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ORIGINAL ARTICLE

CX4945 suppresses the growth of castration-resistant prostate cancer cells by reducing AR-V7 expression

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Abstract

Purpose The aberrant expression of casein kinase 2 (CK2) has been reported to be involved in the tumorigenesis and progression of prostate cancer. The inhibition of CK2 activity represses androgen-dependent prostate cancer cells by attenuating the androgen receptor (AR) signaling pathway. In this study, we examined the effect of CK2 inhibition in castration-resistant prostate cancer (CRPC) cells, in which AR variants (ARVs) play a predominant role.

Methods A newly synthetic CK2 selective inhibitor CX4945 was utilized to study the effect of CK2 inhibition in CRPC cells by CCK8 assay and colony formation assay. Protein and mRNA levels of full-length AR (AR-FL) and AR-V7 were determined by qPCR and western blot, respectively. The nuclear translocation of p50 and p65 was assessed to reflect the activity of the NF-κB pathway.

Results CX4945 reduced the proliferation of CRPC cells in a dose-dependent and time-dependent manner. AR-V7 rather than AR-FL was downregulated by CX4945 in both the mRNA and protein level. Furthermore, CX4945 could restore the sensitivity of CRPC cells to bicalutamide. The analysis of possible mechanisms demonstrated that the inhibition of CK2 diminished the phosphorylation of p65 at ser529 and thus attenuated the activity of the NF-κB pathway.

Conclusion The inhibition of CK2 by CX4945 can repress the viability of CRPC cells and restore their sensitivity to anti-androgen therapy by suppressing AR-V7. This finding presents a potential option for the treatment of prostate cancer, especially CRPC.

Keywords Castration-resistant prostate cancer · CX4945 · AR-V7 · NF-κB · Bicalutamide

C. Deng and J. Chen contributed equally to this research.

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Introduction

Prostate cancer (PCa) has become the most commonly diagnosed cancer in males and accounted for the second largest number of male cancer-related deaths in the United States [1]. Given the crucial role of the androgen/androgen receptor (AR) signaling axis in PCa, androgen-deprivation therapy (ADT) is the first-line treatment for relapsed or advanced prostate cancer patients. Unfortunately, despite the initial response for a median time of 18–20 months, almost all patients will suffer from recurrence, progressing into a lethal state of castration-resistant

prostate cancer (CRPC) [2]. It is now widely acknowledged that even if with low levels of serum testosterone, AR signaling remains active and indispensable in CRPC [3]. Based on this new understanding of CRPC, second-generation AR-targeted therapies, abiraterone and enzalutamide, have been developed and approved for the treatment of CRPC by the US Food and Drug Administration (FDA) [4, 5]. However, despite the exciting success of these drugs, inherent or acquired resistance still remains a major clinical challenge and requires further investigation [6].

It has been demonstrated that complex mechanisms contribute to the development of CRPC, including AR mutations [7], intraprostatic testosterone and DHT synthesis [8], AR bypass signaling pathway activation [9] and the expression of ligand-independent AR splice variants (ARVs) [10]. Among these, constitutively active ARVs, which lack the ligand-binding domain, represent an emerging crucial mechanism responsible for CRPC. One of the best characterized ARVs is AR-V7, also known as AR3 [11]. In vitro and in vivo studies have demonstrated that selective knockdown of AR-V7 expression inhibited androgen-independent cell growth and restored responsiveness to androgen and anti-androgen therapy [11, 12]. Therefore, it is believed that targeting AR-V7 may be a potential strategy against CRPC.

Protein kinase 2, formerly called casein kinase 2 (CK2), is a multifunctional, highly conservative, ubiquitously expressed and constitutively active protein kinase. This enzyme is a holoenzyme complex composed of two catalytic subunits (CK2α or CK2α′) and two regulatory CK2β subunits [13]. Cumulative evidence has demonstrated that CK2 can phosphorylate a large number of substrates involved in cellular growth, apoptosis and angiogenesis. All of the above functions suggest its potential role in carcinogenesis and tumor progression [14, 15]. Certain studies have indicated that elevated CK2 expression and activity were closely associated with the development of prostate cancer and poor prognosis [16, 17]. Accordingly, our previous research illustrated that the inhibition of CK2 attenuated AR function and cell proliferation in prostate cancer cells [18]. Hessenauer et al. [19] found different responses in hormone-sensitive and hormone-refractory prostate cancer cells to the inhibition of CK2. However, the cells they used in that study were LNCaP (AR-dependent) and PC-3 (AR-negative). No AR-independent and ARV-driven CRPC cells were included. Therefore, we sought to investigate whether CK2 also plays a role in ARV signaling. Herein, we utilized a highly selective inhibitor of CK2, CX4945, which is currently in Phase I and II clinical trials (ClinicalTrials.gov Identifier: NCT02128282). We found that the inhibition of CK2 by CX4945 reduced the proliferation of 22Rv1 and VCaP cells and restored their sensitivity to bicalutamide under ADT conditions. Mechanistically, CX4945 does not influence the expression of full-length AR (AR-FL) but downregulates AR-V7 at both the mRNA and protein level. These findings implied a potential and effective approach for the treatment of CRPC, which is a major challenge in clinic.

