

## REVIEW

## Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches

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Prostate cancer is the second-leading cause of cancer-related mortality in men in Western societies. Androgen receptor (AR) signaling is a critical survival pathway for prostate cancer cells, and androgen-deprivation therapy (ADT) remains the principal treatment for patients with locally advanced and metastatic disease. Although a majority of patients initially respond to ADT, most will eventually develop castrate resistance, defined as disease progression despite serum testosterone levels of <20 ng/dl. The recent discovery that AR signaling persists during systemic castration via intratumoral production of androgens led to the development of novel anti-androgen therapies including abiraterone acetate and enzalutamide. Although these agents effectively palliate symptoms and prolong life, metastatic castration-resistant prostate cancer remains incurable. An increased understanding of the mechanisms that underlie the pathogenesis of castrate resistance is therefore needed to develop novel therapeutic approaches for this disease. The aim of this review is to summarize the current literature on the biology and treatment of castrate-resistant prostate cancer.

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## INTRODUCTION

The mainstay of therapy for patients with locally advanced prostate cancer, metastatic prostate cancer and biochemically recurrent disease after failure of localized treatments is androgen-deprivation therapy (ADT) with gonadotropin-releasing hormone analogs and anti-androgens.<sup>1</sup> Gonadotropin-releasing hormone analogs like leuprolide cause continuous stimulation of the pituitary gland, leading to 'chemical castration' with suppression of testosterone production from the testes, whereas anti-androgens directly inhibit the androgen receptor (AR).

ADT is known to provide remission of the disease, best evidenced by a decline of prostate-specific antigen (PSA) in about 90% of patients.<sup>2</sup> After a mean time of 2–3 years, however, the disease progresses despite continuous hormonal manipulation. This type of cancer is known as castrate-resistant prostate cancer (CRPC).<sup>2</sup> Metastatic castration-resistant prostate cancer (mCRPC) is associated with a poor prognosis and mean survival time of only 16–18 months.<sup>3</sup>

Docetaxel and cabazitaxel are the only United States Food and Drug Administration (FDA)-approved chemotherapies for the treatment of mCRPC (Table 1). These tubulin-binding taxanes have been proven to decrease PSA levels and palliate symptoms but survival benefits are modest.<sup>4</sup> Another agent, sipuleucel-T (Provenge; Dendreon Corp., Seattle, WA, USA) is a cellular immunotherapy that has been shown to increase overall survival time by 4.1 months on average but not progression-free survival time for patients with mCRPC (Table 1).<sup>5–7</sup>

The AR is believed to remain active in CRPC, and several new strategies to inhibit AR signaling have recently been developed. Abiraterone acetate (Zytiga; Janssen Biotech, Inc. Horsham, PA, USA) is an FDA-approved inhibitor of androgen biosynthesis, which blocks cytochrome P450-c17 (CYP17), leading to suppression of androgens derived from the adrenal glands, the prostate tumor and the tumor microenvironment (Table 1).<sup>8</sup> This novel therapy increased survival time by almost 4 months, increased the time to PSA progression, and was relatively well tolerated by patients who had failed chemotherapy.<sup>8</sup> Moreover, according to a phase III clinical trial, abiraterone acetate led to increased radiographic progression-free survival and overall survival in patients with chemotherapy-naïve mCRPC.<sup>9</sup> MDV3100 (now known as enzalutamide), an AR antagonist that prevents nuclear translocation and chromatin binding, has produced similar results in recent clinical trials (Table 1).<sup>10,11</sup>

Although novel cytotoxic agents, AR-blocking agents and immunotherapies represent effective therapy strategies for mCRPC, important clinical questions remain. First, the absence of any reliable biomarker, apart from serum PSA, for AR-targeted therapies does not allow clinicians to decide which patients will benefit from these treatments or when to alter or terminate treatment. In addition, despite the effective blocking of androgen biosynthesis<sup>12</sup> and AR signaling, all patients eventually progress.<sup>8,13,14</sup> Furthermore, the optimal sequencing of these therapies remains unknown.

The general aim of this review is to summarize new evidence about mechanisms of castrate resistance and novel

**Table 1.** Therapeutic agents in clinical oncology development for castrate-resistant prostate cancer

| <i>Therapeutic agent</i> | <i>Mechanism of action</i>  | <i>Clinical trial status</i>   | <i>Therapeutic efficacy</i>   | <i>References</i> |
|--------------------------|---|--|---|-------------------|
| Docetaxel                | Stabilization of tubulin, induction of cell cycle arrest and inhibition of cell proliferation                           | FDA approved   | Overall survival benefit and palliation of cancer-associated symptoms   | 4                 |
| Cabazitaxel              | Stabilization of tubulin, induction of cell cycle arrest and inhibition of cell proliferation                           | FDA approved for patients after failure of docetaxel   | Overall survival benefit and palliation of cancer-associated symptoms   | 4                 |
| Sipuleucel-T (Provenge)  | Enhancement of patients' autologous antigen-presenting cells to induce cytotoxic response against prostate cancer cells | FDA approved   | Increase in overall survival but not progression-free survival  | 5–7               |
| Abiraterone acetate      | Irreversible inhibition of CYP17 and subsequent androgen synthesis  | FDA approved in the pre- and post-docetaxel settings   | Increase in overall survival (almost 4 months), radiographic progression-free survival, time to PSA progression, and palliation of cancer-associated symptoms | 8,9               |
| MDV3100 (Enzalutamide)   | AR antagonist preventing nuclear translocation and binding to chromatin   | FDA approved in the post-docetaxel setting   | Increase of overall survival (4.8 months), radiographic progression-free survival and time to PSA progression.  | 10,11             |
| BEZ235                   | Inhibition of PI3K  | Phase III clinical trial in comparison with placebo in chemotherapy-naïve patients   | Results pending   |                   |
| RAD001 (Everolimus)      | Inhibition of mTOR  | Phase I/II clinical trials in combination with Abiraterone acetate (NCT01717898)   | Results pending   |                   |
| Dovitinib (TK1258)       | Inhibition of FGFR  | Phase II clinical trial in combination with bicalutamide (NCT00630344)   | Failure to show increase in time to progression   | 86                |
| Cabozatinib (XL184)      | Inhibition of c-MET   | Phase II clinical trial in patients after failure of docetaxel-based chemotherapy (NCT01741116)  | Results pending   |                   |
|                          |   | Phase II clinical trial in patients with mCRPC (NCT01428219)   | Reduction of soft tissue lesions, resolution of bone scans, increase of progression-free survival   | 140               |
|                          |   | Phase III clinical trial in comparison with prednisone in patients previously treated with docetaxel and abiraterone or MDV3100 (COMET-1, NCT01605227) | Results pending   |                   |
|                          |   | Phase III clinical trial in comparison with mitoxantrone and prednisone (COMET-2, NCT01522443)   | Results pending   |                   |

