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Original Articles

Targeting the androgen receptor (AR) with AR degradation enhancer ASC-J9® led to increase docetaxel sensitivity *via* suppressing the p21 expression

Jie Luo[a,b,1], Jing Tian[c,1], FuJu Chou[a], Changyi Lin[a], Emily Zixin Xing[a], Li Zuo[a,d], Yuanjie Niu[c], Shuyuan Yeh[a], Chawnshang Chang[a,e,*]

[a] George Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, The Wilmot Cancer Center, University of Rochester Medical Center, Rochester, NY, 14642, USA
[b] Biology Department, University of Rochester, Rochester, NY, USA
[c] Chawnshang Chang Sex Hormone Research Center, Tianjin Institute of Urology, Tianjin Medical University, Tianjin, 300211, China
[d] Department of Urology, Changzhou Second People's Affiliated Hospital of Nanjing Medical University, Changzhou, 213003, China
[e] Sex Hormone Research Center, China Medical University and Hospital, Taichung, 404, Taiwan

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ABSTRACT

Chemotherapy with docetaxel remains the effective therapy to suppress castration resistant prostate cancer (CRPC) in some patients. However, most chemotherapy with docetaxel eventually fails with the development of docetaxel resistance after 18-weeks of treatment. Here we found docetaxel treatment might have an adverse effect of increasing the androgen receptor (AR) protein level in the CRPC cells, and combining docetaxel with anti-AR therapy using AR-shRNA or the AR degradation enhancer ASC-J9® may increase docetaxel sensitivity to better suppress the CRPC cell growth. Mechanism dissection found docetaxel might have the adverse effect of increasing the AR protein stability *via* suppressing the AR ubiquitination due to the increased AR phosphorylation. The consequence of such increased AR protein may then lead to increase p21 expression *via* transcriptional regulation. Preclinical studies with *in vitro* cells lines also demonstrated that targeting AR with ASC-J9® led to suppressing the AR-increased p21 expression to improve the docetaxel sensitivity in the CRPC cells that already developed docetaxel resistance. Together, these results suggest that a combined therapy of docetaxel and ASC-J9® is a novel therapy to better suppress CRPC in patients that already developed docetaxel resistance.

1. Introduction

Prostate cancer (PCa) is the most prevalent cancer among males in United States with the 3rd highest mortality rate [1–3]. For decades, the treatment of PCa, especially at later stages, has mainly relied on the androgen depravation therapy (ADT) with antiandrogens to reduce or prevent androgens from binding to the androgen receptor (AR) [4]. However, most of such ADT with antiandrogens treatment fails in 1–2 years and tumors progress into castration resistant PCa (CRPC) [5]. The FDA standard therapy after CRPC is chemotherapy with various drugs [6,7]. Among them, docetaxel has been the most used and clinical data indicated that chemotherapy with docetaxel could extend CRPC patients survival several more months, before development of the chemotherapy resistance [8–12].

A variety of mechanisms have been linked to the development of chemotherapy resistance [13,14] including up-regulation of multi-drug resistant (MDR) genes [15–17], induction of cellular stress signaling [18,19], and/or protective autophagy [20] in response to chemotherapy. Suppression of cell cycle progression [21,22], or repression of apoptotic machinery [22,23] may also contribute to such chemotherapy resistance.

The p53-p21 axis is the classical cellular stress signaling that dictates the cellular response to genotoxic stress [24]. Activating p53 is generally regarded as essential for cell killing by chemotherapeutics through activation of apoptosis [25]. Furthermore, induction of p21 in response to p53 activation may also allow cells to escape immediate demise through repression of cell cycle progression and/or cellular senescence [26]. In that regard, preventing up-regulation of p21 axis may be able to render tumor cells more sensitive to chemotherapeutic drugs with a consequent increase of efficacy.

* Corresponding author. George Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, The Wilmot Cancer Center, University of Rochester Medical Center, Rochester, NY, 14642, USA.
E-mail address: chang@urmc.rochester.edu (C. Chang).
[1] These authors contribute equally.

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The AR is one of the key factors to influence the PCa progression [27–30], and using the antiandrogens Casodex or Enzalutamide to prevent androgen from binding to AR have been used with success to initially suppress PCa growth [31,32]. However, within 1–2 years patients treated with these antiandrogens may develop castration resistance and have adverse effects including the promotion of PCa cell invasion [33,34]. One of the key puzzles remaining unsolved is the existence of activated AR in CRPC even though androgen concentration at this castration resistant stage is suppressed to a minimum at 1–3 nM [35]. Whether this remaining activated AR in the CRPC patients may still play roles to influence the subsequent chemotherapy also remains unknown.

Unlike the currently used antiandrogens, Casodex or Enzalutamide, which have little impact on the AR protein, the AR degradation enhancer, ASC-J9®, was demonstrated to be able to selectively degrade AR in some cells [36–40]. Results from those studies indicated that ASC-J9® not only could suppress PCa growth, but importantly, it could also suppress PCa cell invasion [33,34].

Here we found that docetaxel, the most widely used chemotherapeutic drug for CRPC, could increase AR protein level *via* modulating the p21 signaling to reduce the docetaxel efficacy. However, combining ASC-J9® with docetaxel can suppress the AR-increased p21 expression to enhance the docetaxel efficacy. These results suggest that a combination of ASC-J9® and docetaxel may provide a more effective therapeutic modality than the single use of docetaxel to better suppress the PCa at the CRPC stage.

2. Materials and methods

2.1. Cell culture

LNCaP, PC3, and CWR22Rv1 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 with 10% FBS. The C4-2 cell line was the gift from Dr. Leland W.K Chung. All cells were maintained in a humidified 5% CO_2 environment at 37 °C.

2.2. Cell growth assay

The cells were seeded in 24-well tissue culture plates in RPMI media containing 10% FBS for 24 h. The cells were then treated with vehicle, 10 μM Casodex, 10 μM enzalutamide or 5 μM ASC-J9® with/without the treatment with docetaxel. The cell growth was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (Sigma, St. Louis, MO). The media containing MTT (0.5 μg/ml) were added into each well at the indicated time points. After a 2 h incubation at 37 °C, all crystals were solubilized by DMSO, and the optical density of the solution was determined spectrophotometrically at 570 nm.

2.3. RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNAs were isolated using Trizol reagent (Invitrogen, Grand Island, NY). One μg of total RNA was subjected to reverse transcription using Superscript III transcriptase (Invitrogen). The qRT-PCR was conducted using a Bio-Rad CFX96 system with SYBR green to determine the mRNA expression level of a gene of interest. Expression levels were normalized to GAPDH level.

2.4. Western blot analysis

Cells were lysed in RIPA buffer and proteins (20–40 μg) were separated on 8–10% SDS/PAGE gel and then transferred onto PVDF membranes (Millipore, Billerica, MA). After blocking membranes, they were incubated with primary antibodies, HRP-conjugated secondary antibodies, and visualized using the ECL system (Thermo Fisher Scientific, Rochester, NY). AR, GAPDH, tubulin, PARP, and GFP antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The p21 antibody was from Cell Signaling Technology (Danvers, MA).

