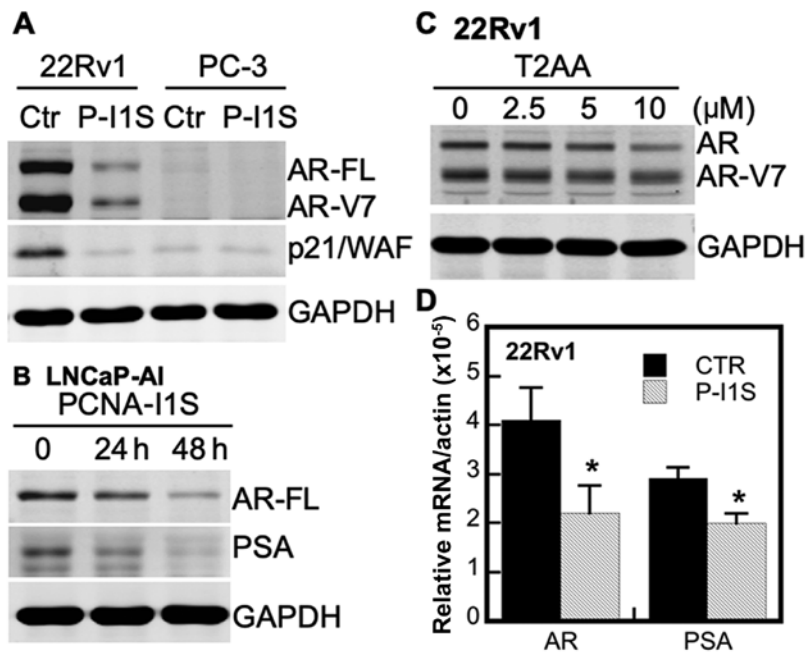


Figure 3. PCNA-IIS (P-IIS) inhibits the expression of AR target genes. Western blot analysis was performed using extracts from control (Ctr) cells and cells treated with (A) 1 μ M PCNA-IIS for 24 h, (B) 1 μ M PCNA-IIS for 24 or 48 h, or (C) T2AA for 24 h. (D) RNA samples from cells treated for 24 h with 0.5 μ M PCNA-IIS were analyzed for the mRNA levels of AR and PSA by RT-qPCR. * P <0.05, PCNA-IIS vs. control. PCNA, proliferating cell nuclear antigen; PSA, prostate-specific antigen.

reduced in PC-3 cells transfected with PCNA siRNA. The knockdown of PCNA expression partially attenuated the DHT-induced promoter activity of the PSA gene (Fig. 5B) and abolished the DHT-induced promoter activity of the p21/WAF gene (Fig. 5C), suggesting that PCNA is a regulator of and promotes AR activity. More importantly, the inhibitory effects of PCNA-IIS on the DHT-stimulated promoter activities of the PSA and p21/WAF genes were abolished in the cells in which the expression of PCNA was knocked down (Fig. 5B and C).

On the whole, these results validate the role of PCNA in the upregulation of AR transcriptional activity and the target specificity of PCNA-IIS on PCNA in the suppression of AR activity.

Additive inhibitory effects of ADT and PCNA-II on the growth of AR-positive prostate cancer cells. Given the significant roles of PCNA in the regulation of AR activity, the present study examined the effects of the concurrent treatment of

