

REVIEW

EMERGING INSIGHTS OF DRUG RESISTANCE IN METASTATIC CASTRATION-RESISTANT PROSTATE CANCER

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ABSTRACT: In recent years, castration resistant prostate cancer patients therapeutic landscape has dramatically changed. The use of taxane-based chemotherapy, new generation hormone therapy, an immunotherapeutic agent Sipuleucel-T, an alpha-emitter Radium-223, and recently PARP-inhibitors has provided a consistent benefit in terms of survival and quality of life. In particular, androgen receptor (AR) remains a key player of prostate cancer progression also in this setting and more effective therapeutic agents are still directed against AR pathway. Unfortunately, not all patients respond to available treatments and almost all will develop a drug resistance. The resistance mechanisms involve not only AR dependent signaling pathways but also AR independent cells survival pathways. In this review we discuss the potential mechanisms of resistance to drugs employed in the treatment of metastatic castration resistant prostate carcinoma patients.

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Key words: mCRPC; drug resistance; new generation hormone therapy; prostate cancer; androgen receptor.

Impact statement: Understanding the mechanisms of resistance in castration-resistant prostate cancer by investigating all agents used in this setting.

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INTRODUCTION

Prostate cancer is the most common tumor in men with a rate of 111.3 new cases per 100,000 men per year and the second leading cause of death among men in the USA with a death rate of 18.9 per 100,000 men per year. About 7% of patients develop metastatic disease with a 5-year survival rate of 30.6% (1). Androgen deprivation therapy (ADT), by inhibiting testosterone synthesis, is the cornerstone of advanced disease systemic therapy. *De novo* metastatic castration sensitive patients with “high volume disease” (defined as having either visceral metastases or a number of >4 bone lesions including at least one outside the vertebral column and pelvis) may benefit from the addition of Docetaxel to ADT giving an improvement in progression free survival

(PFS) (20.2 vs. 11.7 months; HR 0.61; $P < 0.001$) and overall survival (OS) (57.6 vs. 44.0 months; HR 0.61; $P < 0.001$) (2). In addition, clinical studies have demonstrated that the addition of new generation hormonal therapies (NGHT) to ADT in this setting, such as Abiraterone (3, 4) Enzalutamide (5, 6) and Apalutamide (7), provide a benefit in terms of PFS and OS, irrespectively from disease burden. Furthermore, Apalutamide and Enzalutamide have been demonstrated to have a role in both *de novo* (synchronous) and recurrent (metachronous) hormone-sensitive metastatic disease. Despite these brilliant results, the castration sensitivity phase is transient and most patients eventually develop metastatic castration resistant prostate cancer (mCRPC) after a median

of 18-36 months (8). At present, according to the latest guidelines of the European Association of Urology, mCRPC is defined as: (1) serum testosterone <50 ng/dl or 1.7 mmol/L and (2) one of the following conditions including: (i) three consecutive increases in PSA one week apart, with two increases of 50% above nadir and/or (ii) the appearance of new lesions and in particular two or more new lesions on bone scan or the detection of a soft tissue lesion based on the response evaluation criteria in solid tumor (9).

Treatment of mCRPC patients has been revolutionized in the last decade. In particular, the use of chemotherapy (Docetaxel (10) and Cabazitaxel (11)), new generation hormone therapy (Abiraterone (12) and Enzalutamide (13)), Sipuleucel-T (14), alpha-emitters such as Radium-223 (15) and recently the use of PARP-inhibitors (Rucaparib (16), Olaparib (17)) has provided a consistent benefit in terms of survival in this setting (**table I**).

Several trials are currently ongoing evaluating the correct therapeutic sequence of these drugs and possible combination regimens, in order to increase efficacy and personalize treatment. Despite the introduction of these new therapies, several escape mechanisms have been established, conferring resistance to these molecules. The aim of this review is to analyze the pharmacological effect of these drugs focusing on potential mechanisms of resistance.

NEW GENERATION HORMONAL THERAPIES

In recent years, androgen receptor (AR) signaling inhibitors such as Abiraterone and Enzalutamide have been introduced into the standard treatment of mCRPC demonstrating significant survival benefit. Unfortunately, a not negligible portion of patients ranging from 15 to 20% appears resistant *ab initio* or will develop resistance to new generation hormonal therapies due to the outbreak of mechanisms of resistance. Primary resistance is commonly defined as the treatment failure within the first 3 months after the start, as a result of clinical progression, with or without radiological progression (18). According to this conventional definition of primary resistance acquired resistance is considered as a treatment failure that occur later during treatment.

AR-dependent mechanism of resistance

Androgens are sex hormones required for development of the male reproductive system and secondary sexual characteristics. In adult male testosterone (T) and 5- α -dihydrotestosterone (DHT) are mainly produced in the testes. 5%-10% of their synthesis occurs in the adrenal glands.

The androgen receptor is a protein consisting of a DNA-binding domain (DBD), an N-terminal transactivation domain, containing dominant AF-1 transactivation region, an Hinge region (H), which carries a nuclear localization signal and a ligand binding domain (LBD), containing also the AF-2 transactivation region. In physiological conditions androgens such as testosterone and dihydrotestosterone bind to the AR resulting in a conformational change of the receptor. Upon binding to the ligand, AR separates from the heat shock protein 90 complex, undergoes homodimerization, translocates into the nucleus and binds to androgen-specific elements in the promoter regions of androgen regulatory genes. Transcriptional activity is supported by the interaction of other pathways involved in the cellular signaling pathway, including coactivator and suppressor proteins (19). Non-DNA binding dependent pathways may also be involved in the cascade generated by the androgen/AR complex. In particular, the activation of alternative pathways (second messengers) such as ERK, Akt and MAPK have been identified in several cell lines. There is evidence to suggest that some of the non-DNA binding-dependent actions of androgens are mediated by the activation of membrane-bound protein receptors to activate intracellular signaling pathways that can occur even in the presence of low androgen levels. Identification and characterization of cell surface receptors that can mediate the quick non-DNA binding-dependent effects of estrogens and progestins have been documented in a large variety of tissues and cell types. However, until now, membrane-bound AR receptors were not investigated as extensively (20).