2.5. AR protein phosphorylation assay

The cells were lysed by the RIPA buffer. The cell lysates were incubated with AR antibody in 4 °C overnight to pull-down the AR protein. And then the WB assay was performed. The AR phosphorylation levels were determined by the p-Serine and p-Threonine antibody (Abcam, Cambridge, MA).

2.6. Luciferase assay

PCa cells were plated in 24-well plates and transfected with MMTV-luc containing ARE sequence using Lipofectamine (Invitrogen). After transfection, regular media were added with various concentrations of docetaxel and incubated for 48 h. The pRL-TK was used as an internal control. Luciferase activity was measured by Dual-Luciferase Assay (Promega, Madison, WI) according to the manufacturer's manual.

2.7. Chromatin immunoprecipitation (ChIP)

Briefly, protein-DNA complexes were cross-linked by 1% formaldehyde then quenched using 125 mM glycine. Cells were collected in lysis buffer and subjected to sonication. After centrifugation, the supernatant was incubated with AR antibody, and chromatin DNA was purified and subjected to qPCR detection.

2.8. Statistics

The data values were presented as the mean ± SEM. Differences in mean values between two groups were analyzed by two-tailed Student's *t*-test. All experiments were run in triplicate and at least 3 separate times to determine statistical significance. Data are presented as mean ± SD with p value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Chemotherapy with docetaxel increases the AR protein level in CRPC cells

Two recent clinical trials showed better efficacy for the combined antiandrogen treatment with the docetaxel than docetaxel alone [41], suggesting that androgen/AR signals in CRPC may be linked to the docetaxel sensitivity to suppress CRPC cells. Here we first found that docetaxel might increase AR protein level in the CRPC C4-2 cells in a dose dependent manner (Fig. 1a). Similar results were also obtained when we replaced C4-2 cells with CWR22RV1, another CRPC cell line (Fig. 1b). Importantly, when we fixed the docetaxel concentration at 2 nM, we were also able to demonstrate the AR protein level can be increased by docetaxel in a time dependent manner in the C4-2 cells (Fig. 1c).

3.2. Mechanism dissection of how docetaxel increases AR protein level in the CRPC cells: via increasing AR protein stability

To dissect the molecular mechanism of how docetaxel increases the AR protein level in CRPC cells, we then applied the qPCR analysis to assay docetaxel effects on the AR mRNA expression, and result revealed that docetaxel has little effect on AR mRNA level (Fig. 1d). In contrast, we found docetaxel treatment could increase the expression of p21, the AR key target gene in PCa (Fig. 1d). These results suggest that docetaxel may impact the AR expression at the post-transcriptional levels.

We then applied Cycloheximide, an inhibitor to suppress the protein synthesis [42], to examine the AR protein stability after docetaxel

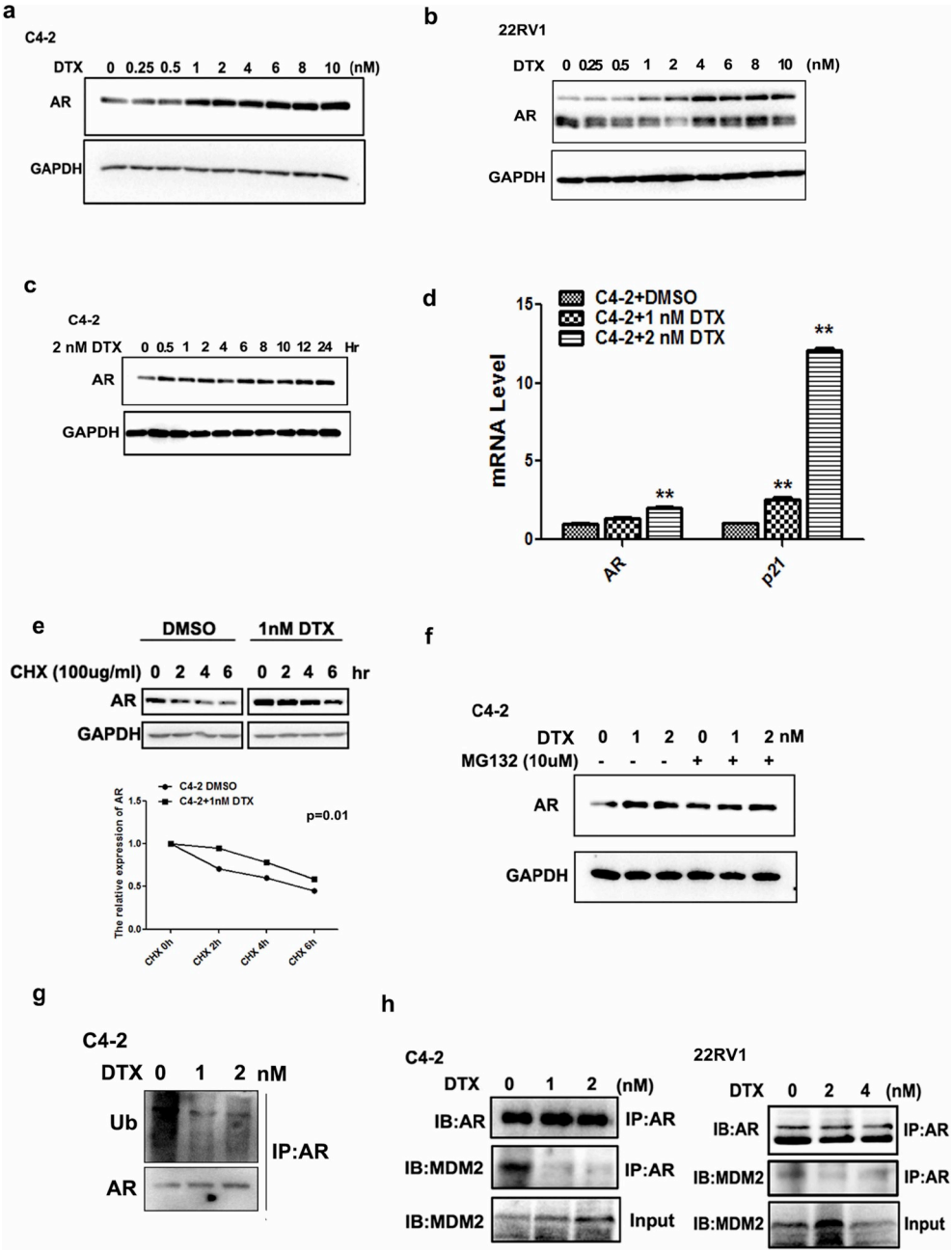

Fig. 1. AR protein level increase after docetaxel treatment.
a Western blot analysis of AR protein level in C4-2 cells. The C4-2 cells were treated with/without different doses of docetaxel (DTX) for 24 h. **b** Western blot analysis of AR protein level in CWR22RV1 cells after treating with/without increasing doses of DTX for 48 h. **c** Western blot analysis of AR protein level in C4-2 cells after treating with/without 2 nM DTX at different time points. **d** The qPCR analysis of AR and p21 expression after DTX treatment compared to control DMSO. Data are presented as mean ± SD, $*p < 0.05$. **e** Western blot analysis of AR protein level. The C4-2 cells with/without 1 nM DTX were treated with 100 μg/ml CHX, and harvested at different time points. **f** The C4-2 cells were treated with DTX and MG132. The AR protein level was analyzed by western blot. **g.** The C4-2 cells were treated with DTX for 24 h. Then the cells were lysed and the AR proteins were pulled-down by the AR antibody. The western blot was performed to detect the Ub interaction with AR. **h** The interaction of AR and MDM2 after treating with/without DTX was analyzed in C4-2 and CWR22RV1 (22RV1) cells.