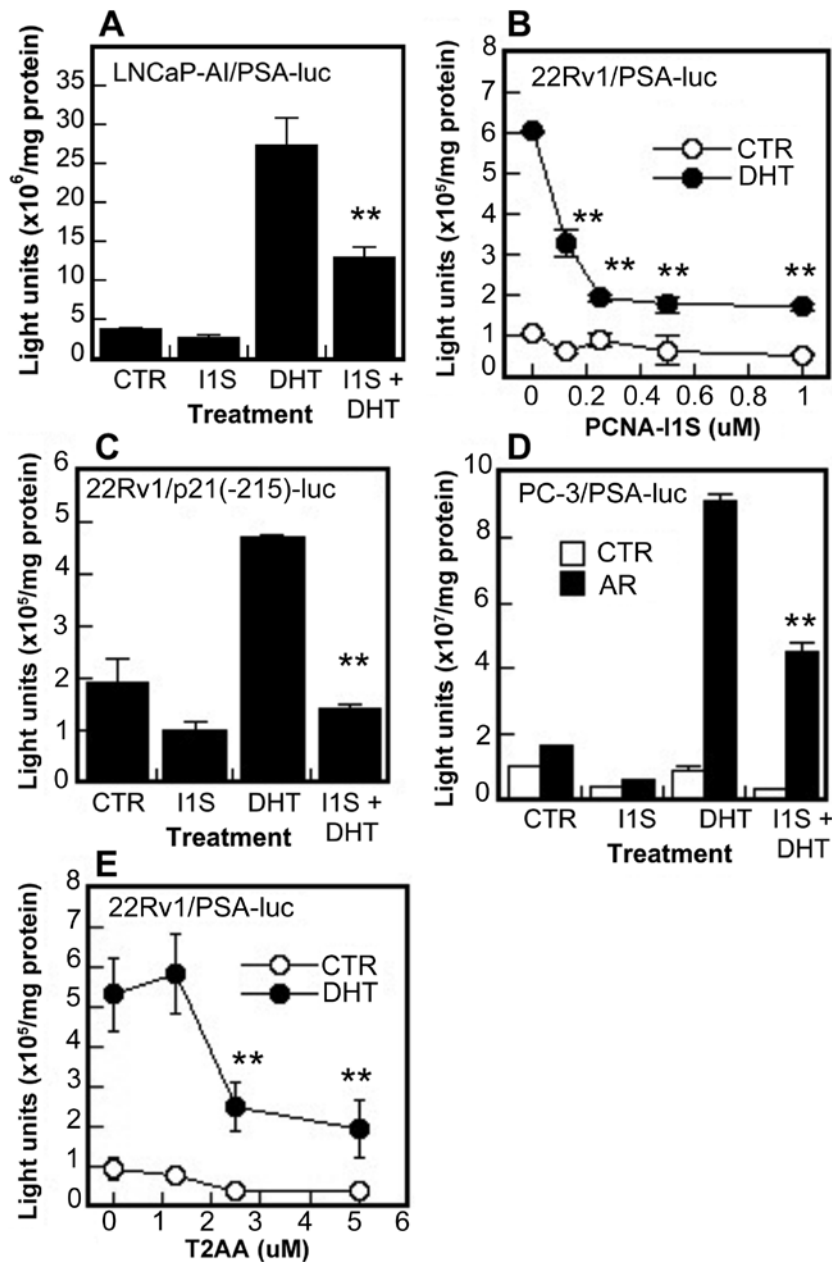


Figure 4. PCNA-IIS (P-IIS) and T2AA inhibit AR transcriptional activity. (A) LNCaP or (B, C and E) 22Rv1 cells (105 cells/well) in 12-well plates in stripped medium containing 10% of charcoal-dextran treated FBS (sFBS) were transfected with PSA-luc or p21(-215)-luc (0.2 μ g/well), and treated with PCNA-IIS at (A) 0.5 μ M, (B) various concentrations, (C) 1 μ M, or (E) T2AA for 24 h in the absence or presence of 10 nM DHT. (D) PC-3 cells transfected with PSA-luc (0.2 μ g/well) without or with AR expression vector CMV-AR (0.2 μ g/well) were treated with 1 μ M PCNA-IIS in the absence or presence of 10 nM DHT for 24 h. Subsequently, luciferase activity was assessed. ** $P < 0.01$, PCNA-IIS plus DHT vs. DHT alone (A-D) and T2AA plus DHT vs. DHT alone. PCNA, proliferating cell nuclear antigen; DHT, dihydrotestosterone.