AR aberrant expression and activity is the key factor in the development of prostate cancer, irrespective of stage or grade. Binding of androgen and AR within prostate cells occurs predominantly in the prostatic stroma and glandular epithelium, regulating several biological mechanisms. The androgen/AR signaling pathway plays an important role in the regulation of prostate

DRUG	MECHANISM OF ACTION	STUDY NAME	SETTING	PRIMARY ENDPOINT	RESISTANCE MECHANISM
Abiraterone	Inhibition of 17 α hydroxylase/C17,20-lyase (CYP17)	COU-AA-302	Abiraterone Acetate vs. Placebo in Docetaxel-naïve mCRPC	OS 34.7 vs. 30.3 mo (HR = 0.81; 95% CI: 0.70-0.93; p = 0.0033) rPFS 16.5 vs. 8.2 mo (HR = 0.52; 95% CI: 0.45-0.61; p < 0.0001)	CYP17A1 upregulation FL-AR AR splice variant AR point mutation EMT PI3K pathway upregulation
			Abiraterone Acetate vs. Placebo in mCRPC previously treated with Docetaxel	OS 14.8 vs. 10.9 mo (HR = 0.65; 95% CI: 0.54-0.77; p < 0.001)	
Enzalutamide	AR inhibitor	PREVAIL	Enzalutamide vs. Placebo in Docetaxel-naïve mCRPC	OS (est) 32.4 vs. 30.2 mo (HR = 0.71; 95% CI: 0.60-0.84; p < 0.001) 12 mo rPFS 65% vs. 14% (HR = 0.19; 95% CI: 0.15-0.23; p < 0.001)	AR splice variants AR point mutations Intratumoral <i>de novo</i> steroid synthesis EMT PI3K pathway upregulation
			Enzalutamide vs. Placebo in mCRPC previously treated with Docetaxel	OS 18.4 vs. 13.6 mo (HR = 0.63; 95% CI: 0.53-0.75; p < 0.001)	
Docetaxel	Inhibition of microtubular depolymerization	TAX -327	Docetaxel plus prednisone vs. mitoxantrone plus prednisone in mCRPC	OS 18.9 vs. 16.5 mo. (HR 0.76; 95% CI: 0.62-0.94; p 0.009)	Tubulin alterations ABC AR overexpression
Cabazitaxel	Inhibition of microtubular depolymerization	TROPIC	Cabazitaxel plus Prednisone vs. Mitoxantrone plus prednisone in mCRPC previously treated with a docetaxel-containing regimen	OS: 15.1 vs. 12.7 mo (HR 0.7; 95% CI 0.59-0.83, p < 0.0001)	MDR1 protein CCL2 oversecretion
PARP inhibitor	DNA damage through synthetic lethality	PROfound	Olaparib vs. NGHT in mCRPC after at least 1 NGHT in mCRPC with Bi- or mono-allelic somatic or germline deleterious HRD	OS: BRCA/ATM: 19.1 vs. 14.7 mo (HR = 0.69; CI 95% 0.5-0.97; p = 0.02) No-BRCA/ATM: 14.1 vs. 11.5 mo (HR = 0.96; CI 95% 0.63-1.49)	ABC Homologous recombination
Radium 223	Cytotoxic DNA double-strand breaks in bone cells	ALSYMPCA	Ra223 vs. placebo in mCRPC previously treated with docetaxel	OS: 14.9 vs. 11.3 mo (HR 0.70; 95% CI, 0.58 to 0.83; p < 0.001)	NHEJ mechanism VEGF, PDGF and endothelin -1 upregulation

Table 1. Efficacy and mechanisms of drug resistance for principal therapeutic agents in mCRPC.

mCRPC: metastatic castration resistant prostate cancer; AR: androgen receptor; FL-AR: full length androgen receptor; EMT: epithelial to mesenchymal transition; HRD: homologous recombination deficiency; NGHT: new generation hormone therapy; ABC: ATP-binding cassettes; MDR1: multidrug resistance 1 protein; NHEJ: non homologous end-joining; VEGF: vascular-endothelial growth factor; PDGF: platelet-derived growth factor.

epithelial cell proliferation, apoptosis and cell growth. Nowadays it is recognized that the shift from castration sensitive disease to mCRPC is not necessarily due to ADT resistance but is closely related to functional abnormalities of the androgen receptor. Prostate carcinoma cells escape to ADT and adapt to a microenvironment with low T levels producing their own androgens or mutate AR gene so that it requires less or no androgens. Several AR-related changes in mCRPC were described including AR overexpression, AR splice variants and AR mutation activation, over expression of molecules promoting AR activity and alternative androgens production. The presence of AR as a tumor promoter was largely showed in mCRPC. About 80% of mCRPC undergo an increase in AR expression, while 20% show AR depletion (21). AR amplification occurs in 20-30% of patients with CRPC. AR gene amplification typically occurs when the tumor progresses to a castration-resistant state and is associated with a two-fold increase in AR mRNA levels.

Splice variants of AR encode for an aberrant variant of the AR protein characterized by the absence of C-terminal LBD, an intact N-terminal domain and a partial or complete DBD able to interact with DNA and AR co-receptors. AR splice variants are constitutively active as transcription factors, promoting the expression of target genes activating downstream AR signaling pathways. Another AR related mechanism in mCRPC is the gain of AR functional mutations through point mutations. Clinically relevant point mutations mainly affect the LBD. However, rather than conveying constitutive activation, functional mutations in AR have been shown to induce increased sensitivity to androgens low levels and affinity for non-androgenic ligands (glucocorticoids, progestins, estrogens, dehydroepiandrosterone). In addition, AR point mutations cause the switch of drug mechanism of action from antagonist to agonist (19).

