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INVITED REVIEW

Prostate Cancer

Current opinion and mechanistic interpretation of combination therapy for castration-resistant prostate cancer

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Recent advances in genomics technology have led to the massive discovery of new drug targets for prostate cancer; however, none of the currently available therapeutics is curative. One of the greatest challenges is drug resistance. Combinations of therapies with distinct mechanisms of action represent a promising strategy that has received renewed attention in recent years. Combination therapies exert cancer killing functions through either concomitant targeting of multiple pro-cancer factors or more effective inhibition of a single pathway. Theoretically, the combination therapy can improve efficacy and efficiency compared with monotherapy. Although increasing numbers of drug combinations are currently being tested in clinical trials, the mechanisms by which these combinations can overcome drug resistance have yet to be fully understood. The purpose of this review is to summarize recent work on therapeutic combinations in the treatment of castration-resistant prostate cancer and discuss emerging mechanisms underlying drug resistance. In addition, we provide an overview of the current preclinical mechanistic studies on potential therapeutic combinations to overcome drug resistance.

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INTRODUCTION

One of the main obstacles to the treatment of advanced prostate cancer is drug resistance. Most patients with early-stage metastatic prostate cancer benefit significantly from androgen deprivation therapy (ADT). However, resistance to ADT develops rapidly. In recent years, the new-generation androgen pathway targeting drugs, including nonsteroidal antiandrogen (enzalutamide) and CYP17A1 inhibitor (abiraterone), have been approved by the U. S. Food and Drug Administration (FDA) for the treatment of castration-resistant prostate cancer (CRPC). Unfortunately, the development of resistance to these new treatments is inevitable. The resistance mechanisms in prostate cancer include androgen receptor (AR) mutations, AR amplification, AR splice variants, AR bypass pathways, and androgen pathway independent alternative pathways.[1] Other general resistant mechanisms include drug efflux alterations and cell death inhibition. Either single or multiple mechanisms can be involved in drug resistance. It is difficult to achieve the complete inhibition of survival pathways with monotherapy since other alternative pathways may be activated in response to treatment and continue to contribute to tumor progression. Thus, there is a theoretical advantage inherent to the use of combination therapy to enhance efficacy through more complete inhibition of a major pro-survival pathway or suppression of multiple pro-survival factors.

Combination therapy has proven to be very effective for the treatment of advanced cancers. The combination of the FDA-approved human epidermal growth factor receptor-2 (HER-2) antibody trastuzumab with chemotherapy has shown significant survival benefits compared to either therapy alone in HER-2-positive breast cancer.[2] Given that androgen signaling is a major driver of prostate cancer development and progression, prostate cancer treatment is predominantly focused on targeting the androgen signaling pathway. Not much effort has been devoted to test therapies inhibiting multi-targets in the treatment of prostate cancer in the past decades. In recent years, there has been an increasing interest in the development of optimal sequential and/or combination treatments using two or more drugs to overcome drug resistance in prostate cancer. We searched the clinicaltrials.gov website and found that nearly a quarter (67 out of 293) of phase 1–4 clinical trials investigating prostate cancer treatment (active, not recruiting, or completed studies with results by September 15, 2018) involve multiple drug combinations. The combination of anti-cancer drugs with distinct mechanisms of action can enhance drug efficacy and efficiency compared with monotherapy. Synthetic lethality caused by combinatorial therapies may also avoid the development of drug cross-resistance. In addition, combination therapy has the potential to lower the dose of each drug required for treatment. Therefore, it may reduce the cytotoxicity and adverse effects caused by the high doses used in monotherapy. The pioneers of clinical studies, such as the Chemo-Hormonal Therapy Versus Androgen Ablation Randomized Trial for Extensive Disease in Prostate Cancer (CHAARTED) and Systemic Therapy in Advancing or Metastatic Prostate Cancer (STAMPEDE), have demonstrated that the

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overall survival of patients treated with multi-therapy combinations is significantly better than those treated with ADT alone.[3–5] Despite the early clinical success of multi-target therapy, the molecular mechanisms underlying more effective combination therapies have yet to be fully understood. The results of combination therapy may not merely reflect the additive effects of the two drugs. The outcome of drug combination could be determined by the cross-talk of signaling pathways that are activated in response to these drugs. Due to space constraints, we will focus on reviewing the preclinical mechanistic studies of some promising combination therapies.

CONCOMITANT TARGETING OF THE AR PATHWAY

ADT has been used as the first-line treatment for metastatic prostate cancer for decades. ADT can reduce prostate cancer growth by either lowering androgen levels or directly targeting AR. Unfortunately, most patients inevitably develop resistance to ADT and progress to CRPC, which is significantly more difficult to cure. During the transition to CRPC, tumor cells change from the androgen-dependent to the androgen-independent state. The new generation of anti-androgen drugs, abiraterone, and enzalutamide, was approved for the treatment of CRPC by the FDA in 2011 and 2012, respectively. Both drugs increase the median survival time for more than 3 months compared with placebo.[6–8] Despite the early success of abiraterone and enzalutamide, clinicians have already noticed the limitations of these drugs. Approximately 20% of patients have no response to the treatment. Most patients who initially respond to the treatment rapidly develop resistance in <2 years.[6–10] Therefore, it is essential to investigate the mechanism by which resistance is acquired. The mechanisms of anti-androgen drug resistance can be categorized into two groups as follows: (1) restoration of AR signaling via upregulated steroidogenesis, AR mutation/amplification, ligand-independent activation of AR, AR crosstalk with other signaling pathways, and constitutively activated AR variants; and (2) activation of bypass pathways independent of AR signaling.