PCNA-II plus ADT on the growth of several human prostate cancer cell lines, including androgen-dependent LNCaP (AR-FL⁺) cells and CRPC LNCaP-AI (AR-FL⁺), 22Rv1 (AR-FL⁺/AR-V7⁺) and PC-3 (AR⁻) cells. As was expected, ADT (in either cultured in stripped medium) (Fig. 6A-C) or treatment with 5 μ M ENZ (Fig. 6D-F), significantly inhibited the growth of LNCaP cells (Fig. 6A and D) and, at a lower potency, also inhibited the growth of CRPC LNCaP-AI and 22Rv1 cells (Fig. 6B, C and E), but not that of AR-negative PC-3 cells (Fig. 6F). PCNA-II (150 nM) inhibited the growth of all cells and exerted additive inhibitory effects with ADT in all AR-positive cells (Fig. 6A-E); however, this effect was not observed in AR-negative PC-3 cells (Fig. 6F).

Development of the AR-specific PIP-box peptide inhibitor, R9-AR-PIP. To further establish the essential role of the AR PIP-box in AR signaling, the effects of a small AR PIP-box peptide inhibitor on AR activity were investigated. It has previously been found that R9-cc-caPeptide, a small peptide with 8 amino acid residues spanning a portion of the IDCL of caPCNA linked with nine arginine residues to enhance the penetration of the peptide into cells and nucleus, inhibits PCNA association to chromatin (30). The peptide also induces cytotoxicity in culture and attenuates tumor growth in mice (30). Using the same structural design, the present study developed the peptide QLGLGRVY-cc-R9 (R9-AR-PIP). It contains eight amino acid residues of the AR PIP-box sequence (Fig. 1) and nine arginine

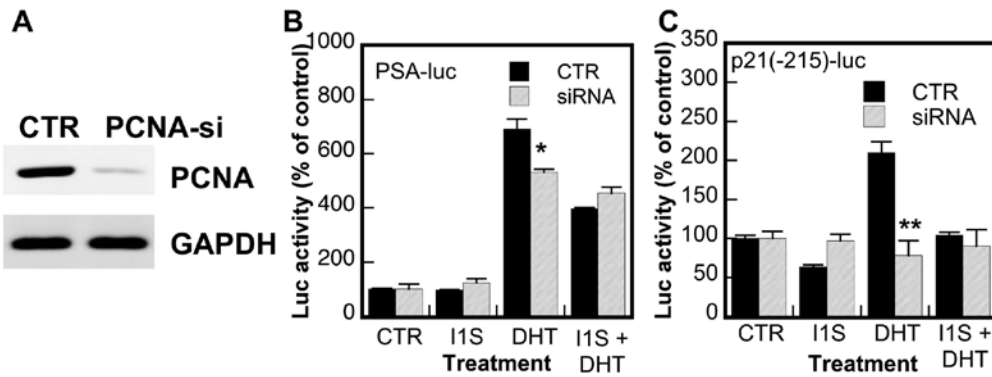


Figure 5. Knockdown of PCNA expression abolishes the inhibitory effects of PCNA-IIS (P-IIS) on AR activity. (A) PC-3 cell extracts transfected with PCNA siRNA (PCNA-si) for three days in stripped medium were analyzed by western blot analysis. PC-3 cells in stripped medium were transfected with PCNA siRNA for three days. During the last 24 h, the cells were co-transfected with (B) CMV-AR and PSA-luc or (C) p21(-215)-luc, respectively, and treated without or with P-IIS (1 μ M) in the absence or presence of DHT (10 nM), followed by luciferase assay. * $P < 0.05$ and ** $P < 0.01$, siRNA plus DHT vs. DHT alone. PCNA, proliferating cell nuclear antigen; DHT, dihydrotestosterone.