treatment. The results from western blot analysis revealed that the docetaxel increased the AR protein stability (Fig. 1e).

Together, results from Fig. 1a–e suggest that docetaxel can increase AR protein level *via* impacting the AR protein stability.

3.3. Mechanism dissection of how docetaxel enhances AR protein stability in the CRPC cells: via inhibiting AR ubiquitination

To further dissect the molecular mechanism of how docetaxel can enhance AR protein stability in the CRPC cells, we then focused the ubiquitination-proteasome signaling, a key signal pathway to control the protein stability [43]. We first treated the cells with docetaxel for 24 h, and then treated the C4-2 cells with MG132, a proteasome inhibitor [44], to suppress the ubiquitination-proteasome signaling. The results from western blot analysis revealed that adding MG132 could then block the docetaxel increased AR protein level (Fig. 1f).

To further examine the ubiquitination of AR upon the docetaxel treatment, the C4-2 cells were treated with 1 nM and 2 nM docetaxel for 24 h, and results from assay of interaction between AR and Ub revealed that adding docetaxel could impact the interaction between AR and Ub (Fig. 1g).

We then examined whether docetaxel could influence the interaction between AR and its E3-ligase, MDM2 [45], and results revealed that in C4-2 and CWR22RV1 cells, the docetaxel could significantly suppress the AR binding to the MDM2 (Fig. 1h).

Together, results from Fig. 1f-h suggest that the docetaxel can increase AR protein level *via* inhibiting the AR ubiquitination to impact the AR protein stability.

3.4. Docetaxel enhances AR protein stability via enhancing AR phosphorylation in CRPC cells

As previous studies indicated that the post-translational modification of AR protein, especially the AR phosphorylation, could influence

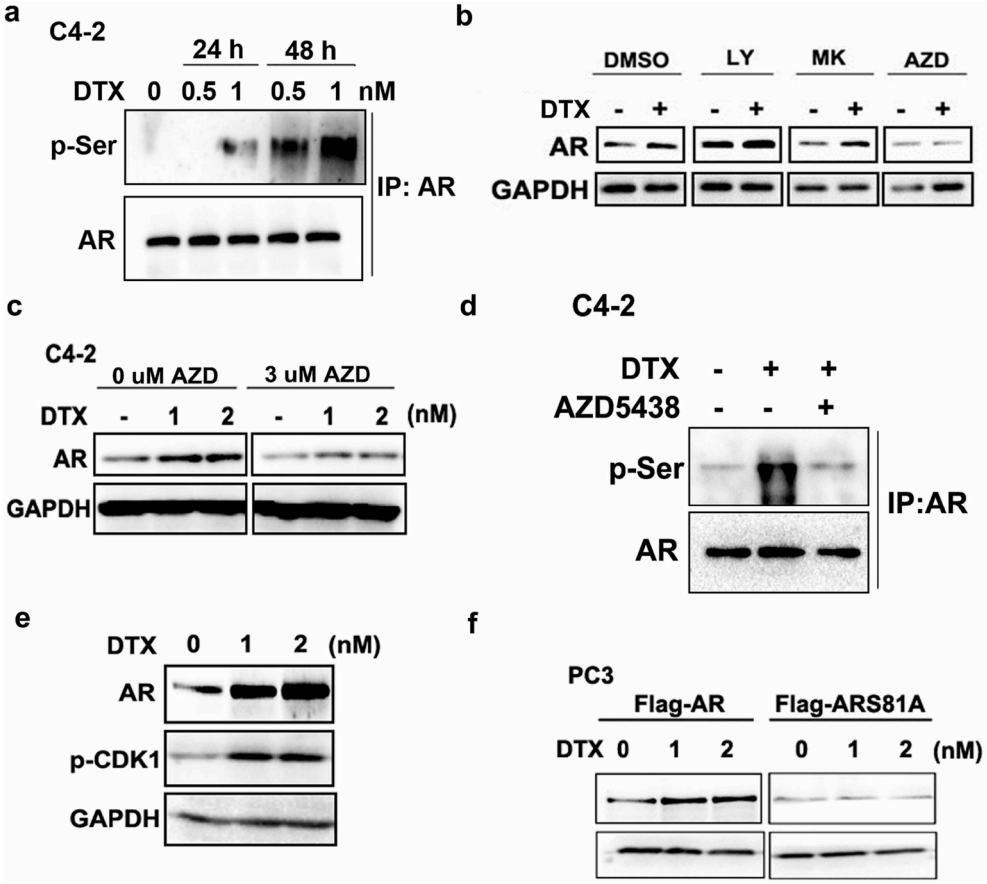

Fig. 2. AR phosphorylation was enhanced by Docetaxel treatment.
a The C4-2 cells were treated with docetaxel (DTX) for 24 and 48 h, the AR proteins were pulled-down, and then serine phosphorylations of AR were analyzed by the western blot. **b** The C4-2 cells were treated with DTX and different kinase inhibitors, (P13F inhibitor Ly29004 [LY]; AKT inhibitor MK2206 [MK]; and CDKs inhibitor AZD5438 [AZD]) and the AR levels were analyzed by western blot. **c** The C4-2 cells were treated with DTX and with/without 3 μM AZD5438, and AR protein level was analyzed. **d** C4-2 cells were treated with 1 nM and 2 nM DTX, and then the T161 phosphorylation of CDK1 was detected by western blot. **e** The C4-2 cells were treated with DTX and AZD5438, and then the AR serine phosphorylation was analyzed by western blot. **f** PC3 cells were infected by lentivirus to overexpress Flag-AR and Flag-ARS81A. The PC3-Flag-AR/Flag-ARS81A cells were treated with different doses of DTX, and then the AR level was detected by western blot.

protein stability [46], we were interested to see if docetaxel could increase AR protein stability *via* altering the AR phosphorylation.