Materials and methods

Cell culture and reagents

The 22Rv1 and VCaP cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were routinely cultured in RPMI 1640 supplemented with 10% FBS (Gibco, Life Technologies), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C with humidified air and 5% CO_2. Unless indicated, cells were transferred to charcoal stripped FBS (csFBS, Biological Industries) medium at the beginning of the experiments.

Cells were treated with CX4945, Bicalutamide or QNZ (Selleck, Shanghai, China) dissolved in DMSO (final concentration ≤0.1%). The control groups of all sets of experiments received DMSO at a concentration equal to that in drug-treated cells.

Western blotting

Cell compartment proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). A total of 20 μg of protein was separated by 10% SDS-PAGE gels and transferred onto a PVDF membrane (Millipore). The membranes were incubated with the following specific primary antibodies: AR, pNF-κB $p65^{ser529}$ (1:500, Santa Cruz Biotechnologies), p50, p65, GAPDH and Histone H3 (1:2000, Cell Signaling Technology). GAPDH was used as an internal control for whole cell lysates, while Histone H3 was an internal control for nuclear fraction protein.

Quantitative real-time PCR

Total RNA was isolated using Trizol™ reagent (Invitrogen). Approximately 1 μg RNA was used for cDNA synthesis using GoScript™ Reverse Transcription System (Promega). QPCR was conducted using the GoTaq® qPCR Master Mix (Promega). Primers for the corresponding target genes are listed as follows:

AR-FL.

5′-GACGACCAGATGGCTGTCATT-3′ (forward).

5′-GGGCGAAGTAGAGCATCCT-3′ (reverse).

AR-V7.

5′-CCATCTTGTCGTCTTCGGAAATGTTA-3′ (forward).

5′-TTTGAATGAGGCAAGTCAGCCTTTCT-3′ (reverse).

PSA.

5′-GTGTGTGGACCTCCATGTTATT-3′ (forward).

5′-CCACTCACCTTTCCCCTCAAG-3′ (reverse).

GAPDH.

5′-TTCTTTTGCGTCGCCAGCCGA-3′ (forward).

5′-GTGACCAGGCGCCCAATACGA-3′ (reverse).

Cell viability assay and colony formation assay

Cells were seeded in 96-well plates the day before CX4945 treatment. Cell viability was evaluated using the Cell Counting Kit 8 (CCK8, Dojindo). To assess the sensitivity of 22Rv1 to bicalutamide, cells were treated with different doses of bicalutamide with or without CX4945 for 3 days under 5% csFBS condition. All the experiments were performed in triplicate.

For colony formation assays, 500 cells per well were seeded in six-well plates and incubated with CX4945 or control reagent (DMSO) for 14 days. Cells were fixed in methanol for 10 min and then stained with Giemsa's solution. Colonies were counted with more than 50 cells under microscopy.

Cell cycle analysis and apoptosis assay

For cell cycle analysis, cells were seeded 4×10^5 cells/well in six-well plates, and treated with CX4945 or DMSO for 24 h. Cell cycle distribution was analyzed using a Gallios flow cytometer (Beckman Coulter).

For apoptosis assay, cells were seeded 2×10^5 cells/well in six-well plates, and treated with corresponding reagents. After 48 h, cells were harvested and incubated with Annexin V-FITC and PI (KeyGen BioTECH) for 10 min and then analyzed by a Gallios flow cytometer (Beckman Coulter).

ECLIA assay

Approximately 4×10^5 cells were seeded in six-well plates and treated with CX4945 for 12, 24 and 48 h. The PSA levels of the conditioned medium were quantified by ECLIA assay using the Elecsys total PSA immunoassay on a Cobas E-602 analyzer (Roche Diagnostics) according to the manufacturer's instruction.

Statistical analysis

All data were presented as the mean ± SD of three independent experiments and analyzed using GraphPad Prism software (version 5 for Windows, GraphPad Software). The significance of the differences between groups was analyzed by one-way analysis of variance (ANOVA) using SPSS software. $P < 0.05$ was considered statistically significant.