Abiraterone Acetate

Abiraterone is a potent androgen biosynthesis inhibitor, which acts inhibiting 17 α -hydroxylase/C17,20-lyase (CYP17). This enzyme is expressed in testicular, adrenal, and prostatic tumor tissues and is a key player in androgen biosynthesis.

It was observed that treatment of human CRPC xenografts with Abiraterone increased the expression of full-length AR (FL-AR) and truncated variants of AR threefold. Furthermore, *in vivo* studies

showed that AR knockdown results in reduced cell growth and AR-related gene expression, leading to delayed tumor growth (22).

AR splice variants play an important role in mCRPC progression. Several AR splice variants have been described, although the most clinically relevant are AR-V7 and ARV567^{es}. The most frequent causes of AR splice variants expression are mRNA aberrations due to splicing events or, less frequently, gene alterations (23). In tumor samples obtained from mouse models of CRPC resistant to Abiraterone the authors observed increased mRNA levels of AR-V7, FL-AR and AR^{V567es}. Additional pre-clinical evidence showed that disease progression in patients treated with NGHT is closely related to an up-regulation of intratumoral CYP17A1. Intratumoral androgen suppression and subsequent resistance to Abiraterone may arise through mechanisms involving upregulation of CYP17A1, as well as induction of AR and splice AR variants expression conferring ligand-independent AR transactivation. In particular, in VCaP castration-resistant xenografts receiving Abiraterone upregulation of CYP17A1 increases androgens-dependent genes expression by approximately twofold in case of tumor recurrence (24).

The T878A mutation seems to occur after treatment with androgen synthesis inhibitors such as Abiraterone. T878A mutations can be strongly stimulated by progesterone, which is generally a moderate agonist of wild-type AR. Interestingly, since progesterone is an upstream substrate of CYP17A1, its level does not decrease with abiraterone treatment. Therefore, inhibition of CYP17A1 picks out tumor clones that harbor progesterone-sensitive AR variants. Other point mutations which activate AR during glucocorticoids and abiraterone therapy have been described (25). Abiraterone treatment needs the concomitant administration of prednisone to reduce side effects induced by CYP17A1 inhibition. L702H seems to be involved in resistance to abiraterone and steroid treatment (26).

Enzalutamide

Enzalutamide is an AR inhibitor which affects multiple step of AR signaling pathway. It is a potent competitive binder of AR. It prevents the translocation of the AR from the cytoplasm to the nucleus. Within the nucleus, it inhibits AR binding to chromosomal DNA, preventing transcription of androgen-related genes.

Several resistance mechanisms to enzalutamide are similar to those described for abiraterone. Yamamoto *et al* demonstrated that high levels of both FL-AR and AR splice variants are present in enzalutamide-resistant LNCaP cells and that these alterations are not present in classical LNCaP responsive to NGHT. AR-V7 overexpression in CRPC cells is associated with resistance to NGHT. These findings were observed in xenograft mouse models with primary resistance to Enzalutamide (27, 28).

In addition, some studies *in vivo* showed that detecting AR-V7 in circulating tumor cells in patients treated with Abiraterone or Enzalutamide could predict cases with lower PSA response rates and shorter PFS (29).

Although point mutations in the AR gene may be sporadically present in castration sensitive disease, it is estimated that there is a higher (>10%) frequency of mutations in CRPC patients, especially in patients who progress to ADT and NGHT (30). AR mutations can generally lead to either a loss or a functional increase in AR activity. However, point mutations of AR seem to be the result of somatic events, mostly affecting the LBD (31). Accordingly, mutations leading to gain of function of the AR-LBD region are most often activated by weak androgens and other hormonal agents pathways such as progestins, glucocorticoids, estrogens and paradoxically antiandrogens. Point mutations which develop in mCRPC aim to overcome the selective pharmacological blockade and to confer a survival advantage in tumor cells by providing the activation of alternative survival pathways within the microenvironment. Despite the most frequently observed mutations are F877L and T878A. The F877L mutation has been studied *in vivo* models and appears most frequently during treatment with Enzalutamide. This mutation favors the conversion of Enzalutamide from an antagonist to a strong agonist (32).

AR independent pathways

Upregulation of the glucocorticoid receptor (GR) may be another potential resistance mechanism wherein blockade of the AR leads to activation of alternative tumor survival signaling pathways. Both AR and GR are members of the class I steroid receptors, which also includes estrogen and progesterone receptors. GR is similarly constituted to AR by three functional domains: the

N-terminal domain, the LDB and the DNA binding domain. For this reason, some GR activators may bind to the promoter regions of AR, identifying GR as a potential overlapping target of activation. Arora *et al.*, showed in murine models of CRPC resistant to Enzalutamide and Apalutamide a significant upregulation of genes encoding for GR. Moreover, suppression of GR in tumor cells derived from resistant cells restores sensitivity to Enzalutamide (25).

The progesterone receptor (PR) may participate in overlap phenomena with AR and consequently may contribute to development of NGHT mechanism of resistance. The expression of PR in stromal fibroblasts and smooth muscle, but not in epithelial cells, provides the appropriate homeostasis of cell growth. It has been suggested that an upregulation of PR as a result of AR blockade due to feedback imbalance may have a role in the establishment of resistance mechanisms to NGHT (44). However, Seritella *et al.* published preliminary data of a phase I/II study evaluating the addition of Mifepristone, a progesterone receptor inhibitor, to Enzalutamide, revealing that there is no delay in PSA progression when Mifepristone is added to Enzalutamide (45).

Other mechanisms of resistance AR-independent includes the epithelial-mesenchymal transition (EMT) and the activation of the PI3K-AKT-mTOR pathway.

The term EMT was used for the first time in 1995 to describe dramatic changes in the extracellular environment impacting on cell polarity (46). Although crucial in the physiological processes of embryogenesis, EMT also plays a crucial role in pathological development such as fibrosis and tumors. EMT development in prostate cancer provides therapy resistance, cancer invasion and impact on patient survival. The switch of epithelial to mesenchymal cells leads to severe structural changes, including impaired cell adhesion, basement membrane degradation, loss of cell polarity and gain of migratory and invasive properties (47). Therefore, EMT is a crucial step for epidermal cancer cells to become invasive and metastasize. EMT provides cells with migratory properties, prevents apoptosis, and delivers stem properties to cells.