We first demonstrated that docetaxel treatment could increase the serine phosphorylation of AR significantly (Fig. 2a), suggesting that the docetaxel may increase AR protein level by up-regulating the AR phosphorylation. Importantly, we found adding CDK1 inhibitor AZD5438 [47], could also abolish the docetaxel increased AR protein level in C4-2 cells (Fig. 2b–c) and docetaxel increased AR phosphorylation in C4-2 cells (Fig. 2d), suggesting that CDK1 is the key kinase to phosphorylate and stabilize the AR protein upon the docetaxel treatments. Furthermore, the analysis of CDK1 activity also revealed that docetaxel treatment could strongly enhance CDK1 activity (Fig. 2e).

Finally, as the amino acid residue 81 (serine, S81) of AR can be phosphorylated by CDK1, CDK5 and CDK9 [46], we also constructed the Flag-ARS81A (serine-alanine) mutation, and found docetaxel had little ability to increase the expression of Flag-ARS81A, however it can still increase the expression of Flag-AR (Fig. 2f), suggesting that docetaxel increased AR protein level through the AR S81 phosphorylation.

Together, these results suggest docetaxel can function *via* enhancing AR phosphorylation to impact the AR protein level (Fig. 2a–f) in the CRPC cells.

3.5. Targeting AR alters the docetaxel sensitivity to suppress the CRPC cells

To further examine if altered AR protein level plays essential roles to impact the docetaxel sensitivity, we then directly targeted the AR using AR-shRNA in C4-2 cells under different doses (from 0 to 8 nM) of docetaxel. The results from MTT assay and cell counting revealed that targeting the AR with AR-shRNA resulted in an increase of docetaxel sensitivity (Fig. 3a & SFig. 1c).

We then applied a different approach *via* adding AR in PC3 cells, which are AR negative. As shown in Fig. 3b & SFig. 1d, we found the PC3 cells with added AR (named as PC3-AR9) had decreased docetaxel sensitivity compared to the parental PC3 cells. Since results in Fig. 2f demonstrated that docetaxel failed to increase the ARS81A expression, we also examined its impact on the PC3 cells, and results revealed that transduction of ARS81A in PC3 cells failed to decrease the docetaxel sensitivity (Fig. 3c).

Together, results from Fig. 3a–c using opposite ways to either knock down or add AR in two different PCa cell lines all demonstrated that altering AR could impact the docetaxel sensitivity to suppress the CRPC cell growth.

We then applied the TUNEL apoptotic assay to examine the docetaxel impact on cell apoptosis [48], and results revealed that knocking-down AR dramatically increased C4-2 cell apoptosis induced by docetaxel (Fig. 3d). In contrast, adding functional AR into PC3 cells decreased the docetaxel induced cell apoptosis (Fig. 3e).

Together, results from Fig. 3a–e suggest that targeting AR can alter the docetaxel sensitivity *via* modulating the cell apoptosis.

3.6. Mechanism dissection of how docetaxel-increased AR can alter the docetaxel sensitivity: via increasing p21 expression

To dissect the mechanism(s) of why docetaxel-enhanced AR can alter the docetaxel sensitivity, we focused on those reported AR-modulated genes related to PCa cell growth, especially to those genes that have been linked to the docetaxel resistance [49,50]. Among several potential candidates, we found the expression of p21 is similar to AR and could be also increased after adding docetaxel in a dose dependent manner in C4-2 cells (Fig. 4a). Importantly, knocking-down AR not only can directly suppress p21 expression, it also attenuated the docetaxel induced p21 protein level, suggesting docetaxel may function *via* modulating AR protein level to influence the p21 signals (Fig. 4b). Similar results were obtained when we replaced the C4-2 cells with PC3