Enhanced inhibition of AR

AR is the most important drug target in prostate cancer treatment. Insufficient inhibition of AR and AR pathway reactivation are major causes of anti-androgen therapy resistance. AR amplification and mutation represent key molecular mechanisms of anti-androgen drug resistance. Somatic mutation of AR can be inherited or acquired in response to anti-AR therapies. Recent studies using next-generation sequencing technology have demonstrated that a mutation in C-terminal ligand binding domain of AR can cause an agonist switch, which results in anti-androgen therapy resistance.[11,12] The *AR* F876L mutation, which confers resistance to enzalutamide, was observed in circulating tumor DNA from anti-androgen therapy-resistant patients.[11] Recent evidence suggests that the combination of anti-androgen therapies could have additive effects on androgen signaling. STAMPEDE is a randomized controlled multi-group and multi-stage study on the effects of the combination of multiple anti-AR therapies. A recently published STAMPEDE report demonstrated an obvious survival benefit of the combination of abiraterone and ADT. In a total of 1917 patients not previously treated with hormone therapy, the 3-year survival was 83% in the combination group compared with 76% in the ADT alone group (hazard ratio [HR]: 0.63, 95% confidence interval [CI]: 0.52–0.76, $P < 0.001$).[8] This encouraging finding warrants further studies of additional anti-androgen drugs in combination. However, not all anti-androgen combination studies have shown a positive outcome. Preliminary data from the PLATO trial (NCT01995513) on the combination of abiraterone and enzalutamide did not show a benefit on progression-free survival of CRPC.[13] Long-term observations are needed for a more objective evaluation.

In addition to conventional AR-targeted therapy, lipid nanoparticle-delivered antisense therapy represents a promising therapeutic direction. Antisense therapy is activated through binding to a specific RNA sequence and inhibiting the function of RNA. Lee *et al*.[14] demonstrated that a small-interfering RNA (siRNA) targeting AR delivered through lipid nanoparticles was able to silence AR expression and decrease serum prostate-specific antigen (PSA) levels in xenograft tumors. Further analysis suggested that AR-targeted siRNA could induce AR mRNA cleavage within the sequence recognized by AR siRNA. Antisense therapy is essentially used to inhibit targets that are undruggable or resistant to conventional therapy. AR reactivation causes anti-androgen therapy resistance, and thus, AR-targeted antisense therapy can potentially be applied in anti-androgen resistant prostate cancer. Constrained-ethyl modified antisense oligonucleotide (Gene 2.5) is a newer generation antisense oligonucleotide with an improved tissue half-life *in vivo*. A study using Gene 2.5 oligonucleotide against both full-length AR and AR-V7 (a.k.a. AR3) demonstrated that this oligonucleotide decreased both AR and AR-V7 mRNA and inhibited the growth of the LNCaP-derived enzalutamide-resistant prostate cancer cell line and patient-derived CRPC xenografts.[15] The oligonucleotide delivery efficiency is a critical factor limiting the usage of this therapy. A recent study has shown that the efficiency of oligonucleotide delivery in the absence of carriers can be improved by a small molecule, 6-bromo-indirubin-3'-oxime (6BIO). Five-fold less AR-target antisense oligonucleotide was required to achieve 50% reduction of AR protein expression in the presence of 6BIO compared with AR-target antisense oligonucleotide alone.[16] The augmenting effect of 6BIO may be caused by its glycogen synthase kinase-3 (GSK-3)α/β inhibition activity. This finding supported by the data showed that the addition of the GSK-3α/β inhibitor CHIR99021 could increase oligonucleotide activity. Till date, the clinical efficiency of antisense therapy as monotherapy is still debated. Recently, investigators have examined the effects of the combined utilization of targeted drugs and antisense therapy. Combining conventional therapies with antisense therapy potentially exerts a higher degree of growth inhibition and lower toxicity. A recent study has shown that the combination of antisense oligonucleotides targeting histone lysine methyltransferase enhancer of zeste homolog 2 (EZH2) with anti-androgen pathway drugs has a more profound effect on the inhibition of prostate cancer growth *in vitro* and *in vivo* compared with the single agent.[17] In this report, the authors also described an altered AR cistrome and enhanced AR dependence following inhibition of EZH2 in CRPC, suggesting a potentially increased efficiency of anti-androgen therapy in the presence of EZH2 inhibitor.[17] The promising results from these preclinical studies provide a rationale to further investigate the clinical effects of the combination of antisense and targeted therapies.