Several transcription factors play an essential role in EMT such as SNAI1 (Snail), SNAI2 (Slug) and ZEB 1/2 which repress the expression of E-cadherin and other genes responsible of the epithelial proliferation regulation. Some studies

have shown that inhibition of AR results in a depression of Snail as an adaptive response, representing a critical mechanism for therapeutic efficacy in CRPC (48). It has also been observed that members of the transforming growth factor beta (TGF-beta) super family play a key role within the transcriptional activation of Snail and Slug. Therefore, both TGF-beta and activation of the protein kinase C (PKC)/Twist1 complex are potential mechanisms of CRPC resistance (49). Indeed, decreased epithelial signalling in AR knock-out tumors may lead to increased expression of mesenchymal transcription factors in preclinical models (50). Phase I and II trials evaluating the potential combination of next-generation hormone therapy with TGF-beta inhibitors are currently ongoing (NCT02452008, NCT03685591). Ultimately, it is currently under investigation the role of Metformin, an oral hypoglycemic agent in combination with enzalutamide. A recent trial demonstrated the role of this drug in restoring sensitivity to new-generation hormone therapy and inhibiting EMT in mouse models, with encouraging results. However, the latest data indicate that the addition of Metformin to new-generation hormone therapy does not lead to an advantage in terms of PSA progression (51).

The PI3K-AKT-mTOR signaling axis is frequently dysregulated in prostate cancer. It is estimated that about 20-40% of prostate cancers have an altered PI3K-AKT-mTOR signaling pathway, up to about 50% in mCRPC patients (52). This pathway is also widely investigated for its interaction with AR signaling. Therefore, alterations within this pathway may be responsible for resistance to current therapies, supporting tumor progression and carcinogenesis (53). Considering the high frequency of activation of this signalling pathway, it was suggested as a potential therapeutic target in inhibiting tumorigenesis and overcoming resistance during therapy. Several inhibitors have been investigated in both monotherapy and combination therapies. The use of pan-PI3K inhibitors and isoform-specific inhibitors have raised particular interest in recent times. Notably, encouraging results were observed with BKM120, a pan-PI3K inhibitor that showed promising outcomes in a phase I trial (54). Unfortunately, these data were not confirmed in phase II trials (55). Isoform-specific inhibitors of PI3K such as BYL719 and MLN117 were able to target selectively the p110alpha catalytic subunit, com-

monly mutated in patients with metastatic prostate cancer, in order to reduce the most common side effects such as hyperglycaemia and insulin resistance. However, both categories of drugs showed that inhibition of both complete and PI3K isoforms leads to hyperactivation of other extracellular pathways, such as AR hyperactivation (56, 57).

Conversely, AKT is involved as a key mechanism in tumor anti-apoptotic regulation. The first AKT inhibitors studied are Ipatasertib and AZD5363, ATP-competitive inhibitors of AKT (58). Preclinical studies have shown that direct inhibition of AKT leads to reduced proliferation and induction of apoptosis and differentiation in prostate cancer cell lines (59). The greatest limitation of this group of drugs is the activation of a negative feedback that activates other different tyrosine kinase receptor pathways (60). Data from the phase 3 study evaluating the efficacy of Ipatasertib in combination with Abiraterone were recently published, showing an OS advantage in patients with a loss of PTEN only.

Finally, mTOR is proposed as a downstream pathway that combines extracellular downstream signals and metabolic processes involved in regulating cell growth. Allosteric mTOR complex 1 inhibitors such as Everolimus and Temsirolimus were the first PI3K-AKT-mTOR pathway inhibitors to be evaluated in clinical trials. However, several trials failed to demonstrate a clinical advantage in patients with prostate cancer (61). A possible explanation is the activation of upstream pathways such as AKT, resulting in an anti-apoptotic and cell growth boost. As a result of this evidence, a role in combination therapies has been hypothesized. The use of inhibitors of this pathway could be useful in overcoming resistance mechanisms to chemotherapeutics such as docetaxel (NCT04404140) or new-generation hormonal therapies (NCT03072238) (**figure 1**).

ROLE OF LIQUID BIOPSY

The need to overcome some traditional markers over time has enabled the development of new, minimally invasive methods such as liquid biopsy, which can provide essential information on prognostic stratification, molecular characterisation and monitoring response to therapy in prostate cancer. Cell-free-DNA (CfD-