Abbreviations: FDA, Food and Drug Administration; PSA, prostate-specific antigen.

therapeutic approaches directed at this complex and enigmatic disease state.

### THE ROLE OF AR SIGNALING IN CRPC

The expression of PSA is mediated by androgen response elements. This suggests that the increasing PSA during ADT reflects activation of AR transcriptional activity.<sup>15</sup> Consistent with this hypothesis, various recent findings have supported the notion that one of the most important mechanisms in CRPC development is the continuous activation of AR in prostate cancer cells.<sup>3</sup> Several cellular and molecular alterations are related to this post-castration activation of the AR, including incomplete blockade of AR-ligand signaling, AR amplifications, AR mutations, aberrant AR co-regulator activities and AR splice-variant expression.<sup>16</sup>

Recent data suggest that despite castration, which decreases serum androgen levels, intratumoral levels of testosterone and dihydrotestosterone in patients with mCRPC are similar to those found in hormone therapy naïve.<sup>2</sup> Evidence suggests that upregulation and stimulation of enzymes involved in androgen biosynthesis such as AKR1C3 occur within the tumor microenvironment<sup>17–20</sup> while several studies evaluating androgen

levels and mRNA levels of relevant enzymes suggest that prostate cancer bone metastases can convert adrenal androgens to testosterone and dihydrotestosterone.<sup>19,20</sup> Interestingly, according to a recent report by Chang *et al.*,<sup>21</sup> mCRPC is dependent on conversion of androstenedione to 5 $\alpha$  androstenedione, which is then converted to dihydrotestosterone, bypassing testosterone. Of note, metastatic prostate cancer may amplify the HSD17B3 gene and sustain copy numbers loss of the HSD17B2 gene, leading to decreased conversion of testosterone to the less active androstenedione.<sup>22</sup> In another recent study, Mitsiades *et al.*<sup>23</sup> found significant heterogeneity in the expression of various steroidogenic enzymes in the tumor microenvironment among patients with mCRPC, suggesting that the combination of enzymatic blockage and potent anti-androgens is a reasonable therapeutic approach for patients with mCRPC. Moreover, Efsthathiou *et al.*<sup>24</sup> recently showed that abiraterone acetate effectively suppresses testosterone concentrations in both blood and the tumor microenvironment to less than picograms per milliliter. Despite this degree of efficacy, patients receiving abiraterone acetate will eventually show evidence of disease progression.

Enzalutamide is a novel AR antagonist that overcomes resistance to conventional anti-androgens by inhibiting nuclear

localization and chromatin binding of AR.<sup>13</sup> According to a recent phase I/II study in patients with mCRPC, the use of enzalutamide is safe, elicits PSA and radiographic response, and results in a median time to progression of 47 weeks.<sup>10</sup> The superiority of enzalutamide over placebo was confirmed in a phase III clinical trial that showed increased overall survival (4.8 months) and improvement in all secondary end points in patients with mCRPC after chemotherapy.<sup>11</sup> Despite these encouraging results, after a period of remission that is characterized by significant variation in therapeutic response, these tumors eventually progress.

#### AR amplification

CRPC is associated with increased expression of AR attributed to gene amplification<sup>25</sup> and other mechanisms, which include decreased retinoblastoma protein (RB) and increased E2F activity leading to increased AR expression<sup>26</sup> and anti-androgen-mediated de-repression of AR expression and signaling.<sup>27</sup> Interestingly, an early study showed that 80% of the tumors acquiring AR amplification also demonstrated higher levels of AR protein.<sup>28</sup> Furthermore, studies utilizing fluorescence *in situ* hybridization have shown that while AR gene amplifications are observed in 20–25% of CRPC<sup>29</sup> and in many cancer cell lines derived from these patients, they are very rare in primary tumors.<sup>30</sup> These gene amplifications, which are heterogeneous among cancerous cells in the same tumor,<sup>31</sup> are related to sensitization of the AR signaling pathway to lower levels of androgens. On the other hand, AR and AR-regulated genes have been shown to be upregulated in prostate cancer xenografts after castration without any increase in the number of gene copies,<sup>32,33</sup> suggesting that AR amplification is not the primary mechanism of the paradoxical upregulation of certain AR target genes after castration in these models.<sup>32</sup>