Targeting AR and AR variants

The deregulation of AR splicing variants is another important mechanism of anti-androgen therapy resistance. AR variants lacking the C-terminal ligand binding domain exhibit nuclear localization and are constitutively active in the absence of androgens.[18–20] AR-V7 is one of the most well-studied AR variants in preclinical and clinical studies. AR-V7 expression in circulating tumor cells is strongly associated with resistance to enzalutamide and abiraterone therapies in prostate cancer patients.[10] The clinical findings are supported by evidence showing that knockdown of AR-V7 expression in an androgen-independent prostate

cancer cell line restores the response to anti-androgen therapy.[21] Given that currently available anti-androgen drugs mainly target the C-terminal ligand binding domain of AR and are expected to be ineffective for inhibiting AR variants, how to block the activity of AR variants has become an attractive topic in recent years. One possible strategy is to develop inhibitors targeting conserved regions of both AR and AR variants. The N-terminal activation function-1 (AF-1) domain of AR is essential for both full-length AR and AR variants. Thus, the N-terminal domain of AR is a promising drug target to overcome AR variant-induced anti-androgen drug resistance. EPI belongs to a set of small molecules that target the N-terminal domain of AR. EPI-001 was found to bind to AR and block the protein-protein interaction of AR and coactivators, in turn inhibiting the transcriptional activity of both AR and AR variants.[22,23] However, the excessive high pill burden caused early termination of phase I/II trials of EPI-506 in CRPC with progression after enzalutamide or abiraterone (NCT02606123). Notably, the excessive adverse effects may be solved by the addition of a secondary anti-androgen therapy. Theoretically, the dose of each drug can be lowered in combination to achieve a similar efficacy. The AR N-terminal targeting antagonist sintokamide A (SINT-1) is shown to block the protein-protein interactions with AR functional partners and inhibit the activity of both AR and AR variants.[24] Although SINT-1 and EPI compound both targets the N-terminal part of AR, these drugs may exert anti-androgen functions via different mechanisms. EPI was observed to inhibit AR expression at both mRNA and protein levels, and it was able to block interleukin 6 (IL-6)-induced transactivation of AR. In contrast, SINT-1 did not affect AR expression and was able to significantly inhibit forskolin-induced AR transactivation.[24] Importantly, this report demonstrated an additive inhibitory effect on PSA luciferase activity in cells treated with the combination of EPI and SINT-1 compared with either drug alone.

The DNA-binding domain (DBD) is another conserved region in both AR and AR variants. Researchers have identified a surfaced exposed pocket on DBD, which can serve as a potential target site. DBD targeting compounds VPC-14228/14449 have been shown to diminish the interaction of AR with androgen response element; therefore, VPC-14228/14449 can effectively block the transcriptional activity of AR and AR variants at a low sub-micromolar concentration.[25] Unlike enzalutamide, VPC-14228/14449 does not affect the nuclear localization of AR and AR variants, making it a promising agent for targeting drug-resistant nuclear-localized AR and AR variants. The proteasome-dependent protein degradation pathway has also been utilized to decrease the AR and AR variant level. The AR degradation enhancer 5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one (ASC-J9) and niclosamide have been shown selectively degrade AR variants, resulting in decreased AR variant transcriptional activity.[26–28] ASC-J9 disrupts the interaction between AR and the AR coregulators ARA55 and ARA70 and enhances the interaction between AR and phosphorylated murine double minute protein 2 (Mdm2).[26] This AR-Mdm2 interaction causes AR ubiquitination and increases the susceptibility of AR to degradation. The combination of niclosamide and enzalutamide demonstrates a strong growth inhibitory effect on enzalutamide-resistant cells both *in vitro* and *in vivo*.[27] The expression of the AR variant is regulated by both gene transcription and splicing factor recruitment to pre-mRNA.[29] Recently, researchers have discovered that the splicing factors heterogeneous nuclear ribonucleoprotein (hnRNP), alternative splicing factor/splicing factor 2 (ASF/SF2), and the 65 kDa subunit of U2 small nuclear ribonucleoprotein particles auxiliary factor (U2AF65) are important for AR-V7 splicing.[29] A natural compound, quercetin, has been shown to bind to and retain hnRNPA1 in the cytoplasm, thus decreasing the AR-V7 expression level.[30] Specifically, the down-regulation of AR-V7 production may result in higher AR dependency and potentially increase the AR-targeted enzalutamide response. A recent study has shown that the combination of quercetin and enzalutamide synergistically reduces AR and AR-V7 expression in enzalutamide-resistant C4-2B-Enza-R and 22Rv1-Enza-R cell lines.[30]

Blockade of androgen signaling via an alternative route

AR activity can be regulated by other pro-survival factors and pathways. Inhibition of the transcriptional activity of AR and AR variants can also be achieved through different routes other than direct targeting of AR. Lipid kinase phosphatidylinositol-4-phosphate 5-kinase type 1 alpha (PIP5K1α) is an essential coactivator for both AR and AR variants. According to a recent report, AR-V7, PIP5K1α, and cyclin-dependent kinase 1 (CDK1) physically form a complex and cooperatively promote tumor progression.[31] This report demonstrated that the PIP5K1α inhibitor ISA-2011B disrupts AR and AR-V7 protein stability and suppresses tumor growth and invasiveness in an AR-V7 overexpression tumor xenograft model. The same study also showed that the combination of ISA-2011B and enzalutamide diminishes AR and AR-V7 protein levels in both the nucleus and cytoplasm in androgen-independent 22Rv1 cells more efficiently than either drug alone. Signal transducer and activator of transcription 3 (STAT3) activation has been shown to enhance the recruitment of AR to androgen response elements and increase the androgen-independent activity of AR.[32] IL-6-induced activation of STAT3 promotes androgen-independent growth and is one of the potential enzalutamide resistance mechanisms. Simultaneous targeting of AR and AR coactivator by enzalutamide and STAT3 inhibitor AG490 can reverse IL-6-induced enzalutamide resistance in an LNCaP-s17 IL-6-overexpressing cell model.[32]