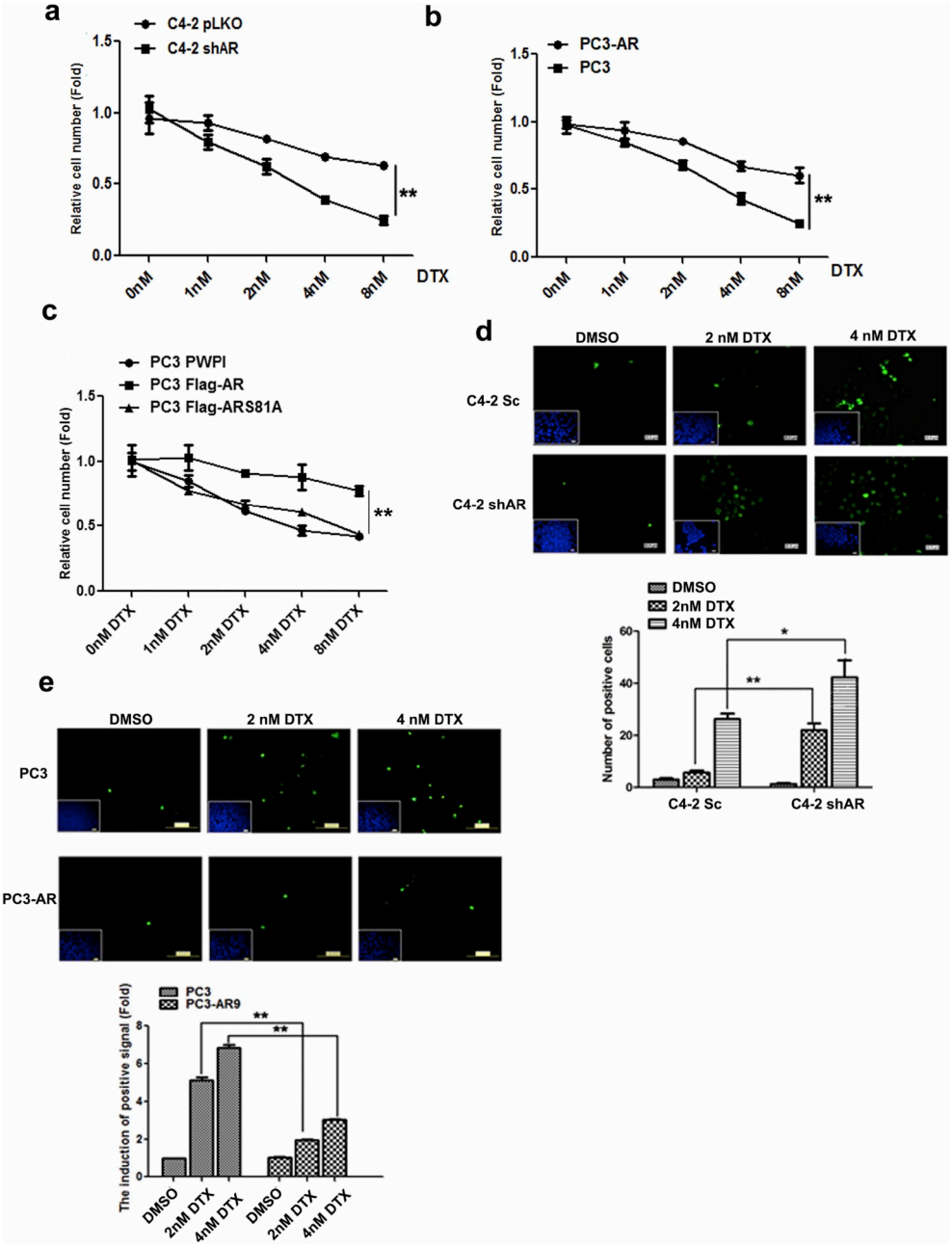

Fig. 3. Targeting AR increases the docetaxel efficiency.
a The MTT assay of docetaxel (DTX) effect on C4-2 scramble (Sc) control (PLKO) and shAR cells growth. 5×10^4 cells were seeded in the plates, and were treated with different doses of DTX. The cells were harvested in 24 h. **b** The MTT assay of DTX effect on PC3 and PC3AR9 cells growth. 5×10^4 cells were seeded in the plates, and were treated with different doses of DTX. The cells were harvested in 24 h. **c** The PC3 cells were infected by viruses to overexpress Flag-AR and Flag-ARS81. The DTX sensitivity of the cells was analyzed by MTT assays. **d** The TUNEL assay of the DTX effect on C4-2 Sc and shAR cells apoptosis. The cells were treated with 2 nM and 4 nM DTX and cell apoptosis was analyzed by the TUNEL kit. **e** The TUNEL assay of the DTX effect on PC3 and PC3AR9 cells apoptosis. The cells were treated with 2 nM and 4 nM DTX, and the cell apoptosis was analyzed by the TUNEL kit. For a-d data are presented as mean ± SD. For **d** and **e**, quantitation are in the lower panels. *p < 0.05.

and PC3AR9 cells (Fig. 4c).

These findings are important and in agreement with early reports showing docetaxel could induce p53 signaling [51] and its downstream targets including the p21 [50]. Therefore the linkage from docetaxel to AR to its downstream target gene p21 further strengthened the key role of the AR in the CRPC during docetaxel treatment even though the remaining AR is no longer sensitive to ADT [52].

3.7. Mechanism dissection of how docetaxel-increased AR can increase p21 expression: via transcriptional regulation

To further dissect the molecular mechanism of how AR increases p21 expression, we first found knockdown of AR could suppress p21 expression at both the protein level (Fig. 4d) and the mRNA level (Fig. 4e) in the C4-2 cells, and docetaxel could increase p21 mRNA level *via* an AR dependent manner (Fig. 4f). Importantly, treating with the CDK inhibitor, AZD, not only attenuated the docetaxel capacity to increase AR protein level (See Fig. 2b), it could also suppress the docetaxel-increased p21 expression (Fig. 4g), which further supports that docetaxel increases p21 *via* up-regulating AR (Fig. 4g).

We then applied the luciferase assay (Fig. 4h) to examine if AR can increase p21 expression *via* transcriptional regulation. We first identified an androgen-response-element (ARE-5-GGTACAnnnTGTTCT-3) located at 200 bases upstream of the transcriptional starting site of p21. We then constructed the luciferase reporter plasmid which contains the 2000 bp region of the p21 promoter, and results revealed that AR was able to transactivate the p21 promoter (Fig. 4i). The chromatin immunoprecipitation (ChIP) assay with AR antibody in control C4-2 cells or C4-2 cells treated with docetaxel also confirmed that AR could bind directly to this ARE and this binding was increased in cells after treating with docetaxel (Fig. 4j). Importantly, in PC3 cells, knocking-down p21 could then reverse the AR inhibiting effect on the docetaxel sensitivity (Fig. 4k).

Since we proved that docetaxel increased AR S81 phosphorylation, we were interested to investigate whether the S81 phosphorylation is important for the increase of p21 expression. We then treated the cells

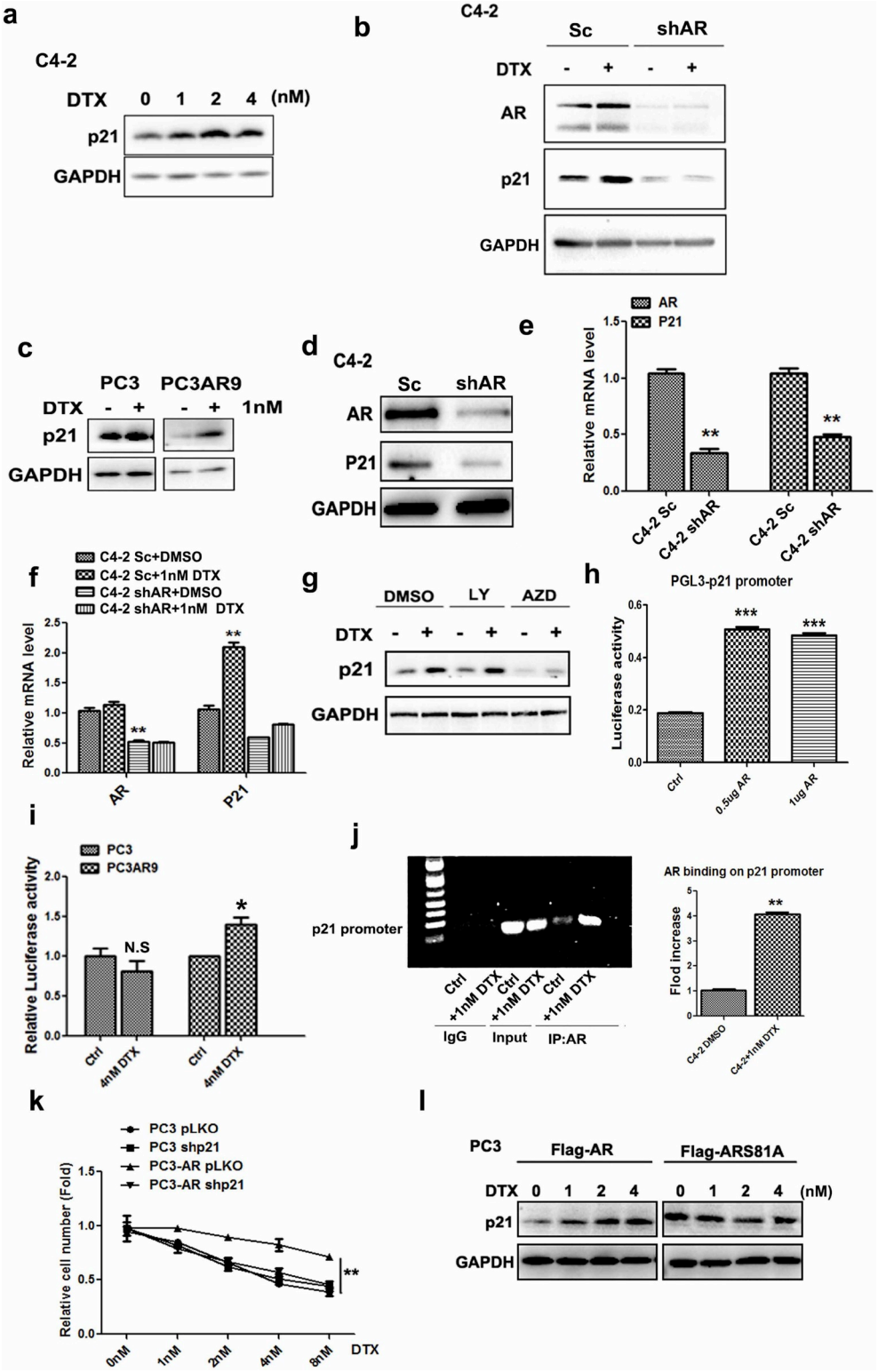

Fig. 4. AR regulates p21 to influence the docetaxel efficiency.