Results

Suppressive effect of CX4945 on CRPC cell lines

To determine whether CX4945 exerted any effect on CRPC cell lines, different doses of CX4945 were added to 22Rv1 and VCaP, two AR-V7-positive CRPC cell lines. In addition, different incubation times of 3, 5 and 7 days were used to examine the time-dependent effect. As shown in Fig. 1a, CX4945 reduced cell viability of 22Rv1 and VCaP in a dose-dependent and time-dependent manner. To define the mechanisms by which CX4945 repressed 22Rv1 cells, we analyzed the cell cycle status as well as the percentage of apoptotic cells by flow cytometry. As shown in Fig. 1b, treatment of CX4945 caused a significant G2/M cell cycle arrest. Apoptosis assay showed that CX4945 increased both early and late apoptotic populations compared with the control (Fig. 1c). For the observation of long-term toxicity, colony formation was performed with vehicle or 2.5 μM CX4945. As seen in Fig. 1d, CX4945 (2.5 μM) significantly inhibited the colony formation ability of 22Rv1.

CX4945 inhibits PSA gene transcription and secretion

Prostate-specific antigen (PSA) is the most well-recognized biomarker and is widely used in clinics for diagnosis and treatment surveillance. Remarkably, elevated PSA levels after ADT implicate the emergence of recurrence and progression to CRPC. Although AR-V7 lacks the ligand-binding domain compared to full-length AR (AR-FL), previous research has shown that AR-V7 regulates the canonical AR target genes, including the PSA gene [20]. QPCR analysis of PSA mRNA (Fig. 2a) indicated that CX4945 could significantly inhibit the transcription of the PSA gene in 22Rv1. In addition, we detected the secreted PSA level in the conditioned culture medium of 22Rv1 cells. As shown in Fig. 2b, treatment with CX4945 significantly decreased the secreted PSA.

CX4945 downregulates AR-V7 expression and nuclear translocation by inhibiting NF-κB activity

Because AR-V7 plays a crucial role in CRPC cells under androgen depletion conditions, we conducted a series of experiments to assess the expression level and nuclear translocation of AR-V7 and AR-FL. As shown in Fig. 3a, CX4945 significantly downregulated the AR-V7 protein level both in 22Rv1 and VCaP, whereas the level of AR-FL