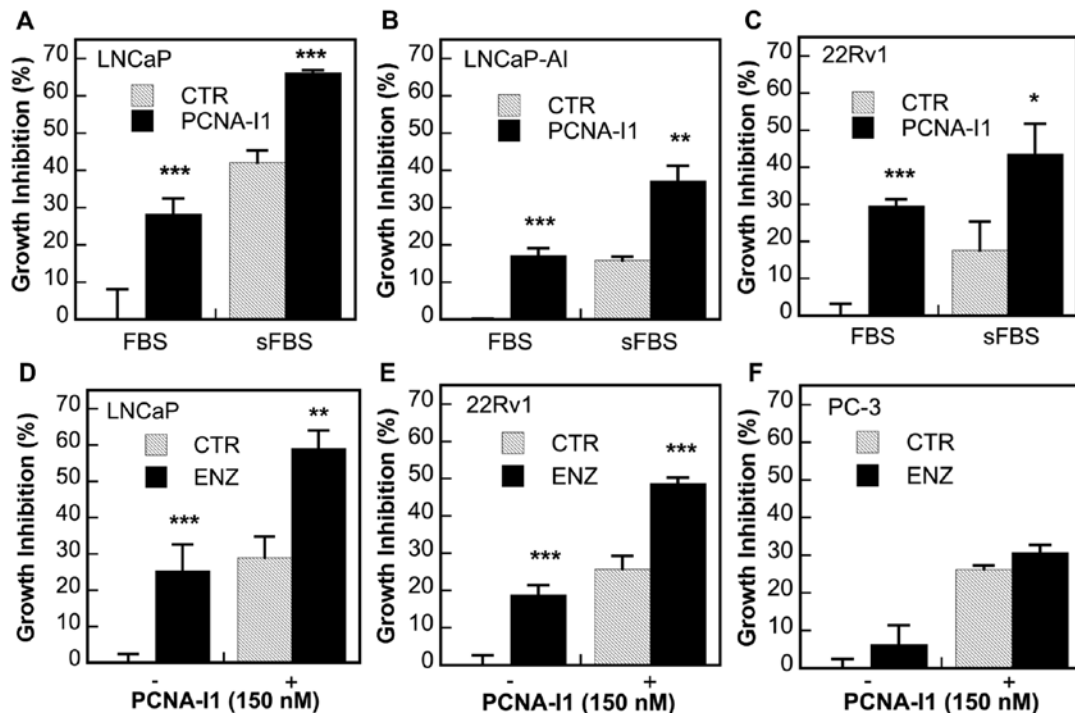


Figure 6. Effects of ENZ and PCNA-I1 (P-I1) on cell growth. (A-C) Cells were treated with PCNA-I1 (150 nM) in complete medium with FBS or stripped medium with sFBS for four days, followed by MTT staining. (D-F) The cells in complete medium were treated with PCNA-I1 and/or ENZ (5 μ M) for four days followed by MTT assay. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, PCNA-I1 vs. control (A-C) and ENZ vs. control or ENZ plus PCNA-I1 vs. PCNA-I1 alone. (D-F) ENZ, enzalutamide; PCNA, proliferating cell nuclear antigen.

residues linked with two cystine residues. The nine arginine residues are in 'all-D' configuration to minimize proteolysis.

To measure the binding and affinity of R9-AR-PIP to PCNA, the lysates of PC-3 cells containing either GFP-PCNA (34,46) or GFP (control) were incubated with increasing concentrations of R9-AR-PIP and the apparent dissociation constant (K_d) was determined by MST. R9-AR-PIP was found to bind to GFP-PCNA at K_d of $2.73 \pm 2.18 \mu$ M ($n=5$) and not to GFP (Fig. 7A). The data presented in Fig. 7B demonstrated that treatment with R9-AR-PIP reduced both the basal and DHT-induced expression of AR-FL and PSA proteins in LNCaP cells and AR-FL, AR-V7, and PSA proteins in 22Rv1 cells. Moreover, R9-AR-PIP inhibited both the basal and DHT-induced

promoter activity of PSA gene in a concentration-dependent manner in AR-negative PC-3 cells co-transfected with an AR expression vector (Fig. 7C). Finally, R9-AR-PIP inhibited the growth of androgen-dependent LNCaP cells both in the absence and presence of DHT with an IC_{50} value of 8-10 μ M (Fig. 7D). By contrast, the growth inhibitory effects of the peptide on AR-negative PC-3 cells were more modest at the same concentrations (Fig. 7D).