#### AR mutations

Multiple early studies evaluating the prevalence of AR gene mutations have demonstrated variable results.<sup>34–36</sup> Most mutations are located in the ligand-binding domain, providing a mechanistic explanation for the development of resistance to anti-androgen therapy. The results of more recent studies provide support for the hypothesis that the absence of androgens or the presence of anti-androgens acts as selective pressure for emergence of mutations in the AR gene. Consistent with these results, it has been established that AR mutations are rare, but treatment with AR antagonists increase their incidence selecting for mutant ARs.<sup>37,38</sup> AR mutations documented in mCRPC are related to decreased specificity of AR–ligand interaction, allowing AR activation by alternative steroidal molecules, including estrogens, corticosteroids and progesterone.<sup>39,40</sup> The NH2 terminal region, which is known to be critical for the interaction of AR with co-regulators, has also been shown to harbor mutations.<sup>41</sup> Moreover, mutation analysis identified a 5-amino-acid core sequence located in the NH2 terminal region, <sup>435</sup>WHTLF<sup>439</sup>, which can mediate androgen-independent AR activation.<sup>42</sup>

#### AR splice variants

Identification of AR splice variants in cell lines and tumor tissues derived from patients with CRPC provides an additional mechanistic explanation for the development of CRPC.<sup>43,44</sup> Many of these variants result from insertion of cryptic exons downstream of the coding sequences for the DNA-binding domain or from deletion of exons coding for the ligand-binding domain, leading to the formation of an AR molecule lacking the ligand-binding domain.<sup>45,46</sup> The regulation of variant AR expression is poorly understood, although suppression of ligand-binding domain by an androgen antagonist is known to cause increased expression of AR variants in prostate cancer cell lines.<sup>47</sup> Sun *et al.*<sup>46</sup> suggested that

activation of AR variants leads to upregulation of steroidogenic enzymes, providing prostate cancer cells with higher levels of AR ligands. AR variants are also believed to increase the expression of AR-regulated genes in the absence of any ligand, providing an explanation for the activated AR signaling pathway in CRPC.<sup>46,47</sup>

Hu *et al.*<sup>47</sup> recently used LNCaP95 and VCaP prostate cancer cells to show that inhibition of AR by either small interfering RNA or enzalutamide leads to upregulation of the ARV7 ligand, one of the most frequently observed AR variants in clinical specimens. Of note, full-length AR and ARV7 are expressed differently in each cell line. Full-length AR activates pathways related to biosynthesis, metabolism, and secretion, whereas ARV7 increases the expression of cell-cycle genes, including the activator of the M-phase check point UBE2C.<sup>47</sup> Moreover, when LuCaP35CR xenografts were treated with abiraterone acetate, both full-length AR and ARV7 were upregulated, but the expression of UBE2C paralleled expression of ARV7. This relationship was also demonstrated in clinical prostate cancer samples following ADT.<sup>47</sup> Inhibition of AR in LNCaP and CWR22Rv1 cells did not induce activation of AR variants, providing an explanation for why some xenografts respond to AR suppression with tumor growth inhibition. In a recent report by Li *et al.*,<sup>48</sup> a 48-kb deletion in AR intron 1 was identified in a subset of cells in the heterogeneous CWR-R1 cell line, and was related to the expression of the AR1/2/3/CE3 splice variant (AR-V7/AR3). Following enzalutamide treatment, the AR-V7/AR3-expressing clone expanded and the presence of this variant contributed to tumor growth during ADT. Of interest is that treatment of AR-V7/AR3-enriched LNCaP cells with high concentrations of dihydrotestosterone caused upregulation of PSA but downregulation of M-phase genes, including UBE2C, CDCA5 and CCNA2. These results implicate expression of AR splice variants in the pathogenesis of CRPC, especially after enzalutamide treatment.<sup>48</sup> Finally, genomic deletion of exons 5–7 has been related to increased expression of ARV12 in prostate cancer cell lines<sup>44</sup> while AR-antagonists targeting the N-terminal domain are believed to be effective against these variants.<sup>49</sup>

#### Post-translational modifications of AR

Other possible mechanisms related to persistent AR transcriptional activity during ADT are post-translational alterations of AR, especially phosphorylation. Guo *et al.*<sup>50</sup> suggested that Src-induced phosphorylation of ARY534 can lead to both AR sensitization to low levels of androgens and androgen-independent activation of AR. Moreover, it is believed that Etk, a non-receptor tyrosine kinase and downstream effector of Src and phosphatidylinositol 3 kinase (PI3K), is upregulated during ADT and phosphorylates AR at Y534 and Y551/552, stabilizing it and promoting its activity under androgen-depleted conditions.<sup>51</sup> Finally, Tyr284 phosphorylated and activated ACK1 has been related to increased Tyr267 phosphorylation of AR, disease progression and decreased survival of PCa patients.<sup>52</sup>

#### AR co-regulators and collaborating factors

Another critical mechanism implicated in the development of CRPC is the interaction between AR and co-regulators. Wang *et al.*<sup>53</sup> showed in a castrate-resistant LNCaP derivative that AR directly regulates M phase genes including CDC20, CDK1 and UBE2C, inactivating the M-phase check point and promoting tumor growth in xenografts. Chromatin immunoprecipitation analysis showed enhanced activities of the AR co-stimulator MED1 and of the FoxA1 and GATA2 AR collaborating factors in castration-resistant cells, whereas silencing of these factors decreased UBE2C mRNA levels.<sup>53</sup> Results from this study indicate that the distinctive pattern of AR transcriptional activity in castrate-resistant cells is determined to a large extent by coactivator stimulation and accompanying chromatin modifications. It is interesting that interleukin 6-mediated nuclear receptor

coactivator 1 (NCoA1, or SRC-1) phosphorylation promotes AR-dependent transcription in a ligand-independent manner, whereas mitogen-activated protein kinase (MAPK)-mediated phosphorylation of this coactivator may increase its affinity for AR, contributing to disease recurrence and CRPC.<sup>54</sup>