It is well-known that AR signaling is closely associated with epigenetic factors, including DNA methyltransferases, histone modulators, microRNA, nonlong-coding RNA, and other epigenetic cofactors.[33,34] Bromodomain proteins (*e.g.*, the bromodomain and extra-terminal [BET] family) can bind acetylated lysine residues in histone and associate with AR on chromatin, resulting in increased expression of AR-regulated pro-survival factors. It has been reported that elevated AR activity increases BET protein expression-enhanced BET-mediated chromatin opening in advanced prostate cancer.[35] Inhibition of the BET family protein bromodomain containing 4 (BRD4) by the small molecule inhibitor JQ1 decreases AR and AR variant expression and disrupts the recruitment of AR to the target genes in chromatin.[36,37] A recent study has shown that a BET inhibitor could restore drug sensitivity in enzalutamide-resistant cells. In that study, the combination of enzalutamide and JQ1 blocked AR signaling and exhibited enhanced tumor growth inhibition in enzalutamide-resistant LNCaP-AR and VCaP cells.[38] These findings are corroborated by the observation that the combination of enzalutamide and JQ1 is more effective in AR-overexpressed LNCaP cells than in parental LNCaP cells.[35] Interestingly, a very recent study on BET inhibitor resistance has demonstrated that AR signaling is reactivated via CDK9-mediated phosphorylation in BET inhibitor-resistant cells.[39] BET inhibitor-resistant cells exhibit enhanced sensitivity to enzalutamide treatment. These studies suggest that combining anti-androgen therapy and BET targeting treatment may potentially avoid the development of resistance to either therapy.

TAXANE-BASED COMBINATION THERAPY

Taxanes are a class of mitotic inhibitors that function through stabilizing GDP-bound tubulin in the microtubule. The taxane compounds docetaxel and cabazitaxel are FDA-approved chemotherapies for

CRPC. Docetaxel is widely used in various types of cancer, including prostate cancer. More recent evidence suggests that docetaxel can reduce prostate cancer progression through downregulating AR transcriptional activity in addition to its mitotic inhibitory function.[40] The microtubule motor protein dynein may play an essential role in AR nuclear transportation. Taxane compounds can disrupt the microtubule structure, and thus, inhibit dynein motor function. AR nuclear translocation on ligand binding is a necessary process for the transcriptional activity of AR. Targeting AR nuclear transportation by the taxane compound reduces AR activity. In LNCaP cells, taxane treatment inhibits AR nuclear translocation and subsequently blocks AR signaling activation.[40] Docetaxel has also been shown to have roles in the regulation of antiapoptotic factors and cell cycle regulators.[41] Thus, docetaxel affects multiple targets simultaneously and has served as a successful multi-target therapy for many years. Although docetaxel provides a significant overall survival benefit to CRPC patients, as compared to mitoxantrone treatment (17.5 months *vs* 15.6 months, $P = 0.02$ by the Log-rank test), it is noteworthy that, in the same study, a large portion of CRPC patient did not respond to docetaxel very well. Even among those who responded initially, the progression-free survival rate at 24 months after enrollment declined and was similar to those treated with mitoxantrone.[42] Both prostate-cancer-specific and cancer-in-general mechanisms could be involved in taxane resistance. AR signaling re-activation is a prostate-cancer-specific mechanism that confers taxane resistance. There is evidence to suggest that the AR signaling pathway is restored in docetaxel-resistant cells, and AR activation could desensitize docetaxel treatment.[43] The presence of AR variants plays important roles in AR signaling re-activation-induced taxane resistance. A preclinical report has shown that taxane is unable to inhibit the transcriptional activity of AR-V7 and AR^{V567es}.[44] This phenomenon can be explained by the observation that microtubule binding of AR is mediated by the C-terminal region of AR.[45] Thus, AR-V7, which lacks the C-terminus, does not cosediment with microtubules. The nuclear localization and function of AR-V7 may not be impaired by microtubules targeting taxane drugs. NonAR-driven taxane resistance is another major challenge. The complete AR-independent property makes anti-androgen agents nonfunctional. neuroendocrine prostate cancer (NEPC) is the major subtype of "complete AR-independent prostate cancer".[46] Although NEPC merely represents <2% of all prostate cancer cases, NEPC potentially causes approximately 25% lethality in prostate cancer.[47,48] A recent report has shown that enzalutamide treatment promotes prostate cancer cell neuroendocrine differentiation through the recruitment of mast cells.[49]