Discussion

PCNA is an evolutionally very well conserved multifunctional protein (53,54). Native PCNA, which is located mainly

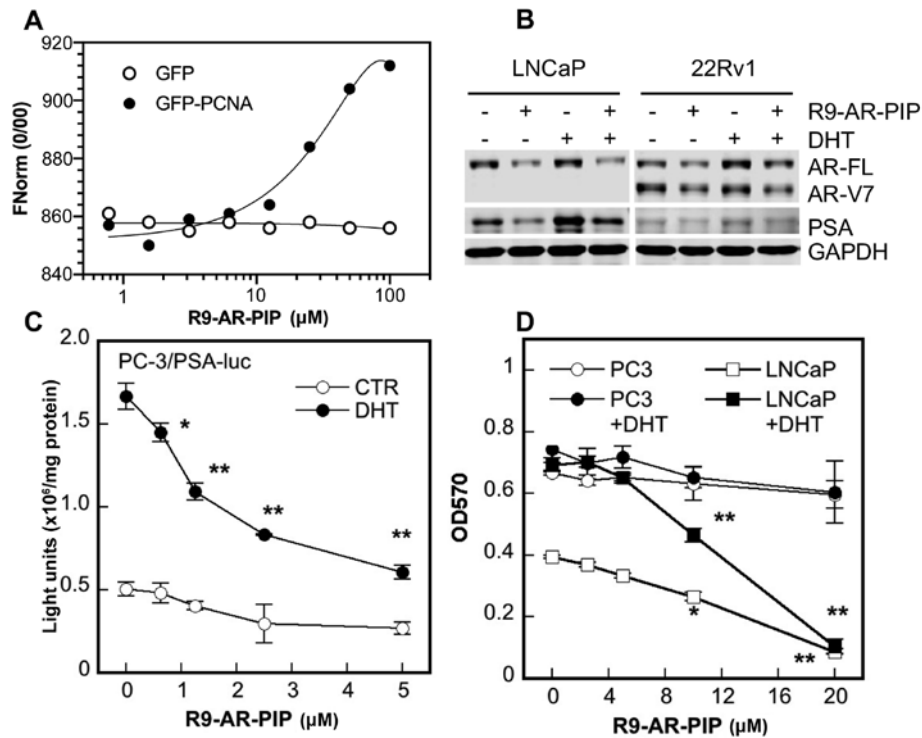


Figure 7. R9-AR-PIP inhibits the expression of AR target genes and cell growth. (A) Determination of the affinity of R9-AR-PIP to GFP-PCNA and GFP by thermophoresis with temperature jump. (B) Cells were treated without or with 20 μM R9-AR-PIP in the absence or presence of 10 nM DHT for 48 h, followed by western blot analysis. (C) PC-3 cells transfected with PSA-luc without or with CMV-AR were treated with R9-AR-PIP in the presence of 10 nM DHT for 24 h, followed by luciferase assay. (D) Cells were treated for five days with R9-AR-PIP in the absence or presence of 10 nM DHT, followed by MTT assay. * $P < 0.05$ and ** $P < 0.01$, R9-AR-PIP vs. control without the small peptide treatment.