a Western blot analysis of p21 expression in C4-2 cells after 1 nM docetaxel (DTX) treatment for 24 h. **b** The p21 protein level was detected in C4-2 sc or shAR cells after treating with different doses of DTX. **c** Western blot analysis of p21 expression in PC3 and PC3AR9 cells after 1 nM DTX treatment for 24 h. The cells were harvested and protein was extracted for the western blot analysis using p21 specific antibody. **d** Western blot analysis of AR and p21 expression in C4-2 shAR cells using AR and p21 specific antibody. **e** The qPCR analysis of the p21 mRNA level in C4-2 Sc and shAR cells. **f** The qPCR analysis of p21 mRNA level in C4-2 sc and shAR cells after treating with DTX. **g** The p21 protein level was analyzed in C4-2 cells after treating with DTX and different kinase inhibitors. **h** The luciferase assay for the ARE in the p21 promoter. **i** the luciferase assay for the ARE in p21 promoter after DTX treatment in PC3 and PC3AR9 cells. **j** The chip assay to analyze the AR binding to the p21 promoter region after treating C4-2 cells with DTX for 24 h, we used AR antibody to precipitate the AR protein, and then PCR to analyze the AR binding to the p21 promoter region. **k** PC3 pLKO/shp21 and PC3AR9 (PC3-AR) pLKO/shp21 cells were treated with different doses of DTX and cell viability was analyzed by MTT assay. **l** PC3-Flag-AR and PC3-Flag-ARS81A cells were treated by docetaxel and p21 expression was analyzed by WB. For **e**, **f**, **h**, **i**, **j**, and **k**, data are presented as mean ± SD * p < 0.05, **$p < 0.005$, ***$p < 0.001$, N.S = Not significant.

with docetaxel after overexpressing Flag-AR and Flag-ARS81A in the PC3 cells. The results from western blot analysis revealed that adding docetaxel can only increase the p21 expression in PC3-Flag-AR cells, but not in PC3-Flag-ARS81A cells (Fig. 4l), suggesting that the increasing AR phosphorylation after docetaxel treatment is the key process to promote the p21 expression.

Taken together, mechanism dissection results from Fig. 4d–l demonstrate that AR can directly bind to the ARE located on the p21 promoter to increase p21 expression at the transcriptional level to alter the docetaxel sensitivity in CRPC cells.

3.8. Targeting AR to re-sensitize the docetaxel resistant cells

All above data from Figs. 1–4 conclude that docetaxel can function *via* altering the AR/p21 signals to impact the docetaxel sensitivity. Next, we were interested to see if targeting this newly identified AR/

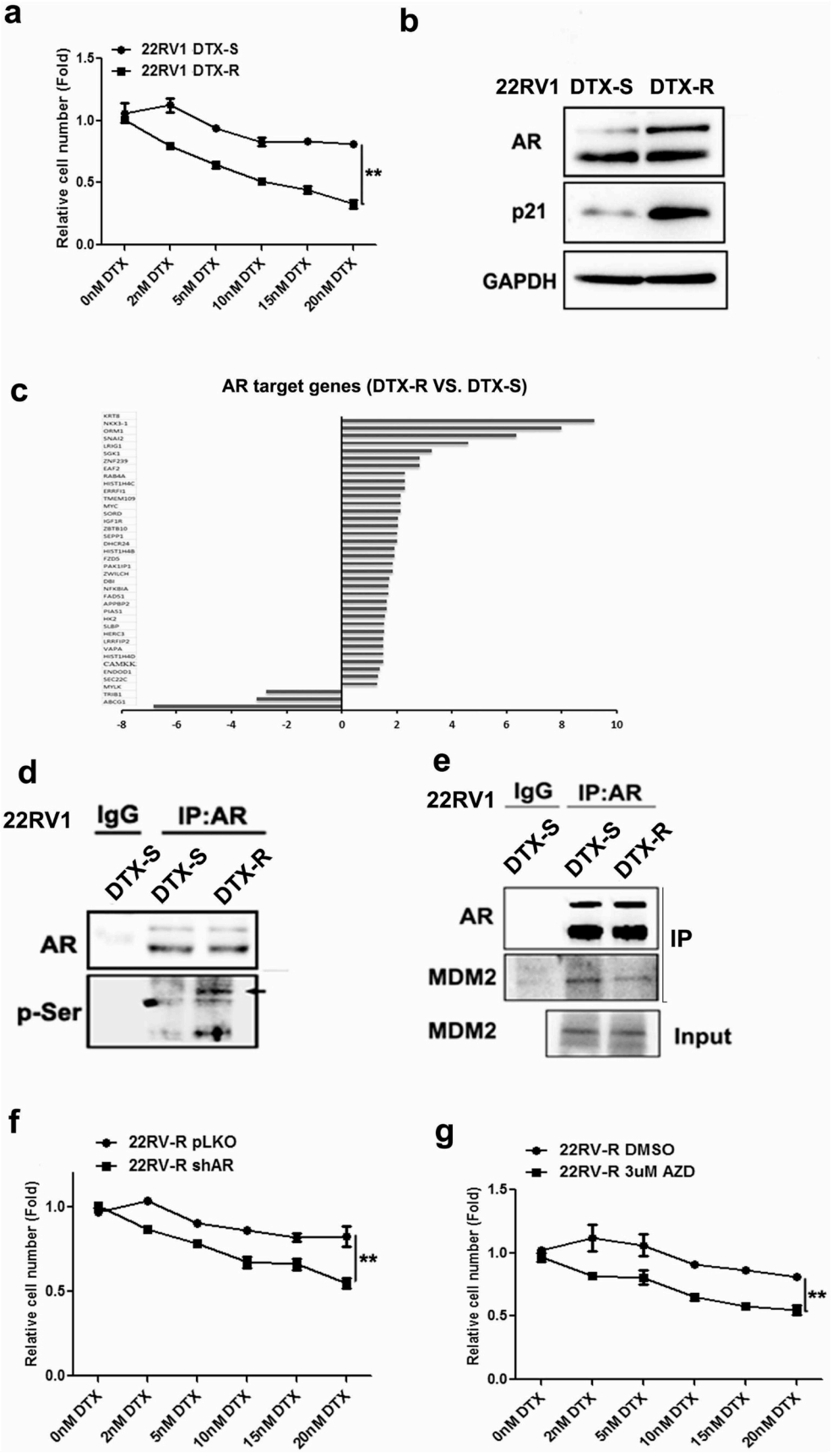

Fig. 5. Docetaxel resistant cells have higher AR protein level.