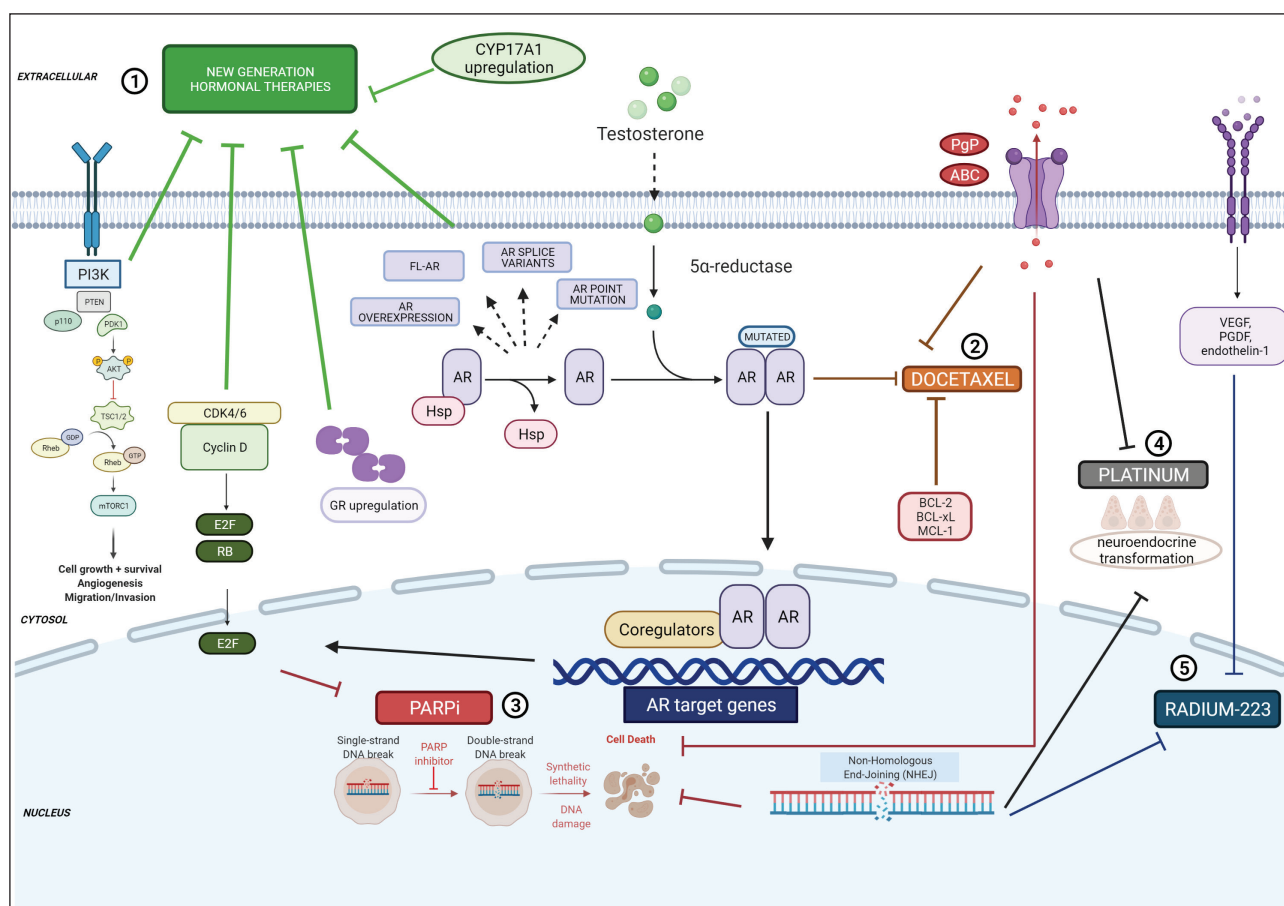


Figure 1. Main mechanisms of therapeutic resistance in mCRPC.

1) Mechanisms of resistance to new generation hormonal therapies. AR-related and non-AR-related alterations such as glucocorticoid receptor (GR) overexpression, cyclin-dependent kinase pathway (CDK4/6), phosphatidylinositol-3-kinase (PI3K).

2) Docetaxel resistance. Mechanisms preventing drug engagement (efflux pumps) and parallel mechanisms interfering with drug efficacy (AR signaling, inhibition of apoptosis via bcl-2/mcl1).

3, 4) Resistance to PARPi and platinum-based chemotherapy. Enhancement of DNA repair mechanisms and activation of efflux pumps, as well as activation of alternative mechanisms such as CDK 4/6 for PARPi alone.

5) Resistance to radiolabeled therapy with Ra-223. Enhancement of single helix repair mechanisms and stimulation of growth factors such as VEGF, PDGF, Endothelin-1.

NA) and circulating tumor cells (CTCs) may be representative of disease burden. In fact, they may reflect characteristics of the entire tumor, and may also be used as biomarkers to define prognosis and response to therapy. In particular, the IMMC-38 study defined the cut off <5 CTCs in 7.5 ml of blood to distinguish a favorable from an unfavorable prognosis (CTCs >5) in patients with mCRPC with significant impact in terms of OS (20.7 months in patients with favourable counts compared to 9.5 months for patients with unfavourable counts) (33). The use of cfDNA has also proved to be even superior to PSA variation in predicting OS (34). Some clinical studies have shown that the presence of AR-V7 variants on cfDNA in mCRPC patients suggests poor response to NGHT (35, 36). Conversely, the

presence of these variants does not suggest OS changes when using taxanes in subsequent lines (37). Although currently no method has received Food and Drug Administration (FDA) approval, multiple methods have been developed to identify AR-V7 variants on liquid biopsy. Scher *et al.* reported that the presence or absence of the AR-V7 variant may have an impact on second-line therapy choice, discriminating the use of taxanes from NGHT using a decision-making algorithm (38). Brown *et al.* emphasize that the detection of the AR-V7 variant should be the prerogative of high-risk mCRPC after progression to first-line hormonal therapy (39). According to current ESMO guidelines, the detection of the AR-V7 variant is of limited value in clinical practice (40). Furthermore, genetic mutations resulting in defi-

ciencies of the DNA repair mechanism are associated with resistance to NGHT and poor prognosis in mCRPCs (41). As will be discussed below, the most frequent mutations are characterized by loss of function in BRCA1, BRCA2 and ATM, which encode for proteins involved in the homologous recombination repair mechanism (HRR), a pathway involved in DNA double helix repair. Detection of homologous recombination deficiency (HRD) in cfDNAs is useful in stratification of patients eligible for PARPi. Current NCCN guidelines recommend the use of liquid biopsy for HRD detection in mCRPCs if tumour tissue is not sufficient for genetic testing. The usefulness of these tests, in addition to the use of target therapies, could have an impact on the therapeutic sequence (42). The PROREPAIR-B study showed that patients with carrier mutations for BRCA2 have worse outcomes when treated with taxanes followed by NGHT rather than NGHT followed by taxanes (43). Despite the use of cfDNA has shown great promise in monitoring the presence of reverting mutations associated with PARPi resistance, further studies are needed to validate the usefulness of the majority of HRR genes correlated with worse prognosis outcomes (e.g. CDK 12) when using NGHT.