in the nucleoplasm as ‘free form PCNA’, is a ring-shaped homotrimeric protein joined together through head to tail interaction (21-23,53,54). To be functional, PCNA must be linearized or monomerized and relocalized, and serves as platforms for interaction with its partner proteins containing several motifs, including the PIP box (22,24,25). The present study identified a PIP-box consensus sequence at the N-terminus of AR and found that PCNA complexed with AR-FL, as well as AR-V7 and ARv567es, and this was attenuated by the PIP-box inhibitor, T2AA. PCNA-IIS and PCNA-II are small-molecule PCNA inhibitors that bind to PCNA trimers at the interfaces of two monomers, stabilize the trimer structure, interfere with PCNA linearization or monomerization, and attenuate PCNA relocalization and chromatin association (33-36). The present study found that PCNA-IIS attenuated AR transcriptional activity and the expression of the AR target genes, PSA, p21/WAF, AR-FL and AR-V7. The knockdown of PCNA expression abolished the inhibitory effects of PCNA-IIS on AR transcriptional activity. Similarly, AR transcriptional activity and the expression of AR target genes were also inhibited by T2AA. Moreover, the additive growth inhibitory effects were demonstrated by targeting PCNA with PCNA-II plus ADT by either the deprivation of androgen or treatment with antiandrogen enzalutamide only in prostate cancer cells expressing AR, but not in AR-negative prostate cancer cells. Finally, an AR PIP-box mimicking peptide inhibitor (R9-AR-PIP) was developed, and it was found that it binds to PCNA at K_d of approximately 2.73 μM and inhibited the expression of AR target genes, AR transcriptional activity and the growth of AR-expressing cells in a concentration-dependent manner.

By contrast, the growth inhibitory effects of the peptide on AR-negative PC-3 cells were more modest at the same concentrations. These data strongly suggest that PCNA interacts with AR through the PIP-box and enhances AR-mediated signaling.

The exact mechanisms through which PCNA promotes AR activity in prostate cancer cells remain to be elucidated. One of the potential mechanisms is that chromatin-associated PCNA serves as a platform for AR and facilitates AR binding to chromatin. This notion is supported by several observations: i) Co-immunoprecipitation assay revealed that PCNA binds to AR and several other PCNA partner proteins at the S-phase of the cell cycle, but not at the G1 phase of the cell cycle when PCNA mostly presents as ‘free-form’ in the nucleosome (37); ii) PCNA-II and PCNA-IIS binds to PCNA and reduces PCNA chromatin association (34-36), and inhibits AR transcriptional activity and the expression of AR target genes; iii) the small-molecule PIP-box specific inhibitor, T2AA, inhibits PCNA interaction with partner proteins including AR-FL and AR-V7, and suppresses AR transcriptional activity and the expression of AR target genes; and iv) targeting the PCNA-AR interaction with AR PIP-box-specific peptide inhibitor R9-AR-PIP inhibits AR transcriptional activity, the expression of AR target genes and the growth of AR-positive cells.

In a previous study by the authors, it was demonstrated that PCNA-II inhibited the growth of tumor cells of various tissue origins at IC50 concentrations ~9-fold lower than those in normal cells (36). One of the important findings of the present study is that PCNA-II enhanced the growth inhibitory effects of ADT, either by androgen ablation or the anti-androgen, ENZ, in both androgen-dependent prostate

cancer cells and CRPC cells. The additive growth inhibitory effect appears to be AR-dependent, since it was not observed in AR-negative prostate cancer cells. The potential underlying mechanisms for this observation are that, in addition to inhibiting androgen-dependent AR-mediated signaling, PCNA-II suppresses PCNA chromatin association (35,36), interrupts the PCNA-AR interaction and hence attenuates AR activity via the androgen-independent AR-mediated pathway, such as AR phosphorylation by several tyrosine and serine protein kinases (55), the overexpression of Vav3 functioning as a co-activator (56) and constitutively active AR-Vs (5-7), as well as other cellular processes critical for cell growth (21-23).

In conclusion, the present study found that PCNA interacted with both AR-FL and AR-Vs very likely through the PIP-box at the N-terminus of AR and promoted AR transcriptional activity. In addition, targeting PCNA plus ADT exerted additive inhibitory effects on the growth of AR-positive prostate cancer cells. Given that PCNA is preferentially overexpressed in replicating tumor cells and CRPC overexpresses AR-FL and/or AR-Vs, targeting the PCNA-AR interaction by R9-AR-PIP to block AR-FL- and AR-V-mediated signaling may prove to be an innovative and selective therapeutic strategy against CRPCs, particularly those overexpressing constitutively active AR-Vs.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Both authors (SL and ZD) participated in the study design, conducted the experiments, performed the data analysis, and wrote or contributed to the writing of the manuscript. Both authors confirm the authenticity of all the raw data, and have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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