a The MTT assay of docetaxel (DTX) resistant CWR22RV1 (DTX-R-22RV1) cells and parental 22rv1 cells treated with different doses of DTX. **b** The AR and p21 expression in DTX-R-22RV1 cells and DTX sensitive CWR22RV1 (DTX-S-22RV1) parental cells. **c** AR downstream genes expression in DTX-R-22RV1 and DTX-S-22RV1 cells. **d** The AR serine phosphorylation level in DTX-R-22RV1 cells and DTX-S-22RV1 cells. **e** The interaction between AR and MDM2 in DTX-R-22RV1 cells and DTX-S-22RV1 cells. **f** The MTT assay of 22RV1 DTX-R sc or shAR cells after treating with different doses of DTX. **g** The MTT assay of DTX-R-22RV1 cells after treating with DTX and AZD5438. For **a**, **f**, and **h**, data are presented as mean ± SD, $^{**}p < 0.005$. ($^{**}p < 0.005$).

p21 axis can improve/restore the docetaxel sensitivity in the CRPC cells that already developed docetaxel resistance. We first generated the docetaxel resistant CWR22RV1 cells (named as DTX-R-22RV1), and applied the MTT assay to confirm the DTX-R-22RV1 cells indeed are resistant to docetaxel treatment (Fig. 5a). As expected, we found that DTX-R-22RV1 cells had higher expression of AR and p21 compared to the parental docetaxel sensitive CWR22RV1 (DTX-S-22RV1) cells (Fig. 5b). Results from RNA sequencing to analyze the transcriptomes in DTX-R-22RV1 and DTX-S-22RV1 cells also revealed that several AR target genes were increased in the DTX-R-22RV1 cells (Fig. 5c), and much higher AR phosphorylation level was detected in DTX-R-22RV1 compared to the DTX-S-22RV1 cells (Fig. 5d). Importantly, the interaction of AR and MDM2 was reduced in DTX-R-22RV1 cells than the original parental cells (Fig. 5e).

Together, results from Fig. 5a–e suggest that the higher AR/p21 signaling was found in the docetaxel resistant cells.

To explore if targeting AR can restore the docetaxel sensitivity in DTX-R-22RV1 cells, we then knocked down AR in the AR-shRNA in the DTX-R-22RV1 cells and examined the docetaxel efficacy. The results from MTT assay revealed that the knocking-down AR could re-sensitize the DTX-R-22RV1 cells to docetaxel treatment (Fig. 5f), and adding CDK inhibitor could also re-sensitize the DTX-R-22RV1 cells to docetaxel treatment (Fig. 5g).

Taken together, results from Fig. 5f–g suggest that targeting AR could restore the docetaxel sensitivity in the docetaxel resistant cells.

3.9. Preclinical studies with targeting AR with ASC-J9® to better suppress docetaxel resistant CRPC cells

All above data demonstrate targeting AR can increase docetaxel sensitivity during docetaxel chemotherapy and also re-sensitize the docetaxel resistant cells. To link these *in vitro* results to the future clinical application, we then applied the preclinical studies by targeting AR using the AR degradation enhancer ASC-J9® [33,34,40,53–55], but not AR-shRNA due to the *in vivo* delivery difficulty for the AR-shRNA molecule in C4-2 cells. We first proved that the current available antiandrogen Casodex failed to enhance the efficacy of docetaxel in C4-2 cells in the presence of 1 nM DHT, a condition that mimics CRPC under ADT [33]. In contrast, treatment with both the more powerful antiandrogens enzalutamide and ASC-J9® resulted in significant enhancement of cytotoxicity of docetaxel, and ASC-J9® showed even better effect than enzalutamide (Fig. 6a). Importantly, increased cytotoxicity of docetaxel is accompanied with ASC-J9® ability to target the AR that results in decreased AR protein level (as well as its target gene p21) in C4-2 cells (Fig. 6b). Further titration of ASC-J9® also confirmed its ability to suppress p21 expression in a dose dependent manner in C4-2 cells (Fig. 6c), and suppressed AR-p21 expression by ASC-J9® led to re-sensitize the DTX-R-22RVq cells (Fig. 6d).

Taken together, results from Fig. 6a–d confirm the above *in vitro* findings from Figs. 1–5 showing ASC-J9® can function through targeting AR to suppress AR and p21 to increase docetaxel chemotherapy efficacy and also restore the docetaxel sensitivity in the docetaxel resistant cells.

4. Discussion

After the patients under-going ADT developed antiandrogen resistance, chemotherapy is one of the next therapies to further treat the CRPC patients [56]. Since chemotherapy is widely used when when the CRPC cells already metastasized to the distant organs, many patients may eventually develop the chemotherapy resistance even though the initial response to the chemotherapy can be very positive. How to improve the chemotherapy efficacy and prevent the CRPC cells from developing the chemotherapy resistance becomes an urgent issue in the later CRPC stage.

In most cases, docetaxel is the first choice for PCa chemotherapy. Docetaxel can block the de-polymerization of the tubulin and disrupt the cytoskeleton, which may then lead to cellular apoptosis [57]. However, most CRPC patients who received docetaxel therapy may develop the docetaxel resistance and tumors recur/progress. Several mechanisms have been applied to explain the development of docetaxel resistance. For example, alterations of the cancer stem cells [58], the drug efflux genes [59], the tumor microenvironment [60], and the anti-apoptotic mechanism [61]. But the aberrant AR is still the key factor behind the drug resistance [62]. In a recent report, the AR splice variant, ARv7, has been identified as a critical factor to confer the antiandrogen enzalutamide resistance [63]. The AR mutant, ARF876L, may also contribute to the enzalutamide resistance [64]. Furthermore, the AR amplification may represent another key mechanism to increase the PCa cells resistance to the ADT [65]. The direct linkage of AR to the docetaxel resistance, however, remains unclear.

Here we demonstrated that docetaxel might have the adverse effect of increasing AR/p21 signaling to increase the docetaxel resistance, and mechanistic studies revealed that docetaxel treatment can enhance the AR protein level *via* increasing AR phosphorylation. This is the first report that links the docetaxel resistance to the AR protein level. Importantly, targeting the AR with AR-shRNA or ASC-J9® can re-sensitize the docetaxel resistant cells to further suppress the CRPC cells that already developed docetaxel resistance.