TAXANES

Currently, taxanes are the only family of chemotherapeutic agents demonstrating an OS benefit in mCRPC (12, 11). Docetaxel, a taxane chemotherapeutic, was approved for the treatment of mCRPC men in 2004, according to the positive results of the TAX 327 trial, and is now standard of care for mCRPC (62). In addition, recent evidences demonstrated that *de novo* metastatic castration sensitive prostate cancer patients benefit from docetaxel administration, especially in case of high volume disease (2).

Docetaxel is an antineoplastic drug with an antimitotic function able to bind tubulin beta subunits in microtubules, stabilizing them and preventing depolymerization to complete mitosis (63). Taxanes also disrupt mitotic spindle formation, promoting apoptosis. The mechanisms of resistance to taxanes have been thoroughly investigated in recent years. There are two main strands of thought regarding taxanes resistance mechanisms (64). The first group sug-

gests that resistance is driven by a loss of drug target engagement whereby microtubules are not stabilized and microtubule bundles are not formed. Altered microtubules may inhibit taxane stabilization. In this regard, beta-tubulin mutations identified in preclinical models were not confirmed *in vivo* experiments (65). Other tubulin alterations such as disruption of microtubule-associated proteins and acetylation may impair drug binding and suppress stabilization. Another mechanism of resistance described is the aberrant activation of efflux pumps which reduce drug accumulation and prevent target engagement such as ATP-binding cassettes (ABC). In particular, P-glycoprotein expression is closely related to Docetaxel resistance in prostate cancer (66, 67). This mechanism of resistance underlies the efficacy of Cabazitaxel in Docetaxel refractory patients, due to the reduced affinity of Cabazitaxel to this transporter protein (68). However, assessment of the ABC protein has not shown in clinical practice (69) (**figure 1**). One alternative hypothesized mechanism is the ERG overexpression due to aberrant activity of the TMPRSS2:ERG gene fusion, which occurs in 50% of prostate cancer patients. ERG binds to soluble tubulin destabilizing the microtubules, shifting the dynamic balance between soluble and polymerized tubulin towards soluble. Since the microtubule polymer is the most active substrate for taxane binding, overexpression of ERG results in taxane resistance (70).

The second group analyses resistance mechanisms induced by pathways downstream of target engagement, establishing a so-called 'tolerance to microtubule stabilization'. This process directly involves BCL-2 or MCL-1 overexpression, p53 functional loss, Notch upregulation, NFkB activation or Gata2-IGF-2 increase (71-74).

There is also a close correlation between AR and taxanes (75). Microtubules integrity and dynamism is crucial for nuclear translocation of AR. In a phase II study enrolling taxane-treated mCRPC patients the overexpression of nuclear AR in circulating tumor cells (CTC) was a predictive resistance marker to taxane treatment (76).

Cabazitaxel, a second-generation taxane, was developed to overcome resistance to Docetaxel. In the phase III trial Cabazitaxel has been shown to elicit clinical responses and provide improved OS, compared with Mitoxantrone, in mCRPC patients previously treated with docetaxel (11).

The underlying mechanisms of resistance to Cabazitaxel are still largely unknown. However, a role of the multidrug resistance gene 1 (MDR1) has been suggested (77). Interestingly, the expression of chemokines and especially the secretion of CCL2 upon AR inhibition induces increased activity of transcription activator 3 (STAT3), leading to Cabazitaxel resistance due to increased migration and invasion of prostate cancer cells (78).

PARP-INHIBITORS

In the last decade poly (ADP-ribose) polymerase inhibitors (PARPi) were approved as a treatment in several forms of cancer. PARPi are a group of antineoplastic agents that induce the so-called synthetic lethality, a mechanism that occurs when PARPi and either another agent or an underlying genetic alteration together led to an unfixable DNA damage and consequently to the cell death.

These drugs showed promising results in patients with BRCA1/2 mutations and have currently become part of the standard treatment for breast and ovarian cancer.

According to NCCN guidelines (79) two PARPis - Rucaparib and Olaparib - have received approval as monotherapy by Food and Drug Administration (FDA) for the treatment of metastatic castration-resistant prostate cancer (mCRPC). Both agents are indicated for tumors with BRCA1/2 alterations, Olaparib is also indicated for patients with other homologous recombination deficiency (HRD) gene alterations.

Although the encouraging activity shown by PARPi in prostate cancer, their use is limited due to the relatively low frequency of BRCA germline mutations, accounting for about 20% (80). In addition, primary or acquired drug resistance markedly restricts the indications for use of these drugs.

Clinical resistance to PARPi in mCRPC has not been clearly elucidated. Resistance to PARP inhibitors may be innate, due to pre-existing mechanism, or acquired, when PARP inhibitors become ineffective after an initial clinical benefit (81-83).

Knowing the potential mechanism of primary resistance is particularly relevant to optimize treatment sequencing in mCRPC. Simmons (84) and colleagues reported the case of a patient with mCRPC with a germline BRCA2 mutation that was treated with carboplatin and subsequently with the PARP inhibitor rucaparib. Due to the patient's limited re-

sponse to this last drug, they analyzed his genomic profile using next-generation sequencing panel tests on patient's pre-treatment blood samples. They identified 12 different somatic reversion mutations that restored the BRCA2 open reading frame and potentially protein function. They assumed that the limited benefit was likely due to these reversion mutations responsible for the tumor insensitivity to PARP inhibitor treatment.