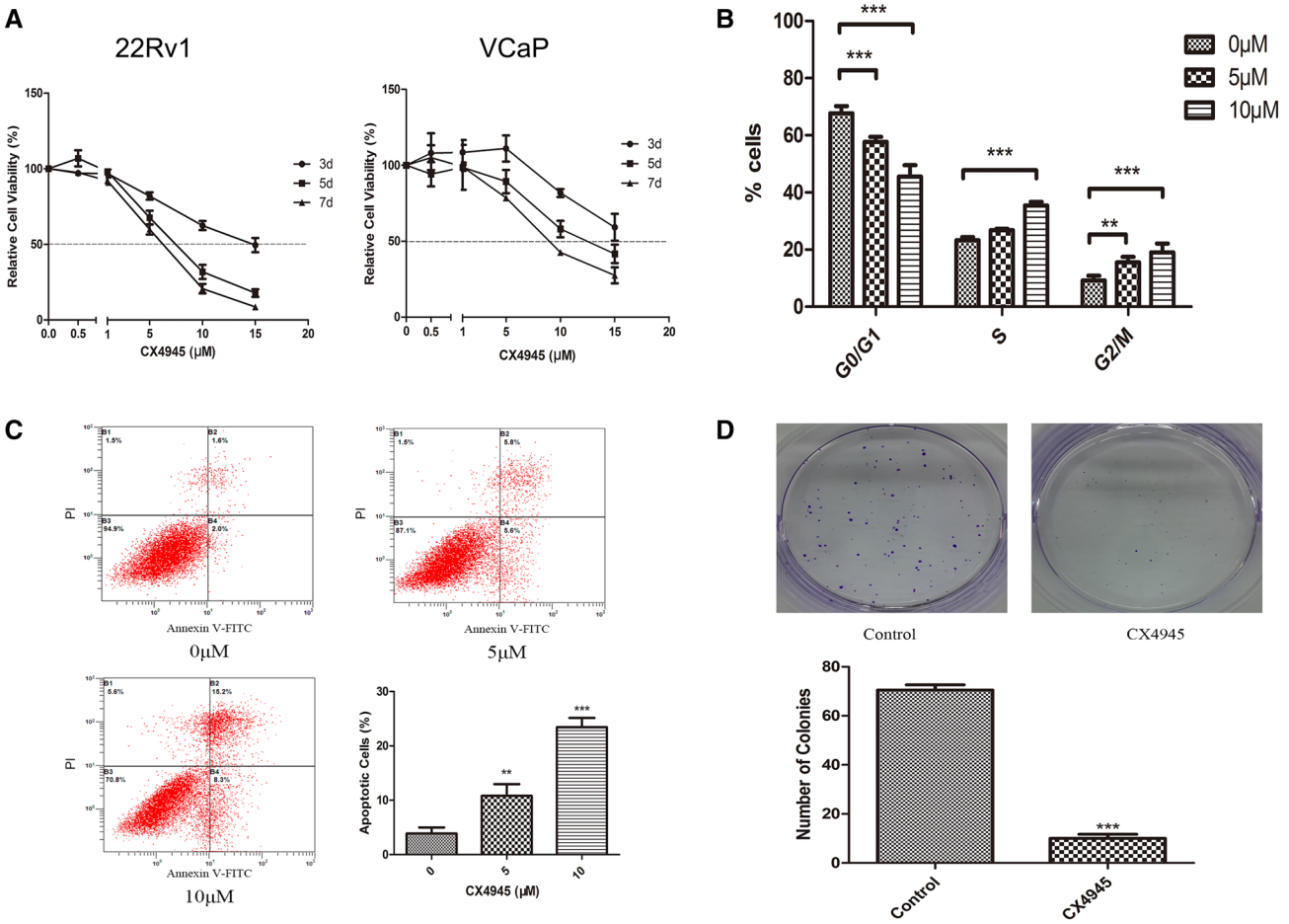

Fig. 1 Suppressive effect of CX4945 on CRPC cell lines. **a** 22Rv1 and VCaP cells were seeded into 96-well plate, cultured with csFBS and then treated with different doses of CX4945 for 3, 5 and 7 days. **b** 22Rv1 cells were treated with CX4945 (0, 5 and 10 μM) for 24 h and then harvested for the analysis of the cell cycle as described in the text. **c** 22Rv1 cells were treated with CX4945 for 48 h and stained with Annexin V-FITC and PI. Apoptotic cells were counted as early (*lower right*) plus late (*upper right*) apoptotic cells. Data are shown as the mean ± SD of three independent experiments. **d** Approximately 500 cells per well of 22Rv1 were seeded in a six-well plate and incubated with CX4945 (2.5 μM) or DMSO for 2 weeks. Colonies were counted with more than 50 cells under microscopy. $^{**}P<0.01$, $^{***}P<0.001$

was barely affected. Consistent with the results of western blotting, qPCR analysis of AR-V7 and AR-FL mRNA levels in 22Rv1 showed that the transcription of AR-V7 rather than AR-FL was significantly suppressed by CX4945 (Fig. 3b). As transcription factors, AR-V7 and AR-FL only exhibit their transcription activities when located in the nucleus. Therefore, we examined whether CX4945 could influence the nuclear translocation of AR-V7 or AR-FL. We showed that CX4945 decreased the nuclear fraction of AR-V7, but not AR-FL in 22Rv1 (Fig. 3c).

Next, we sought to investigate through which pathway CX4945 downregulated AR-V7 expression and nuclear translocation. Previous studies suggested that CK2 could phosphorylate NF-κB p65 at ser529 specifically, and was closely associated with the aberrant activation of NF-κB in several cancers [21, 22]. The classical NF-κB pathway, which includes the p65/p50 heterodimer, has been demonstrated to be involved in prostate cancer development, and the inhibition of NF-κB activity can restore the sensitivity of CRPC cells to traditional ADT therapy [23]. Therefore, we speculated that CX4945 may exert its inhibitory effect on AR-V7 through the NF-κB pathway. Figure 3d shows that CX4945 significantly reduced the phosphorylation of pNF-κB p65 at ser529 in 22Rv1. In addition, the nuclear translocation of p65 and p50 was inhibited by CX4945.