Our results indicated that CDKs are the key regulators to increase the AR protein level and drive the docetaxel resistance. There are three CDKs that can phosphorylate AR at the serine 81 site, including CDK1, CDK5 and CDK9 [47,66,67], and consequence of AR phosphorylation by CDKs may then increase the protein stability to further promote the PCa cell proliferation. The early report demonstrated that the CDK1 can be activated by docetaxel treatment and block the apoptosis in PCa [26]. According to our data, such blockage is due to the activation of the AR signaling and some anti-apoptotic genes expression increase, including the p21. Indeed when we treated the docetaxel resistant cells with the CDK inhibitor AZD5438 we found the cells can then regain the partial sensitivity to docetaxel. This is the direct evidence that CDKs may contribute to the docetaxel resistance.

Interesting, Recent studies reported that docetaxel treatment could inhibit the AR nuclear translocation due to the disruption of the cytoskeleton [68,69]. However, we failed to detect the docetaxel suppressed AR translocation (data not shown), and several possibilities may be able to explain this. In our system, we always performed the studies in the castration androgen levels (near 1 nM DHT), because the docetaxel is usually applied after ADT therapy. And during ADT conditions, the AR translocation is already impaired at the castration androgen level. Another possible reason is the different concentrations of docetaxel used in different systems. The therapeutic dose of docetaxel in patients is 100 mg/m^2, however, the pharmacokinetic study revealed that after 25 h of implantation, the concentration in the patient's serum is lower than 10 nM [8]. Because the implantation of docetaxel is once every 3 weeks, at the time of implantation the CRPC cells are subjected to the high dose of docetaxel, which diminishes to the low dose of docetaxel until the next implantation. This condition may help the PCa to develop the mechanism against the cytotoxicity of docetaxel. Under the low dose of docetaxel, AR will be activated and drive the anti-apoptotic pathway in the PCa.

While our results demonstrated that combining anti-AR therapy with docetaxel therapy can increase the docetaxel sensitivity *via* suppressing the docetaxel adverse effect of increasing the AR/p21 signaling, it is notable that targeting AR with antiandrogens or AR-shRNAs may also raise some adverse effects. For example, accumulating studies indicated that ADT with antiandrogen could also increase the PCa neuroendocrine differentiation, which can cause the poor prognosis and resistant to ADT therapy [70]. ADT with antiandrogen could also increase the PCa cell invasion [34,40] as well as increase the cancer stem cell population [71]. Interestingly, replacing the antiandrogens with ASC-J9®, the AR degradation enhancer, may lead to suppress CRPC cell

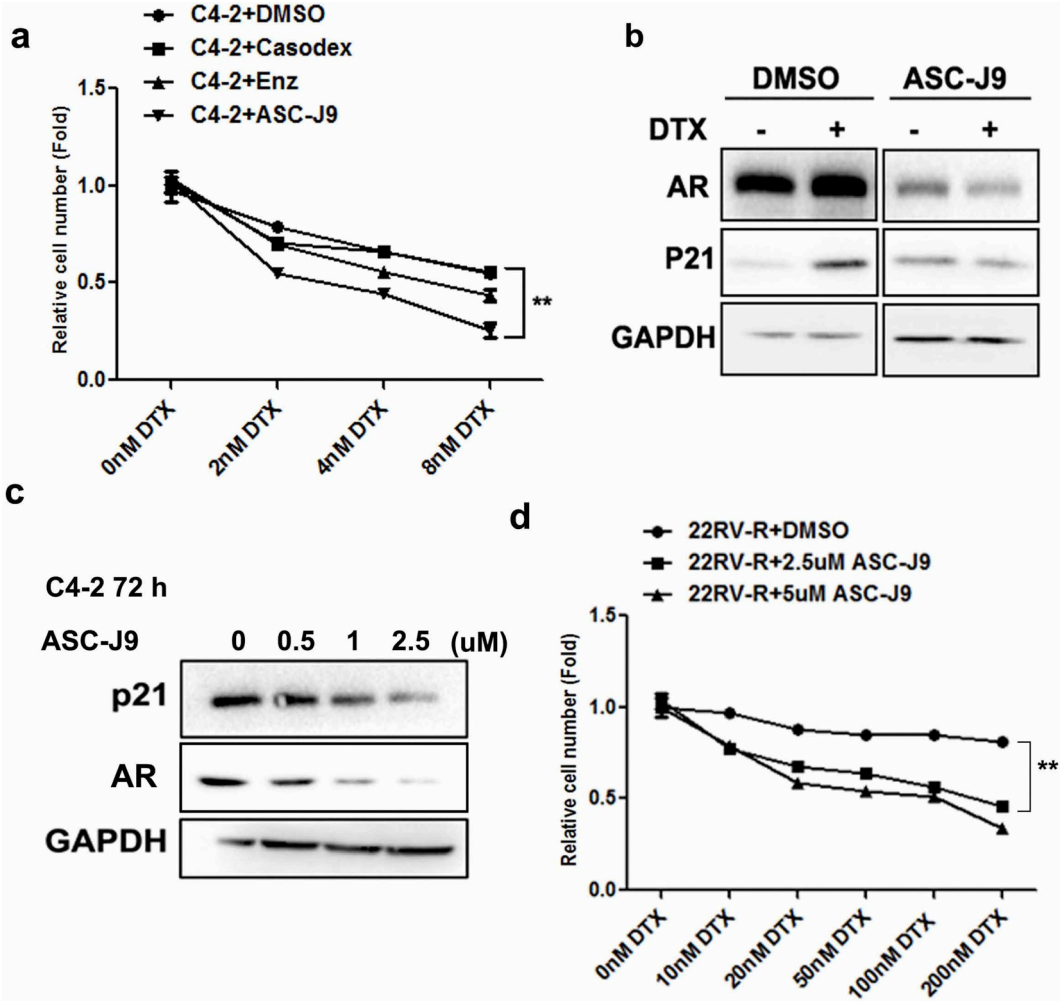

Fig. 6. ASC-J9® improves the docetaxel effect.
a The MTT assay of the C4-2 cells after treating with the different antiandrogens and docetaxel (DTX). The C4-2 cells weretreated with 10 mM casodex, enzalutamide (Enz) and 5 mM ASC-J9® plus different doses of DTX for 24 h. **b** The western blot analysis of AR and p21 after treating C4-2 cells with DMSO or ASC-J9® plus DTX. **c** The western blot analysis of AR and p21 after treating C4-2 cells with ASC-J9®. **d** The MTT assay of DTX-R-22RV1 cells treated with DTX and different doses of ASC-J9®. For **a** and **d**, data are presented as mean ± SD, $^{**}p < 0.005$.

growth with little of these adverse effects [34,53,72,73]. Thus using ASC-J9® with docetaxel here shall allow not only the increase of the docetaxel sensitivity, it may also induce little adverse effects to further promote the CRPC progression.

Conflicts of interest

ASC-J9® was patented by the University of Rochester, the University of North Carolina, and AndroScience Corp., and then licensed to AndroScience Corp. Both the University of Rochester and C. Chang own royalties and equity in AndroScience Corp.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.09.025.

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