Taxanes plus anti-androgen agents

The combination of anti-androgen therapy and docetaxel has received increased attention in recent clinical studies. The synergistic effect of targeting microtubules and AR signaling is one advantage of the taxane plus ADT combination. The initial CHAARTED trial demonstrated a promising outcome of combining docetaxel plus ADT for the treatment of CRPC. A recently reported long-term survival analysis from the CHAARTED trial results showed a median overall survival of patients with high-volume tumors of 51.2 months for docetaxel plus ADT versus 34.4 months for ADT alone (HR: 0.63, 95% CI: 0.50–0.79, $P < 0.001$).[4] The GETUG 12 study conducted in France also demonstrated that docetaxel plus ADT improved relapse-free survival compared with ADT alone in high-risk localized prostate cancer.[50] Recent studies provide a rationale for the addition of second-generation anti-androgen drugs to docetaxel treatment. A recent preclinical study has shown that the addition of dihydrotestosterone (DHT) desensitizes AR-positive LAPC4 cells to docetaxel treatment, which suggests that the re-activation of AR signaling contributes to docetaxel resistance.[43] The combination of enzalutamide with docetaxel dramatically reduced LAPC4 cell growth even in the presence of DHT, demonstrating that docetaxel sensitivity with DHT stimulation is mediated by the H3K4me3 histone demethylase, lysine (K)-specific demethylase 5D (KDM5D). Knocking down *KDM5D* in LNCaP cells caused docetaxel insensitivity in the presence of DHT. Early phase Ib results for enzalutamide plus docetaxel have demonstrated manageable toxicity.[51] Several clinical trials are currently examining the safety and efficacy of the enzalutamide and docetaxel combination (NCT03246347, NCT02685267, NCT01565928). In addition, preliminary results from a phase I/II trial of cabazitaxel plus abiraterone have shown that 12 CRPC patients (46%) achieved a PSA response after receiving docetaxel and abiraterone.[52] This progress warrants further investigation of the anti-cancer mechanisms of the combination of taxane with new-generation anti-androgen agents in preclinical cell and animal models.

In our preliminary study, we found that docetaxel plus enzalutamide had a profound effect on the androgen-independent CWR-R1 cell line. The combination of docetaxel and enzalutamide exerted a greater growth inhibitory effect compared with either drug alone. In addition, the dose of drugs can be significantly lowered for both drugs to achieve growth inhibition. Cell cycle analysis showed that docetaxel plus enzalutamide had a different impact on the cell cycle compared with either drug alone (unpublished data). To further investigate the combinatorial effects of docetaxel plus enzalutamide, we cultured the docetaxel-resistant R1/DTX cells described previously[53] in the androgen-depleted medium for a long duration. The newly established R1-CS/DTX cells did not respond to either docetaxel or enzalutamide alone at clinically relevant doses. This represents a double resistance model for both docetaxel and ADT. Surprisingly, we found that the R1-CS/DTX cells still responded well to the combination of docetaxel and enzalutamide. Our mechanistic study revealed that E2F transcription factor 1 (E2F1) was one of the key mediators of this effect. The E2F1 expression level was dramatically decreased under combination treatment but did not show significant alterations under either drug treatment alone. This finding indicates a unique anti-cancer mechanism for the combination of docetaxel plus enzalutamide. We also observed that the AR-V7 expression level was decreased following E2F1 inhibition. After careful examination, we found that E2F1 and AR-V7 could form a positive feedback loop. The expression levels of both E2F1 and AR-V7 were decreased correlatively under treatment with docetaxel and enzalutamide (unpublished data). The importance of E2F1 along with AR signaling in anti-androgen-resistant cells was supported by a study showing that the suppression of E2F1 and AR by thymoquinone inhibited androgen-independent prostate cancer growth and blocked G1 to S phase progression.[54] Together, our findings indicate that the cancer inhibitory effect of combination therapy may not merely be the additive effects of two or more drugs.

Taxane plus nonandrogen-targeted agents

Additional cancer-in-general mechanisms underlying taxane resistance include tubulin isotype alterations, microtubules/cytoskeleton mutations, drug efflux pump overexpression, and upregulation of pro-survival pathways. The deregulation of β-tubulin isoforms has received accumulated attention in recent years. βIII-tubulin is a β-tubulin isoform that is predominantly expressed in neurons and testis. Although βIII-tubulin expression was rarely observed in early-stage prostate cancer patients, the βIII-tubulin expression is elevated in CRPC patients and associated with taxane resistance.[55,56] A recent

report has demonstrated that βIII-tubulin is a direct target of AR and the expression of βIII-tubulin can be regulated by androgen stimulation in mouse and rat Sertoli cells.[57] Since constitutively activated AR-V7 can also regulate conventional AR signaling pathway targets, AR-V7 may very likely play a role in βIII-tubulin-induced docetaxel resistance. A recent study of high-content screening of a well-characterized clinical compound has demonstrated that the Src-Abl dual kinase inhibitor PD180970 can decrease AR-V7 expression and inhibit androgen-independent cell proliferation.[58] More importantly, Src kinase may play a direct role in regulating the stability and function of βIII-tubulin. Our preliminary study found that βIII-tubulin protein levels can be reduced by the Src inhibitor SU6656 (unpublished data). These findings suggest that the Src inhibitor could be a potential complement to taxane therapy. Dasatinib is an FDA-approved Src family tyrosine kinase inhibitor for children with chronic myelogenous leukemia. Early results for dasatinib in metastatic CRPC have been disappointing. The benefit of dasatinib is minimal, but the adverse effects are not tolerable.[59] A recent clinical trial using docetaxel plus dasatinib failed to show an overall survival benefit for CRPC compared with docetaxel monotherapy. The median overall survival was 21.5 months for docetaxel plus dasatinib versus 21.2 months for docetaxel alone (HR: 0.99, 95.5% CI: 0.87–1.13; $P = 0.90$).[60] Although the study did not demonstrate a statistically significant survival benefit, a case study in this project reported improved bone scans outcomes, a high rate of soft-tissue responses, and decreased bone turnover markers with prolonged dasatinib treatment after combination treatment in a subset of patients.[61] In addition, the combination of dasatinib with docetaxel showed durable 50% PSA declines in 26 of 46 patients (57%) in an earlier phase I/II trials conducted in CRPC patients.[62] In addition to Src family inhibitors, the well-known TK domain tyrosine kinase inhibitor imatinib is another promising therapy that may function together with docetaxel. In a preclinical study, the combination of docetaxel and imatinib enhanced cancer cell apoptosis through inhibition of docetaxel-induced NF-κB activation in anaplastic thyroid cancer cells.[63] Another study has reported that cancer cell growth and invasion ability are reduced in response to docetaxel plus imatinib treatment.[64] Although the preliminary phase II trial of docetaxel plus imatinib in metastatic breast cancer patients shows a low response rate with toxicity issues, a subpopulation of patients (6 of 37) has shown a partial response (NCT00193180). Focal adhesion kinase plays an essential role in docetaxel-resistant CRPC.[65] Cotreatment of docetaxel-resistant AR-negative prostate cancer cell lines with the focal adhesion kinase (FAK) tyrosine kinase inhibitor PF-00562271 and docetaxel attenuates cell growth and induces apoptosis. There are also several ongoing trials investigating taxane plus other nonantiandrogen therapeutic methods such as anti-angiogenesis agents, mTOR inhibitor, and radiation therapy.[66]