Inhibition of NF-κB suppresses CRPC cells by reducing AR-V7

To investigate whether inhibition of NF-κB could suppress the growth of CRPC cells, 22Rv1 cells were treated with selective NF-κB inhibitor QNZ. As shown in Fig. 4a, QNZ

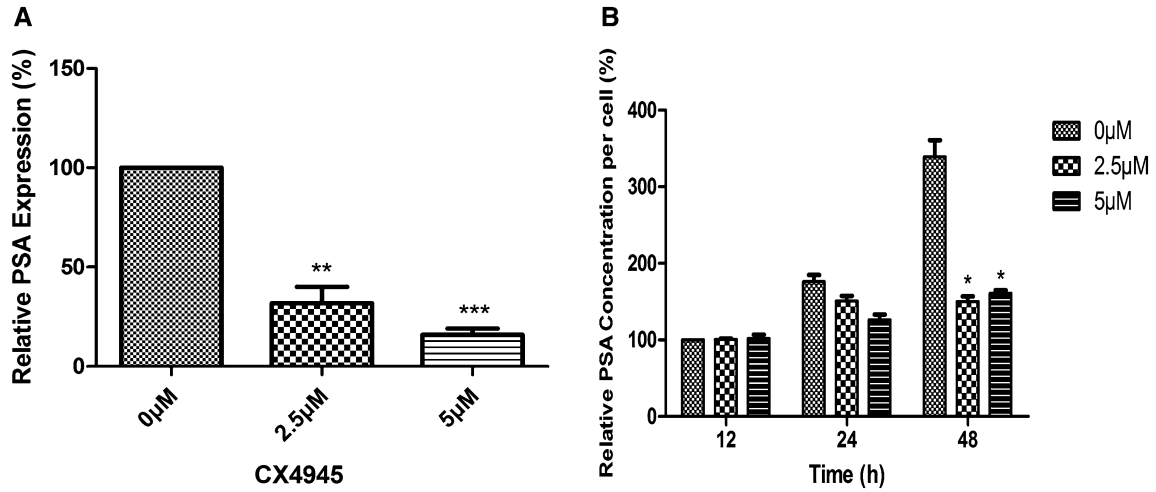

Fig. 2 CX4945 inhibits PSA gene transcription and secretion. **a** 22Rv1 cells were treated with different concentrations of CX4945 (0, 2.5 and 5 μM) for 24 h. The mRNA level of PSA was estimated by qRT-PCR. **b** 22Rv1 cells were treated with CX4945 (0, 2.5 and 5 μM) for 12, 24 and 48 h. The relative concentrations were corrected for the cell number counted by a Beckman Z2 Coulter® Particle Count and Size Analyzer. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

significantly reduced the cell viability of 22Rv1 in a dose-dependent and time-dependent manner. We also measured the change of AR-FL/AR-V7 after treatment of QNZ. We found that inhibition of NF-κB by QNZ could reduce the expression of AR-V7, while little effect was found on AR-FL (Fig. 4b).

CX4945 resensitizes CRPC cells to anti-androgen therapy

As the inhibition of CK2 by CX4945 downregulated AR-V7 expression and nuclear translocation, we next investigated whether CX4945 treatment could restore the sensitivity of CRPC cells to conventional anti-androgen therapy. Bicalutamide alone had little suppressive effect on 22Rv1 and VCaP under androgen-depleted conditions with 5% csFBS (Fig. 5a, b). However, when combined with a sub-dose of CX4945, the viability of 22Rv1 and VCaP cells was significantly suppressed (Fig. 5c, d). The result of apoptotic assay using flow cytometry was in accord with the above cytotoxic experiment (Supplemental Figure S1A and B).

Discussion

CK2 is a highly conservative protein kinase that has a wide range of substrates. Many studies indicate that CK2 is involved in various types of cellular processes in cancer, including the cell cycle, apoptosis, and metastasis. Several types of CK2 inhibitors have been improved, and one of the most specific inhibitors, CX4945, has recently progressed into Phase I and II clinical trials (ClinicalTrials.gov Identifier: NCT02128282). The aberrant activity of CK2 is also closely associated with prostate cancer development and poor prognosis [16, 17, 24]. However, whether this effect of CK2 inhibition exists in ARV-driven CRPC cells remains unknown.

In the present study, we demonstrated for the first time that CX4945 could repress the proliferation ability of 22Rv1 and VCaP under ADT conditions. Both the 22Rv1 and VCaP cell lines express abundant AR-V7, so they are ideal models to study the functions of ARVs [25]. CX4945 caused cell cycle arrest and induced the apoptosis of 22Rv1 cells. Unlike in AR-dependent prostate cancer cells, CX4945 did not influence the expression of AR-FL. Instead, CX4945 downregulated the mRNA and protein levels of AR-V7. As AR-V7 is predominantly located in the nucleus and is constitutively activated, we evaluated the changes in the nuclear fraction of AR-V7 after CX4945 treatment. We showed that CX4945 attenuated the nuclear translocation of AR-V7, while little effect was observed on AR-FL. Moreover, both the transcriptional and secreted levels of PSA decreased after CX4945 treatment. This further confirmed that CX4945 could inhibit the transcriptional activity of AR-V7 under ADT condition. Previous studies implicated that bicalutamide alone could not suppress the growth of CRPC cells, which are ARV positive [26, 27]. Considering the inhibitory effect on AR-V7 expression, we treated 22Rv1 and VCaP with bicalutamide plus CX4945. Significant inhibition of cell growth was observed. All of the above results suggested that CX4945 alone or combined with traditional ADT may be a valuable therapeutic strategy against CRPC.