It is also known that bicalutamide therapy reduces recruitment of the nuclear receptor co-repressor (NCoR) in cells with increased AR protein.<sup>32</sup> According to recent reports, NCoA2 (SRC-2) expression is positively associated with high tumor cell proliferation and early disease relapse.<sup>55</sup> Downregulation of NCoA2, which is amplified in a subset of PCa reduces AR target gene expression and inhibits proliferation of AR-dependent and -independent prostate cancer cells, whereas it is believed that in AR-positive cancer cells, high levels of androgens repress NCoA2 expression.<sup>56</sup> Finally, NCoA3 (SRC-3), which is positively associated with tumor recurrence and PI3K/Akt activation, is known to be critical in the development of poorly differentiated prostate cancer.<sup>57</sup>

#### AR transcriptional activity

During the development of CRPC, AR transcriptional activity is modified and aberrant regulation of numerous genes that promote cell survival and proliferation occurs. Available data indicate that AR-stimulated genes are initially repressed but subsequently rebound during ADT. For example, Holzbeierlein *et al.*<sup>58</sup> reported a gene-expression analysis of prostate tumors during hormonal therapy showing that multiple AR target genes were upregulated during ADT and were associated with clinical resistance to therapy. Moreover, Sharma *et al.*<sup>26</sup> suggested that retinoblastoma protein loss and subsequent E2F transcriptional factor stimulation can lead to deregulation of AR and androgen-independent activation of multiple AR-stimulated genes. Recent reports added support to the concept that multiple alternative oncogenic pathways can contribute to generation of AR signaling that is hypersensitive to low levels of androgens.<sup>59</sup>

During AR signaling, AR can act as either a transcriptional enhancer or repressor for downstream target genes depending on androgen levels. For example, Cai *et al.*<sup>27</sup> found that with increasing androgen levels, AR directly represses transcription of the AR gene in androgen-dependent VCaP cells through the ARBS2 enhancer located within intron 2 of the AR gene. In contrast, with low androgen levels following androgen deprivation, transcription of the AR gene increases. Additional AR target genes involved in androgen biosynthesis, DNA synthesis and repair, cell cycle and proliferation are similarly repressed with high androgen levels and become de-repressed with low androgen levels. Consistent with these activities, expression of androgen-repressed genes is increased in castrate-resistant VCaP-derived VCS2 cells and VCaP xenografts, and in human CRPC samples. These data suggest a model of castrate resistance whereby androgen levels are adequate to enhance expression of positively regulated AR target genes related to cellular metabolism, but are insufficient to recruit AR and lysine-specific demethylase 1 to suppressor elements on genes that are negatively regulated by AR, and are related to DNA replication and cellular proliferation.<sup>27</sup> Thus, a combination of enhanced and de-repressed AR target genes ultimately contributes to castrate-resistant progression (Figure 1).

#### ALTERNATIVE GROWTH AND SURVIVAL PATHWAYS IN CRPC

During the transition from androgen dependence to castrate resistance, cancer cells become driven by alternative growth signaling pathways. Many of these pathways can participate in normal cellular processes, but can also become oncogenic in the early adaptive period after ADT has begun.<sup>60</sup> An increased understanding of the molecular interactions between androgen

signaling and these alternative growth and survival pathways should lead to the identification of novel therapeutic targets.

#### C-myc overexpression

The proto-oncogene c-myc is a known regulator of cell growth and has a critical role in prostate cancer development and progression. Gurel *et al.*<sup>61</sup> showed that c-myc is frequently overexpressed in prostate intraepithelial lesions (PINs) with an incremental increase from normal tissues to low-grade PIN to high-grade PIN. Various mouse models have shown that c-myc overexpression in prostate luminal cells can lead to hyperplasia and PIN lesions but not invasive carcinoma.<sup>62–64</sup> Alternatively, genetically engineered mice that express higher levels of c-myc from the ARR2PB prostate-specific promoter develop PIN that progresses to prostate cancer with variable frequencies.<sup>65,66</sup> Interestingly, specific amplifications of the c-myc gene have been confirmed in up to 72% of CRPC<sup>67</sup> and ADT has been suggested to increase the incidence of these amplifications.<sup>68</sup> Finally, Bernard *et al.*<sup>69</sup> demonstrated that while AR inhibition causes downregulation of c-myc in prostate cancer cells, prostate cancer cells that overexpress c-myc continue to grow under ADT. Together, these data support a role for c-myc activation in the development of prostate cancer and as a potential mechanism for CRPC development.

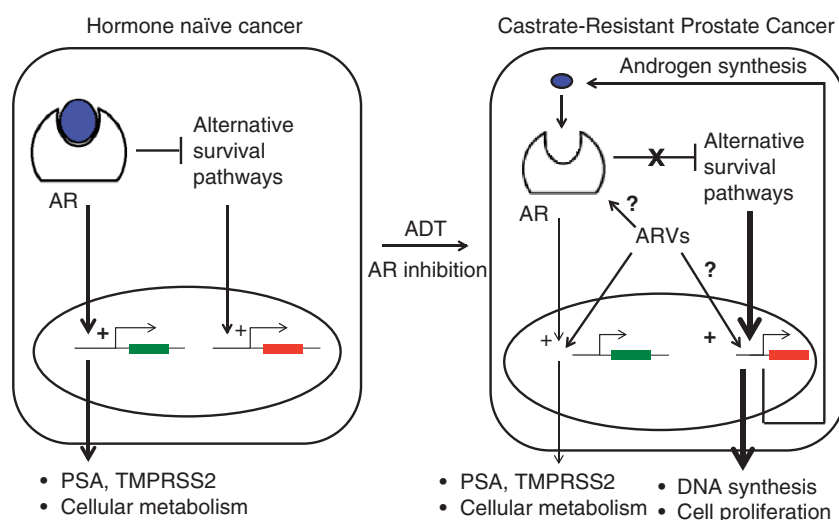
#### The PI3K/Akt/mammalian target of rapamycin (mTOR) pathway