COMBINATORIAL IMMUNOTHERAPY

In the past few decades, researchers have started to reconsider taking advantage of the immune response in cancer treatment after the first wave of "immunotherapy" in the 19th century. Immunotherapy eliminates tumors using the host's own immune system. The advantages of immunotherapy over other therapeutic methods are increased safety, durability, and likely greater efficiency in overcoming diverse pro-survival mechanisms.[67] However, tumors can utilize various mechanisms to escape immune targeting. Molecular events of acquired immunotherapy resistance include gene mutation and deletion, alternative splicing, and oncogenic signaling/epigenetic/microenvironment alterations.[68] A recent study has reported that the Janus kinase (*JAK*)*1*/*2* and beta-2-microglobulin (*B2M*) mutation might lead to a lack of response to interferon (IFN)-γ and loss of surface expression of Class I major histocompatibility complex (MHC), respectively.[69] It is also believed that gene deletion and alternative splicing are involved in immunotherapy resistance. Loss of CD19 antigen in response to CART-19 therapy through CD19 deletion and mRNA splicing make antitumor T cells unable to recognize targets.[70] It is noteworthy that acquired resistance has been previously observed in metastatic melanoma patients who initially responded to immunotherapy.[71] Sipuleucel-T is the only FDA-approved immunotherapy for the treatment of CRPC. In a placebo-controlled phase III trial, CRPC patients receiving sipuleucel-T treatment showed a significantly reduced risk of death compared with the placebo group.[72,73] In a more recent study involving 512 metastatic CRPC patients, the median survival advantage of sipuleucel-T-treated patients was 4.1 months longer than those in the placebo group.[74] Although little is known about the acquired resistance of sipuleucel-T due to the lack of long-term observations, subgroups of patients in that study displayed primary resistance to this therapy with minimal response.

Enhanced immunotherapy efficiency by therapeutic combinations

The success of antibody-based (anti-programmed cell death 1 [PD-1]/programmed cell death ligand-1 [PD-L1] and anti-cytotoxic T-lymphocyte antigen-4 [CLTA-4]) immunotherapies in other cancers makes them extremely attractive in investigations of their efficacy in the treatment of CRPC. However, the preliminary outcome of immunotherapy as monotherapy is disappointing in prostate cancer. Little or no response was observed in these studies.[75,76] The poor response of anti-PD-1/PD-L1 treatment in prostate cancer patients may be due to the lack of antigen expression in prostate tumors. A recent comprehensive immunohistochemical analysis of PD-L1 in prostate cancer specimens has shown that PD-L1 is not expressed in localized and benign prostate hyperplasia, and it is only expressed in a small subset of CRPC specimens.[77] Recovery of antigen expression is required for immune checkpoint therapies in prostate cancer. Fortunately, the antigen can be re-expressed under certain circumstances. According to a recent report, although PD-L1 is rarely expressed in primary tumors, its expression can be upregulated in response to IFN-γ in PC3 prostate cancer cell line.[78] Increases in PD-L1/2$^+$ dendritic cells are observed in enzalutamide-resistant patients compared with responders.[79] In the same study, PD-L1 upregulation in patients was verified in AR-positive/PSA-negative enzalutamide-resistant prostate cancer cells compared with enzalutamide-sensitive cells. Increased immunotherapy efficacy was observed in anti-androgen-resistant patients. In a study of anti-PD-1 pembrolizumab with continued enzalutamide treatment for CRPC patients with enzalutamide resistance, researchers observed increased PD-L1 expression and the presence of CD3$^+$, CD8$^+$, and CD163$^+$ leukocyte infiltration.[80] Thus, the general concept is to utilize a secondary therapeutic method to reduce the immunosuppression-induced resistance or insensitivity and make CRPC re-targetable by immunotherapy.