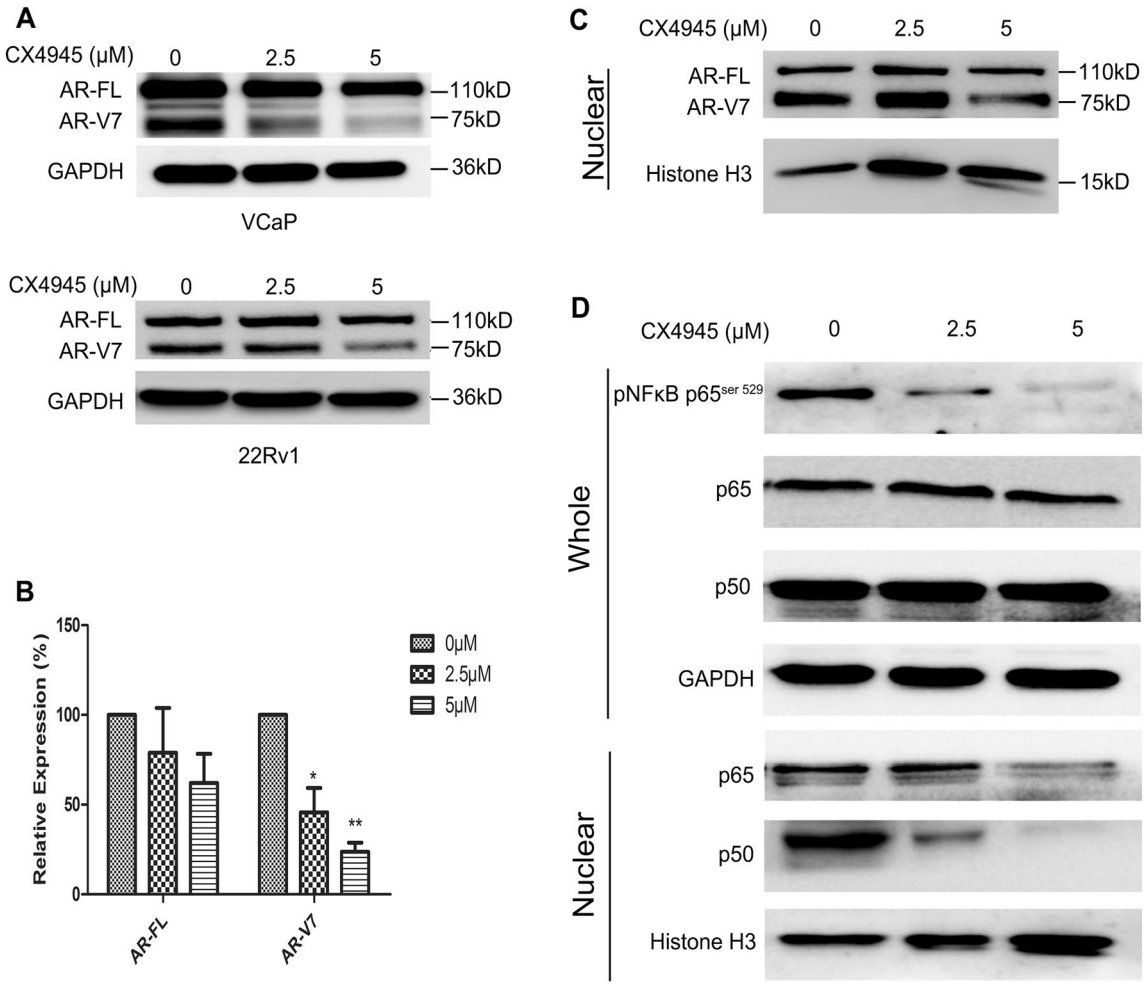

Fig. 3 CX4945 downregulates AR-V7 expression and nuclear translocation by inhibiting NF-κB activity. **a** 22Rv1 and VCaP cells were treated with CX4945 for 48 h. The proteins of the whole cell lysate were extracted and analyzed by western blotting. **b** 22Rv1 cells were treated with CX4945 for 24 h. The mRNA levels of AR-FL and AR-V7 were estimated by qRT-PCR. **c** 22Rv1 cells were treated with CX4945 for 48 h. The nuclear fractions of AR and AR-V7 were analyzed by western blotting. **d** 22Rv1 cells were incubated with CX4945 for 48 h. The whole cell lysates and nuclear proteins were isolated as mentioned in the text. $*P < 0.05$, $**P < 0.01$