The PI3K pathway is one of the most critical in human cancer.<sup>70</sup> Various growth factors, including insulin-like growth factor (IGF) and fibroblast growth factor (FGF), regulate this pathway, leading to activation of PI3K and the formation of PIP3. PIP3 activates AKT via phosphorylation and phosphorylated Akt (pAkt) activates multiple molecules involved in cell survival and proliferation, including MDM2, c-myc, GSK3 $\beta$ , nuclear factor- $\kappa$ B and mTOR. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid phosphatase that functions as the main inhibitor of PI3K/Akt signaling. Genetic alterations of the PI3K signaling pathway occur in 42% and 100% of primary and metastatic prostate cancers, respectively, suggesting this pathway is crucial in the development of CRPC.<sup>60</sup>

In an important study using the PbCre;Pten<sup>loxP/loxP</sup> mouse model characterized by prostate-specific inactivation of PTEN, Wang *et al.*<sup>71</sup> showed that 100% of mice developed invasive adenocarcinoma between 9 and 29 weeks, 5 of 11 had lymph node metastasis between 12 and 29 weeks, and 3 of 11 mice developed lung metastases. Following castration, there was a time-dependent phenotype characterized by an immediate initial increase in prostate cancer cell apoptosis (3 to 6 days post-castration) that was gradually replaced over time with the outgrowth of Pten null castrate-resistant proliferative clones. The results of more recent studies using Pten-knockout mice are generally consistent with the initial reports, yet time to progression to PCa may be variable. In this regard, transgenic mouse models with combined PTEN and p53 knockout lead to rapid PCa development with higher incidence.<sup>72–74</sup>

Results from PI3K, Akt and mTOR inhibitors in clinical trials and xenograft studies<sup>75–77</sup> suggested a potential interaction between AR and PI3K/Akt signaling pathways. Two recent publications reported negative feedback regulation between AR and PI3K/Akt pathways. First, Carver *et al.*<sup>78</sup> initially observed that mice with prostate Pten deletion (Pten<sup>loxP/loxP</sup>) had lower AR levels than their wild-type littermates, whereas treatment with BEZ235, a dual PI3K and mTOR inhibitor, and RAD001, an mTOR inhibitor, rescued AR protein levels. Similar results were achieved in LNCaP cells, demonstrating that PI3K pathway inhibition can upregulate AR and activate AR target gene expression.<sup>78</sup> Subsequent experiments in LNCaP cells and Pten<sup>loxP/loxP</sup> mice showed that





**Figure 1.** Androgen deprivation therapy initiates alterations in gene-expression profiles in prostate cancer cells. AR induces the expression of PSA, transmembrane protease serine 2 (TMPRSS2) and multiple genes related to cellular metabolism. AR signaling also inhibits the activation of alternative survival pathways, that is, PI3K/Akt. ADT results in de-repression of alternative survival pathways inducing the expression of genes related to DNA synthesis and cell proliferation and in recalibration of prostate cancer tissue androgen levels leading to partial restoration of AR transcriptional activity. ADT may also lead to expansion of prostate cancer cells expressing AR variants (ARVs), which may contribute to altered gene expression.

this PI3K–AR interaction is mediated in part by upregulation of HER3 after inhibition of PI3K.<sup>78</sup>

Second, Mulholland *et al.*<sup>79</sup> used the  $PbCre;Pten^{loxp/loxp}$  model to demonstrate that Pten loss suppresses AR transcriptional activity and generates a gene-expression profile resembling that of the castration phenotype. A mechanism for this crosstalk involved negative regulation of EGR1, c-JUN and EZH2 by PTEN. Interestingly, according to a study by Lin *et al.*,<sup>80</sup> Akt phosphorylates AR leading to increased AR ubiquitinylation and degradation suggesting an alternative mechanism for the negative crosstalk between AR and Akt. These reports demonstrate that the PI3K/Akt pathway can inhibit AR transcriptional activity. It should be mentioned that in an earlier study Wang *et al.*<sup>81</sup> found that rapamycin stimulated AR transcriptional activity while the combination of rapamycin and bicalutamide increased apoptosis in prostate cancer cells. The authors attributed this effect to negative crosstalk between Akt-independent mTORC1 signaling and AR in prostate cancer cells. Finally, other studies indicate that specific experimental conditions and/or genetic manipulations may lead to results that are not consistent with negative crosstalk between AR and Akt.<sup>82,83</sup>

Additional experiments showed that after 7 days of enzalutamide treatment of  $Pten^{loxp/loxp}$  mice, despite decreased AR transcriptional activity the tumors had not significantly regressed and were histologically similar to those before treatment, although the treatment was much more effective in transgenic mice with inducible c-myc.<sup>78</sup> Further studies revealed increased Akt phosphorylation at Ser473 in the  $Pten^{loxp/loxp}$  mice and in LNCaP and LAPC4 AR-positive cells after castration and enzalutamide treatment, respectively. The same treatment did not increase pAkt in PC-3 cells, which are AR negative.<sup>78</sup>

Similarly, Mulholland *et al.*<sup>79</sup> used the  $PbCre;Pten^{loxp/loxp}$  model to show that castrate-resistant cancer developed in the regions of the prostate with combined Pten and AR loss. In contrast, AR-positive regions contained lower levels of pAkt with less cell proliferation after castration. These observations led to the conclusion that active AR transcriptional activity can inhibit phosphorylation of Akt to activate a potent oncogenic pathway. Both groups of investigators attributed this interaction to the upregulation of FKBP5 and PHLPP by AR.<sup>78,79</sup> It is known that