Chemotherapy and radiation therapy are also feasible for enhancing the efficacy of immunotherapy. Both therapies are considered to cause a massive cytotoxic effect in rapidly dividing cancer cells. Tumor antigen and other components released from the dead cells may potentially affect the immune response. Radiation-induced cell death results in increased expression and release of tumor antigen and cell signaling proteins, including MHC-I, death receptors, immunomodulatory cytokines, adhesion molecules, and costimulatory molecules.[81] The presence of these components

may induce immunostimulatory effects and enhance the efficacy of immunotherapy. Metastatic urothelial cancer shares a similar low response rate to checkpoint inhibitor monotherapy. In an early clinical trial report, the immune-targeting drug pembrolizumab plus docetaxel showed encouraging anti-tumor activity with improved progression-free survival in metastatic urothelial cancer (NCT02437370).[82] A synergistic response to combinatorial immunotherapy and radiation/chemotherapy has also recently been observed in prostate cancer. A clinical study showed that surviving-specific $CD8^+$ T lymphocytes in peripheral blood were increased in colorectal cancer and prostate cancer patients after radiation therapy.[83] Another phase III clinical trial investigating the addition of CTLA-4-targeted ipilimumab to radiotherapy for docetaxel-resistant bone metastatic CRPC has shown promising results.[84] Despite the absence of a statistically significant improvement in overall survival, *post hoc* subgroup analyses demonstrated that overall survival and progression-free survival were improved for patients with poor prognostic features. In early phase I/II results of this study, researchers also found a subset of patients with a PSA response in the ipilimumab plus radiotherapy group.[85] In addition to promoting an immunostimulatory effect, chemotherapy and radiation therapy may enhance immunotherapy through inhibiting immune suppressors. Myeloid-derived suppressor cells (MDSCs) play an important role in immune evasion in prostate cancer. An *in vivo* study has shown that docetaxel is able to suppress MDSCs while increasing the cytotoxic T lymphocyte response.[86] The chemotherapy drug cyclophosphamide has been shown to suppress immune suppressor cells and stimulate an immune response at a proper dose.[87] Repression of MDSCs can also be achieved through targeted drugs in addition to radiation and immunotherapy. BEZ234 is a multikinase inhibitor that can attenuate MDSC frequency and immunosuppressive activity. An immune checkpoint blocker plus BEZ234 can greatly reduce the tumor mass and metastasis in a novel nongermline CRPC mouse model.[88] Mechanistically, BEZ234 suppresses the secretion of cytokines that promote MDSC activity through the inhibition of PI3K signaling. This process sensitizes immunotherapy-resistant CRPC to immune checkpoint blockers. More recently, a study conducted by the National Institutes of Health is testing the efficacy of the combination of multiple immunotherapies for biochemically recurrent prostate cancer (NCT03315871). In that study, the PSA-targeted immunotherapy PROSTVAC is combined with tumor-associated antigens. The carcinoembryonic antigen (CEA)- and mucin 1 (MUC-1)-targeted immunotherapy CV301 PROSTVAC monotherapy has recently shown disappointing results in a phase III study. The bifunctional fusion protein MSB0011359C is also being tested in that study. This fusion protein is composed of two functional domains, a monoclonal antibody against PD-L1 and the extracellular part of human transforming growth factor-β (TGF-β) receptor II, which "trap" TGF-β. Therefore, MSB0011359C can block both PD-L1 and TGF-β pathways. TGF-β has an essential role in immune suppression in advanced cancers. TGF-β inhibits the function of T and natural killer (NK) cells. Thus, MSB0011359C can be considered as an immunotherapy antibody combined with anti-immune suppressor therapy. A very recent study has shown that MSB0011359C induces $CD8^+$ and NK cell activity and decreases the tumor volume in a breast and colon cancer preclinical model.[89] In the same study, the authors also showed the synergistic activity of MSB0011359C when combined with another immunotherapy Ad-twist family bHLH transcription factor 1 (TWIST) vaccine that targets the tumor-associated antigen TWIST, suggesting an advantage of targeting multiple immune factors. Together, these studies provide evidence of an objective benefit of adding a secondary therapy to improve the sensitivity of CRPC to immune therapy.

NONCANONICAL COMBINATION

The canonical pathway by which AR facilitates prostate cancer growth and survival has been well characterized.[90,91] Recent findings indicate that AR may have novel functions in the regulation of DNA repair signaling. A recent report has shown that 144 DNA repair genes are associated with AR. Seventy-four genes of the 144 genes can be induced by androgen, and 32 genes are direct AR targets.[92] These genes are critical for the DNA repair pathway, including nonhomologous end joining (NHEJ), homologous recombination (HR), mismatch repair (MMR), base excision repair (BER), and the Fanconi anemia pathway. Thus, the addition of AR-targeting agents to conventional DNA damage repair inhibition therapy may have additive or synergistic effects. When treated with ionizing radiation, suppression of AR activity causes increased DNA damage and decreased tumor growth both *in vitro* and *in vivo*.[92,93] The addition of enzalutamide to radiation improves the sensitivity of radiation therapy in LNCaP cells.[94] The prolonged presence of the DNA damage marker histone variant H2AX phosphorylated on serine 139 (γH2AX) and increased radiation-induced apoptosis/senescence were observed in the same study. Cell division cycle 6 (CDC6) is an important DNA replication checkpoint protein. *CDC6* is an AR target gene, and the expression of CDC6 is positively associated with AR expression in prostate cancer tissues.[95] Downregulation of CDC6 by AR knockdown plus the checkpoint kinase (Chk)1/2 inhibitor AZD7762 greatly increases ATM phosphorylation, a DNA damage marker. Combination treatment with enzalutamide and the Chk1/2 inhibitor AZD7762 promotes DNA damage-induced apoptosis and inhibits cancer cell growth *in vitro* and *in vivo*.[95] Poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) inhibitors represent another class of DNA repair blocker that are widely studied in early-onset breast cancer gene (BRCA1)/breast cancer 2, early onset (BRCA2)-mutated cancers. A preliminary study of the PARP inhibitor olaparib has shown an encouraging outcome for metastatic CRPC patients.[96] More importantly, there are several ongoing clinical trials combining olaparib with anti-androgen therapy or other therapies for CRPC patients with or without DNA repair defects. Early results from a phase II trial have illustrated significantly improved progression-free survival of CRPC patients in the olaparib plus abiraterone arm compared with abiraterone alone.[97]