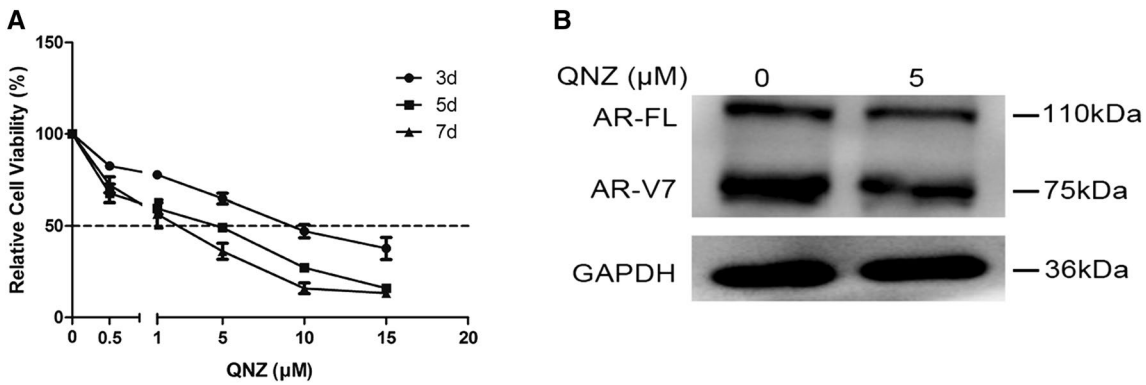

Fig. 4 Inhibition of NF-κB suppresses CRPC cells by reducing AR-V7. **a** 22Rv1 cells were treated with different doses of QNZ for 3, 5 and 7 days. **b** 22Rv1 cells were treated with 5 μM QNZ or DMSO for 48 h