FKBP5 functions as a scaffolding protein for pAkt and PHLPP, promoting pAkt dephosphorylation by PHLPP.<sup>84</sup>

Cross talk between the AR and PI3K/Akt pathways supports the rationale for combining AR and PI3K/Akt inhibitors in CRPC. Although BEZ235 and enzalutamide modestly inhibit LNCaP cell proliferation as single agents, the combination significantly decreased cell numbers via apoptosis *in vitro*. In LNCaP xenografts, the combination produced a greater reduction of tumor volume compared with vehicle-only or single therapy (that is, BEZ235 or castration only).<sup>78</sup> Mulholland *et al.*<sup>79</sup> compared treatment of  $Pten^{loxp/loxp}$  mice and  $Pten^{loxp/loxp}$  mice carrying AR deletion ( $Pten^{loxp/loxp}AR^{L/Y}$ ) with rapamycin only, castration only or the combination. They found significantly enhanced tumor regression in the combination-therapy group relative to that in the two single therapy groups in both models. Despite these encouraging results, recent data indicate complex adaptive resistance pathways to PI3K/mTOR inhibition *in vivo*, and clinical data are lacking.<sup>85</sup> Finally, it should be noted that the efficacy of BEZ235 and RAD001 for mCRPC in combination with bicalutamide are currently being evaluated in phase I and II clinical trials<sup>86</sup> (Table 1).

#### Other growth and survival pathways contributing to CRPC

The RAS/MAPK pathway has also been implicated in prostate cancer development, progression and metastasis. RAS signaling is known to decrease the androgen dependence of LNCaP cells<sup>87</sup> and to promote metastasis in DU145 cells, and tissue specimens from patients with mCRPC display higher levels of pMAPK, a downstream target of RAS signaling.<sup>88</sup> According to a recent report from Mulholland *et al.*,<sup>89</sup> the RAS/MAPK pathway was activated in 43% of primary prostate cancer samples and 90% of metastatic samples. Phospho-MAPK levels were more prominent in tissues derived from patients who had received ADT and were mainly observed in the basal cell compartment. Moreover, although conditionally activated K-ras in the prostatic epithelium of transgenic mice ( $K-ras^{L/W}$ ) is not sufficient to promote prostate cancer development, when  $K-ras^{L/W}$  mice were crossed with  $PbCre;Pten^{loxp/loxp}$  mice, simultaneous deletion of Pten and activation of RAS led to the

development of poorly differentiated carcinoma within 10 weeks.<sup>89</sup> In contrast with the *PbCre;Pten<sup>loxp/loxp</sup>* mice, *PbCre;Pten<sup>loxp/loxp</sup>K-ras<sup>LW</sup>* mice developed lung and liver metastases with 100% penetrance but not bone metastasis, although their expression of AR and AR-targeted genes was much lower. Histologic and gene-expression analysis of cancer tissues showed that expression of mesenchymal markers, including N-cadherin, and cell-cycle genes, including UBE2C, was greater than in *PbCre;Pten<sup>loxp/loxp</sup>* mice, suggesting that RAS pathway activation promotes the de-differentiation of prostate cancer cells.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a secreted cytokine that is implicated in various cellular processes, including cell proliferation and cancer progression.<sup>90,91</sup> Several studies have suggested that in normal epithelial cells and early-stage cancers, TGF- $\beta$  signaling may be growth suppressive.<sup>90,92</sup> In advanced prostate cancer, however, TGF- $\beta$ 1 levels are increased, suggesting a growth promoting role.<sup>93–95</sup> It has also been shown that the expression of an isoform of this cytokine, TGF- $\beta$ 3, is increased in prostate cancer and that TGF- $\beta$ 3 signaling is upregulated in CRPC.<sup>96</sup> Androgens regulate TGF- $\beta$ 1 gene transcription through positive and negative androgen response elements in the TGF- $\beta$ 1 promoter.<sup>97,98</sup> However, AR-mediated TGF- $\beta$ 1 expression in the context of CRPC is not well understood. Recent data support the idea that TGF- $\beta$  cytokines can activate the PI3K pathway, although the role of SMAD molecules in this interaction is not clear.<sup>99,100</sup> One report suggested that the TGF- $\beta$  and PI3K pathways contribute to epithelial to mesenchymal transition (EMT), which is believed to be important in CRPC pathogenesis.<sup>101</sup> Among the most important intracellular mediators of TGF- $\beta$  signaling are the SMAD isoforms. Ding *et al.*<sup>102</sup> used a transgenic mouse model to evaluate the role of SMAD4 in prostate cancer progression and metastatic potential. In prostate intraepithelial neoplastic lesions in *PTEN<sup>loxp/loxp</sup>* mice, TGF- $\beta$  signaling is upregulated and these lesions are characterized by increased SMAD4 expression. Interestingly, SMAD4 was previously reported to be downregulated in human prostate cancer metastasis, and epigenetic silencing of the SMAD4 promoter was associated with advanced disease.<sup>103</sup> Ding *et al.*<sup>102</sup> found that *Pten<sup>-/-</sup>Smad4<sup>-/-</sup>* mice developed earlier and more aggressive invasive adenocarcinoma than *Pten<sup>-/-</sup>* mice, whereas *Smad4<sup>-/-</sup>* mice did not develop prostate cancer. Gene-expression analysis of these models showed that cyclin D1 and SPP1 were upregulated in *Pten<sup>-/-</sup>Smad4<sup>-/-</sup>* tumors, leading to an increased proliferation index.