Many *in vitro* and *in vivo* studies, especially in ovarian and breast cancer patients, have identified several mechanisms of drug resistance (85): an increased drug efflux due to the upregulation of ABC transporters, a stabilization of stalled fork, a reduction of the trapping mechanism and a restoration of homologous recombination (86) (**figure 1**). This last mechanism was previously described in ovarian or breast cancer through the reversal of BRCA2 mutations. Quigley et al tried to detect if the same mechanism could be applied to prostate cancer and whether it can be detected in liquid biopsies. They described two patients which responded and then later relapsed on PARPi therapy (87). In one patient they performed a liver biopsy after progression to Talazoparib and then they analyzed DNA mutations finding the presence of two proteins with shorter lengths polypeptide chain that carried an altered BRC repeat domain 7. This domain would likely restore HRR function leading to treatment resistance. Carneiro *et al.* reported a case of acquired resistance to Olaparib (88). After the identification of two mutations in BRCA2, they treated a patient with Olaparib 400 mg twice per day observing a rapid PSA decrease (from 821 to 300 ng/mL) and a radiological response. The patient subsequently developed a disease progression and a ctDNA analysis was performed showing reversion mutations restoring both the BRCA2 germline mutation and the somatic second-hit loss-of-function mutation on the second allele.

Furthermore, they also investigated using ctDNA tests the prevalence of BRCA2 reversion mutations among a cohort of more than 1500 patients with mCRPC, finding a frequency of 40% in the germline mutation-positive PARP-exposed subgroup.

In this setting become particularly relevant to investigate the role of the liquid biopsy not only as a predictive marker of response but also as a resistance biomarker in metastatic prostate cancer. A prospectively planned analyses of TOPARP-A phase II trial investigated the variations in circulating cell free DNA (cfDNA) using whole-exome

sequencing in mCRPC patients treated with Olaparib (89). Ten of sixteen patients showed an initial tumor response to Olaparib, but then the disease progressed. The analyses of plasma samples collected before Olaparib treatment and at disease progression highlighted mechanisms of secondary resistance to PARP inhibition (like different new mutations restoring the PALB2 reading frame) suggesting the presence of a clonal evolution from treatment-selective pressure.

The challenge for the future is to find strategies to overcome resistance to PARPi.

One option is the combination with CDK inhibitors. In fact, it was demonstrated that the upregulation of the oncogene MYC can lead to double-strand DNA breaks requiring the activation of HR pathway to repair lesions (90). Furthermore, the increased expression of MYC can drive therapeutic resistance to other targeted therapeutic agents (91). A study conducted using triple negative breast cancer (TNBC) cell lines showed that Dinaciclib (a pan CDK 1/2/5/9 inhibitor) re-sensitized cells which had acquired resistance to niraparib and that this strategy combining could be also highly efficacious in ovarian, prostate, pancreatic, colon, and lung cancer cells (92).

Another described mechanism of resistance involves the upregulation of the multidrug efflux transporter P-glycoprotein (PgP) that lead to a reduced availability of PARP inhibitor (93). In some murine tumor models, which have developed PgP-mediated Olaparib resistance, the administration of a next generation PARP inhibitor with poor PgP affinity (AZD2461), demonstrated efficacy (94).

RADIOLABELED THERAPY

Radium-223 (Ra223) is an alpha emitting radionuclide currently approved by FDA and EMA in patients with mCRPC with symptomatic bone metastases and no known visceral metastases, progressing after at least two prior lines of systemic therapy. Alysmpica study showed the efficacy of this radionuclide in this setting in terms of OS (15). The Ra223 mechanism of action is mediated by the binding to the surface of metastatic bone lesions. Upon binding, the radiopharmaceutical emits alpha particles that cause lethal and significant damage to double-stranded DNA (95). At present, there are few data concerning possible mechanisms of resistance during therapy with Ra223. However,

we can distinguish between direct and indirect mechanisms of resistance.

The direct mechanism involves the inability of the drug to generate non-repairable DNA damage due to upregulation of DNA repair mechanisms by positive feedback. It is recognized that dysregulation of DNA repair mechanisms leads to chromosomal translation, chromosomal rearrangements, and high mutation rates, resulting in a growth advantage for the tumor cells (96). In particular, the non-homologous end-joining repair (NHEJ) mechanism plays a key role in radioresistance phenomena. Preclinical studies revealed that some AR genes are directly involved in upregulation of DNA repair genes, in particular NHEJ, conferring increased resistance to treatments requiring DNA double helix damage (97, 98). In support of these findings, it was also hypothesized that patients treated with Ra223 who were deficient in the DNA damage repair (DDR) mechanism could benefit more from such treatment. It has been demonstrated that the absence of DNA repair mechanism deficits results in lower response rates to radiation treatment and worse survival compared with patients with DDR (96). Based on these data, it was suggested that the inhibition of exogenous single helix repair mechanisms may provide a rationale for combination therapies with PARPi. In particular, there are ongoing clinical trials evaluating the efficacy of combination therapies between Ra223 and PARPi. Other direct mechanisms include the inability to deliver the drug to the bone surface due to altered vascularization near the tumor mass and the overexpression of the multidrug resistance protein (MDR).

Indirect mechanisms are related to the capability of tumor cells in stimulating the bone tumor microenvironment. In this regard, the overexpression of some angiogenesis related proteins released by prostate cancer cells within the bone because of the damage induced by radionuclide therapy, leads to an escaping of DNA damage at the double helix, which further drives tumor proliferation. The main proteins involved in this process are vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and endothelin 1 (99, 100) (**figure 1**)

Recently, the FDA approved beta-emitting radioisotope ^{177}Lu conjugated with a small molecule of PSMA-617 (^{177}Lu -PSMA) for men with PSMA-positive mCRPC previously treated with NGHT. However, a percentage of patients showed both

innate and acquired resistance to PSMA-radio-labelled therapy. The mechanisms of resistance to ^{177}Lu -PSMA therapy are still under investigation. Tumor heterogeneity still remains one of the main limitations in the efficacy of ^{177}Lu -PSMA therapy. In mCRPC tumors most metastatic lesions keep reasonable expression levels of prostate epithelial markers such as PSMA. Nevertheless, loss of prostate-specific epithelial markers with increased phenotypic plasticity as well as increased neuroendocrine differentiation may occur in some cases, affecting the efficacy of radiometabolic therapy. Disease distribution may also impact on treatment efficacy. Indeed, we know that metastatic lymph nodes show superior responses compared to bone sites of disease in patients receiving Lu-PSMA therapy. Liver metastases, on the other hand, show a lower chance of response and poorer survival outcome. In contrast, lung metastases show encouraging response rates with Lu-PSMA and do not confer a negative survival outcome. These changes in response may also be due to different tumor heterogeneity according to the examined metastatic sites. Finally, insufficient dose delivery may also contribute to the establishment of resistance mechanisms in Lu-177-PSMA therapy. The most frequent pattern of progression after Lu-PSMA therapy is diffuse tumor infiltration, likely due to a small volume of disease receiving an inadequate effective radiation dose (101).