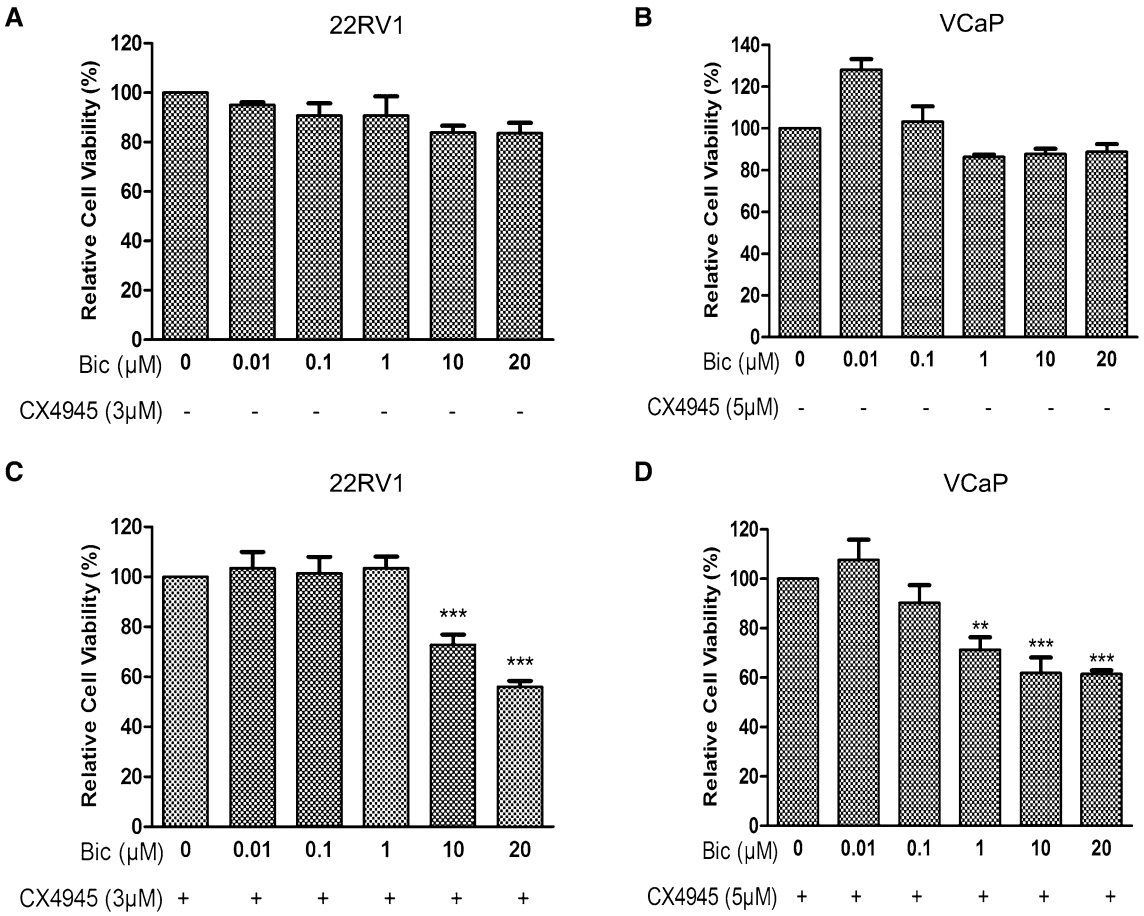

Fig. 5 CX4945 resensitizes CRPC cells to anti-androgen therapy. **a, b** 22Rv1 and VCaP cells were treated with indicated concentration of bicalutamide (Bic) without CX4945. **c, d** 22Rv1 and VCaP cells were treated with indicated concentration of bicalutamide (Bic) with a subdose of CX4945 (3 or 5 μM). CCK8 assay was performed at 72 h to measure the cell viability. $^{**}P < 0.01$, $^{***}P < 0.001$

Multiple mechanisms have been demonstrated to be involved in CRPC progression and increasing attention has been paid to the ARVs, which are believed to be a driving force in CRPC development [12]. As these splice variants do not contain the LBD, traditional ADT that targeting AR LBD has little effect in CRPC patients. By far, the exact mechanism that contributes to ARV expression remains unknown. Sunita et al. [28] conducted an integrative microarray analysis of pathways dysregulated in metastatic prostate cancer. They found that the NF-κB (p50/p65) pathway was the most significant one. Other studies also implied that the aberrant activation of the NF-κB pathway was closely correlated with the progression to castration-resistant growth and metastasis in prostate cancer [29]. The inhibition of NF-κB signaling could suppress AR-V7 expression and restore responsiveness to anti-androgen treatment in CRPC cells [23]. In addition, previous studies implicated that CK2 could phosphorylate NF-κB p65 at ser529 specifically, and was closely associated with the aberrant activation of NF-κB pathway in several cancers [21, 22]. Therefore, we supposed that the CK2 inhibitor CX4945 may exert its suppressive effect by modulating NF-κB signaling. Consistently, CX4945 treatment reduced the phosphorylation of p65 at ser529 and inhibited the nuclear translocation of p50 and p65. In addition, 22Rv1 cells treated with selective NF-κB inhibitor, QNZ, showed significant reduction in the cell viability and AR-V7 reduction similar to CX4945 did. These results indicated that CX4945 downregulated AR-V7 expression by inhibiting NF-κB activity (Fig. 6). Considering the small changes in AR-FL mRNA and protein levels, we supposed that some splice factors may have been downregulated by the inhibition of CK2 through the NF-κB pathway. Meanwhile, the exact mechanism by which NF-κB modulates AR-V7 expression requires further investigation.

In spite of AR-V7, there are other AR variants, such as ARV567es that contribute to CRPC progression [30]. However the mechanism of the generation of these ARVs is still not clearly demonstrated. Therefore, whether inhibition of

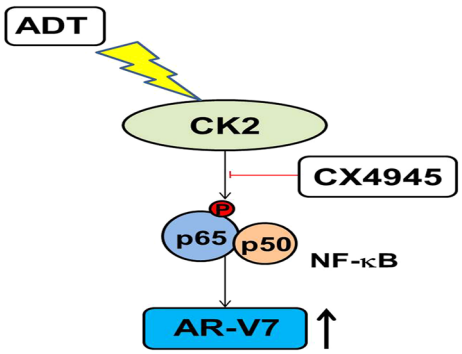

Fig. 6 Regulatory mechanism of AR-V7 by CK2 through NF-κB pathway. CX4945 inhibits the activity of CK2, reducing the phosphorylation of p65. As a result, the upregulation of AR-V7 is attenuated

CK2 can reduce the expression of other ARVs requires further investigation.

In summary, our data suggest that the inhibition of CK2 by CX4945 can repress the viability of CRPC cells and restore their sensitivity to anti-androgen therapy by suppressing AR-V7. This finding presents a potential therapy target for CRPC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

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