The Wnt/ $\beta$ -catenin pathway is dysregulated in several types of cancer, including colorectal, liver and prostate cancer.<sup>104,105</sup> In one study, abnormal  $\beta$ -catenin expression was observed in 23% of tumor samples derived from radical prostatectomies and in 38% of CRPCs and was found to be related to high Gleason scores.<sup>106</sup>  $\beta$ -Catenin activates T-Cell factor/lymphoid enhancer factor-1 (TCF/LEF-1) transcriptional activity and upregulates genes such as MYC, MMP7 and vascular endothelial growth factor. On the other hand,  $\beta$ -catenin is an important component of cadherin cell adhesion complexes, which have a critical role in the development of EMT and CRPC.<sup>107</sup> A functional relationship between Wnt/ $\beta$ -catenin signaling and EMT has also been suggested.<sup>108</sup> Among the Wnt/ $\beta$ -catenin target genes are the transcriptional factors Twist-related proteins 1 and 2 and the zinc-finger protein SNAI2, which downregulate E-cadherin, potentially contributing to EMT.<sup>109</sup>

Despite extensive research, there are conflicting results regarding the crosstalk between AR and Wnt/ $\beta$ -catenin signaling. Some studies suggest that AR activation leads to increased  $\beta$ -catenin nuclear translocation and transcriptional activity.<sup>110,111</sup> Other studies indicate that stimulation of Wnt/ $\beta$ -catenin signaling leads to increased AR expression via TCF/LEF-1-binding sites on the AR promoter<sup>112</sup> and also upregulation of AR target genes.<sup>102,103</sup> However, AR has also been shown to compete with TCF/LEF-1 for  $\beta$ -catenin binding and therefore inhibit  $\beta$ -catenin/

TCF-dependent signaling.<sup>113,114</sup> Consistent with the latter findings, Chesire and Isaacs,<sup>115</sup> in an early study involving experiments in prostate and non-prostate cancer cells, showed that stimulation of AR by androgens can lead to AR-mediated transcriptional suppression of  $\beta$ -catenin/TCF transcriptional activity, whereas activation of TCF may inhibit the expression of AR-regulated genes. Administration of anti-androgens alleviated this AR-mediated suppression of TCF transcriptional activity. Finally, in a recent study, nuclear localization of  $\beta$ -catenin was found in 10 of 27 human tissue specimens derived from bone metastases of mCRPC. This localization was inversely associated with AR expression, suggesting that reduced AR expression enables  $\beta$ -catenin signaling.<sup>116</sup>

The IGF system has been implicated in growth regulation, apoptosis resistance and invasion in a number of human malignancies,<sup>117–119</sup> but its role in the development of CRPC remains controversial. Studies in prostate cancer cells have shown that IGF-1 may increase cancer cell proliferation and glucose consumption, whereas inhibition of the IGF-1 receptor (IGF-1R) suppresses prostate cancer cell invasiveness.<sup>120,121</sup> Xenograft studies demonstrated that increased IGF-1R and IGF-1 can lead to androgen-independent tumor growth.<sup>122</sup> Pandini *et al.*<sup>123</sup> found that AR stimulation leads to increased expression and phosphorylation of IGF-1R in AR-positive cancer cells, enhancing their proliferation and invasiveness. Importantly, this increased AR activity was only partially blocked by anti-androgens such as bicalutamide.

IGF is known to mediate the activity of insulin and IGF-1 and -2 in normal cells, thereby mediating increased glucose uptake and glycolysis resulting in ATP and CO<sub>2</sub> formation via oxidative phosphorylation. In contrast with normal cells, most cancer cells rely on aerobic glycolysis—the Warburg effect. Aerobic glycolysis increases the uptake of nutrients that are critical for cell proliferation and tumor growth, including nucleotides, amino acids and lipids. At the same time, aerobic glycolysis via the pentose phosphate pathway alleviates cellular oxidative stress, providing cancer cells with a survival advantage.<sup>124</sup> According to another recent study, the administration of aerobic glycolysis inhibitors such as 3-bromopyruvate can overcome resistance to trastuzumab in breast cancer<sup>125</sup> and induce cell death in myeloma cells that overexpress hexokinase II.<sup>126</sup> Several studies substantiate the idea that prostate cancer cells activate glycolysis through AR and PI3K/Akt. In particular, Moon *et al.*<sup>127</sup> showed that androgen administration activates glycolysis and lipogenesis in LNCaP cells through Protein kinase A (PKA)-mediated phosphorylation of CREB, which leads to upregulation of hexokinase II. In parallel, liganded AR directly stimulates the expression of PFKFB2, a known isoform of phosphofructokinase 2 (PFK2), and PI3K/Akt signaling is implicated in the phosphorylation of PFKFB2, promoting its kinase function. On the other hand, recent data have shown that AR stimulates AMPK,<sup>128</sup> which is a known negative regulator of Warburg effect.<sup>129</sup> Interestingly, AR-negative cells such as PC-3 cells exhibit lower PFK activity, higher LDH activity and consume less oxygen than LNCaP (AR-positive) cells.<sup>130</sup> Ros *et al.*<sup>131</sup> demonstrated that LNCaP, DU145 and PC-3 prostate cancer cells are more dependent on glucose than non-malignant RWPE1 cells. Furthermore, inhibition of PFKFB4, another isoform of PFK2 that functions as the phosphatase of fructose 2,6-bisphosphate and promotes the pentose phosphate pathway, led to decreased tumor growth in PC-3 xenografts. By inducing the pentose phosphate pathway, this enzyme provides cells with sufficient nicotinamide adenine dinucleotide phosphate for biomass synthesis and for alleviation of oxidative stress. The implication is that aerobic glycolysis, driven by multiple oncogenic pathways, contributes to prostate cancer progression and in the context of CRPC, may reveal potential markers of disease progression and therapeutic targets.