Dysregulation of protein nuclear/cytoplasm localization plays important roles in cancer progression. Nuclear/cytoplasm translocation inhibitors have been under investigation in clinical trials as either stand-alone therapy or part of combination treatment. The selective nuclear export inhibitor selinexor is being tested in multiple cancer types in clinical trials.[98] The combination of docetaxel and selinexor is being evaluated in a clinical trial for nonsmall cell lung cancer with the K-ras (*KRAS*) mutant. Thus, modulation of nuclear/cytoplasm translocation is a potential therapeutic method combined with other therapies for prostate cancer. A recent study has shown that POM121 transmembrane nucleoporin (POM121), a component of nuclear pore complexes, regulates the nuclear import of the cancer progression mediators E2F1, MYC, and AR through its interaction with importin β.[99] POM121 is elevated in lethal prostate tumors, and POM121 promotes tumorigenesis, proliferation, and resistance to standard therapies such as taxanes, DNA-damaging agents, and radiotherapy, both *in vitro* and *in vivo*. The importin β inhibitor importazole was observed to decrease E2F1, MYC, and AR nuclear localization, and suppress tumor proliferation in a drug-resistant prostate cancer cell model and

patient-derived lethal prostate cancer model. More importantly, the combination of importazole with docetaxel or mitoxantrone decreased growth in resistant and lethal prostate cancer models compared to either treatment alone. This study also demonstrated prolonged survival in combination-treated xenograft animals. It is important to remember that AR variant nuclear localization and transcriptional activity do not rely on androgen stimulation and cannot be blocked by microtubule-targeting agents. The nuclear localization of AR variants also does not seem to depend on the heat shock protein complex, which is required for full-length AR nuclear translocation.[100] It is very interesting to examine the effect of importin β inhibitor on AR variant intracellular translocation. A preliminary study has demonstrated that AR-V7 and AR^{v567es} nuclear import can be blocked by importazole in a COS-7 cell AR-V7 or AR^{v567es} overexpression model.[44] These studies warrant further investigation of the efficacy of combining importazole with anti-androgen or chemotherapy in the treatment of prostate cancer.

PERSPECTIVE

Due to space constraints, we can only discuss some potential therapeutic combinations. In addition to the information discussed in the main text, many possible treatment options are under investigation in preclinical studies. For example, recent findings for autophagy can support the growth of enzalutamide-resistant prostate cancer.[101] A small molecule autophagy inhibitor has been shown to reverse the resistance and re-sensitize resistant cancer cells to therapies. To develop a more effective combination therapy, we must resolve several tough challenges. One urgent need is to identify the best drug combination for subgroups of patients with different molecular characteristics. It is often true that only a subset of patients has shown a positive outcome in a clinical study. For example, in the CHAARTED trial, no significant overall survival benefit was found for patients with low volume disease treated with docetaxel plus ADT compared with ADT alone, while significant benefits were observed in patients with more aggressive tumors.[4] In another clinical study, GETUG-AFU 15, no significant difference in overall survival was observed comparing docetaxel plus ADT versus ADT alone.[102] The lower PSA level and lower average Gleason score in the GETUG-15 patient cohorts compared with those in the CHAARTED trial may be attributed to the disparity in response to combination therapy.[103] These findings suggest that the efficacy of combination therapy should be carefully examined in different subgroups of tumors with different molecular characteristics. Emerging new technology may enable us to better define prostate cancer subtypes. A recent comprehensive molecular analysis of prostate cancer patients conducted by The Cancer Genome Atlas (TCGA) network has revealed that most primary prostate cancer tumors can be characterized into seven subtypes by specific molecular events. The seven subtypes are defined by the E26 transformation-specific (*ETS*) gene fusion status and mutations in speckle-type BTB/POZ protein (*SPOP*), forkhead box A1 (*FOXA1*), and cytosolic $NADP^+$-dependent isocitrate dehydrogenase (*IDH1*).[104] Future investigations will focus on studying the molecular mechanisms of combination therapies for specific subgroups of prostate cancer patients. The emergence of pharmacogenomics is of great help in determining effective therapeutic options for a certain group of patients. Unexpected adverse effects are another major cause of early termination of clinical trials. Combination therapy has theoretic potential to lower the dose of each drug required for treatment. Therefore, for those drugs with promising results in preclinical studies but failure in early clinical trials due to intolerable side effects, further studies are needed to evaluate the possibility of dose reduction in combination with currently available drugs.

AUTHOR CONTRIBUTIONS

JX performed literature search and wrote the draft of the review. YQ participated in construction, writing and editing of the review. Both authors read and approved the final manuscript.

COMPETING INTERESTS

Both authors declare no competing interests.

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