CARBOPLATIN BASED CHEMOTHERAPY AND NEUROENDOCRINE TRANSFORMATION

Neuroendocrine differentiation (NE) in prostate cancer is an event that rarely occurs *de novo* and appears most commonly in patients with CRPC (102). Some patients may also present with an aggressive variant of the disease with similar features to small cell prostate cancer (SCPC) although without clear NE differentiation. Both the abovementioned variants are highly sensitive to platinum-based therapy though for a limited time (103).

Actually, platinum-based therapy is not generally used for the treatment of mCRPC because phase 3 studies have failed to show a survival benefit in unselected patients. Nevertheless, responses to single-agent chemotherapy with a platinum analogue have been reported (104).

Approximately 20% of patients with CRPC will present as having NE differentiation, whereas only 2% of patients experience *de novo* NE differentiation. There are several theories about the possibility of NE differentiation in mCRPC. The most recent hypothesis is that a divergent clonal evolution from CRPC-adenocarcinoma to CRPC-NE results in an AR-dependent to AR-independent condition (108). Another hypothesis suggests that prostate cancer cell plasticity events during AR suppression induces overexpression of transcription factors such as SOX2,11 and retinoblastoma 1(Rb1), TP53 and PTEN tumor suppressor gene alterations (105, 106). In this regard, Rb1 is the most investigated target in overcoming resistance during new generation hormone therapy, as the interaction between RB1 and AR transcription genes is widely known. Loss of function of Rb1 stimulates the tumor microenvironment to increased proliferation and to uncontrolled cell growth, promoting the development of NE transformation (107). Cyclin-dependent kinase inhibitors can prevent RB1 phosphorylation and inactivation, restoring sensitivity to hormonal treatment (105).

As previously reported, patients with neuroendocrine differentiation may benefit from platinum-based therapy, albeit with limited survival (108). Patients treated with platinum-based therapy may develop early resistance to treatment due to a progressive inability to induce apoptosis. Apoptosis is the final step in the chemotherapy mechanism of action, resulting in induced cell death. Platinum-based antineoplastic drugs enhance the ability to activate apoptosis pathways, inducing direct DNA damage. Conversely, defects in activation of the apoptosis pathway result in ineffectiveness of these agents, resulting in the development of drug resistance (**figure 1**). In this regard, it is known that activation of the tumor suppressor gene TP53, having a DNA repair function through production of the p53 protein, is closely involved in platinum resistance. Platinum-resistant tumor cells usually exhibit defects in the apoptosis induction as a result of overexpression of anti-apoptotic proteins or deficient mitochondrial signaling. Notably, pro-apoptosis signaling pathways such as MAPK, ERG, PI3K, NF- κ B, the tumor microenvironment, and epigenetic regulatory factors play a crucial role in these unexpected phenomena.

A further mechanism of platinum-related resist-

ance is the lack of intracellular accumulation of the drug, resulting in a loss of lethal effect on DNA. In this case, reduced drug uptake at the intracellular level due to inefficient diffusion mechanisms and subsequent endocytosis leads to decreased drug viability in the intracellular compound, with subsequent drug resistance.

These resistance mechanisms are usually simultaneously activated in platinum-resistant tumor cells, generally leading to a poor outcome in platinum-resistant patients (109, 113).

It is important to notice that in ovarian cancer resistance to platinum-based chemotherapies has historically been considered a strong predictor for PARPi resistance, indicating possible shared mechanisms (17). On the other hand, it was observed that reversion of the HR defect conferring PARPi resistance will not always confer cross-resistance to platinum, providing a rationale for a subsequent platinum-based therapy in patients who have progressed on PARP inhibitors therapy (110). In fact, in ovarian cancer patients the objective responses to chemotherapy in the post-PARPi setting were observed regardless of pre- PARPi platinum sensitivity (111).

CONCLUSIONS

Treatment options in the mCRPC setting have substantially increased in recent years with the introduction of both target therapies and classical cytotoxic agents.

Although there are several effective drugs available that have dramatically increased patient survival and quality of life in this setting, the efficacy of these agents is deeply limited by the occurrence of resistance mechanisms.

A deeper understanding of mechanisms of both primary and acquired resistance to treatment, is crucial to optimize mCRPC treatment sequence and to identify predictive biomarkers of response. Patients treated with new generation hormonal therapies (Abiraterone or Enzalutamide) or taxanes (Docetaxel or Cabazitaxel) may present with primary resistance, or eventually develop resistance during treatment. Mechanisms of resistance to AR-targeted agents includes: intratumoral androgen production, amplification, mutation, or expression of AR splice variants, increased steroidogenesis, up-regulation of signals downstream of the AR, and development of androgen-independent tumor cells.

Known mechanisms of resistance to chemotherapy are distinct, and include: tubulin alterations, increased expression of multidrug resistance genes, TMPRSS2-ERG fusion genes, kinesins, cytokines, and components of other signaling pathways, and epithelial-mesenchymal transition. Several trials testing molecules targeting the different identified AR and non- AR-driven pathways involved in the drug resistance mechanisms are currently ongoing. Future research will have to focus on the identification of predictive markers of response and on the evaluation of treatment sequencing and combination able to overcome drug resistance (**table I**).

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Availability of data and materials

The data underlying this article can be shared just before a reasonable request to the corresponding author.

Authors' contributions

All authors contributed equally.

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N/A.

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