The FGF pathway is believed to have a critical role not only during prostate organogenesis but also during prostate cancer development. FGF is known to be secreted by both stromal and cancer cells in the tumor microenvironment, acting by paracrine and autocrine mechanisms, respectively.<sup>132</sup> Consistent with these data, FGF receptor 1 is not expressed in benign prostate epithelium, whereas it is upregulated in 40% of poorly differentiated adenocarcinomas.<sup>133</sup> By using a lentiviral vector to alter stromal FGF expression, Memarzadeh *et al.*<sup>134</sup> showed that increased stromal FGF can lead to the enhanced epithelial proliferation associated with AR upregulation. Moreover, when stroma-overexpressing FGF and epithelium-expressing activated Akt were combined, tumor growth was more prominent. Acevedo *et al.*<sup>135</sup> demonstrated the development of a spectrum of prostate malignancies, including adenocarcinoma, which were positive for nuclear AR and negative for synaptophysin, with lymph node and liver metastasis in mice with conditional overexpression of FGF receptor 1 in the prostate epithelium. There was a high incidence of sarcomatoid carcinoma with relatively diffuse expression of E-cadherin, which is a characteristic of EMT. A clinical trial with Dovitinib (TKI258), a multi-tyrosine kinase inhibitor with high-specific activity against FGF/FGFR, is currently underway in patients with mCRPC (ClinicalTrials.gov Identifier: NCT01741116) (Table 1).

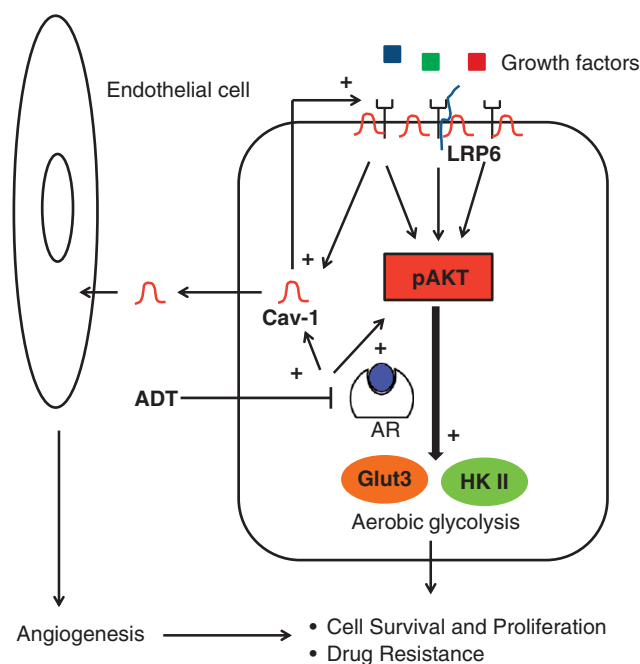
Hepatocyte growth factor and its receptor, c-Met, have been implicated in the regulation of cell growth, cell motility, morphogenesis and angiogenesis by autocrine and/or paracrine mechanisms.<sup>136,137</sup> c-Met is highly expressed in AR-negative prostate cancer cell lines such as PC-3 and DU145 and in basal and intermediate cells in the prostate epithelium but only minimally expressed in AR-positive prostate cancer cells, including LNCaP and CWR22, and in luminal cells in the prostate epithelium.<sup>138</sup> Of note, increased c-Met expression has been found in prostate cancer tissues with a greater incidence of staining in metastatic tumor samples.<sup>138</sup> Finally, Verras *et al.*<sup>139</sup> showed that AR downregulates c-Met expression by interfering with Sp1, a known transcriptional factor that stimulates c-Met expression. These data support the idea that c-Met signaling is negatively regulated by AR and is potentially de-repressed during the development of castrate resistance. In a recently reported study, Cabozantinib (XL184), a multi-tyrosine kinase inhibitor with high-specific activity against c-Met, produced significant responses in patients with mCRPC characterized by improvements in bone scan, lymphadenopathy, and bone pain<sup>140</sup> (Table 1).

Recent data substantiate the concept of negative crosstalk between AR- and EMT-promoting pathways and that overexpression of self-renewal pathways can be sufficient for tumor progression during ADT. Sun *et al.*<sup>141</sup> recently reported that castration can induce EMT in normal mouse prostate, characterized by increased expression of N-cadherin and vimentin and by low expression of E-cadherin and the acquisition of stem cell characteristics. They confirmed these results in LucaP35 xenografts, which showed increased TGF- $\beta$ , IGF1, FGFR2 and platelet-derived growth factor signaling after ADT. The authors also found negative crosstalk between AR and Zeb1, which may be related to EMT. Zhu *et al.*<sup>142</sup> found that long-term ADT can promote EMT and metastasis through downregulation of AR expression, suggesting that intermittent ADT is a promising option for patients with late disease. Finally, Jeter *et al.*<sup>143</sup> showed that LNCaP cells and xenografts overexpressing NANOG, a known self-renewal and pluripotency gene, are resistant to ADT and characterized by c-myc and Ki-67 upregulation and AR and PSA downregulation, suggesting that NANOG can mediate castrate-resistant progression.

Caveolin-1 (Cav-1) is a major structural component of caveolae that is known to interact with critical components of multiple oncogenic pathways, including receptor tyrosine kinases, serine/threonine kinases, phospholipases, G-coupled protein receptors

and Src family kinases. These proteins are located in caveolar membranes, where they interact with Cav-1 through the Cav-1 scaffolding domain.<sup>144,145</sup> Elevated expression of Cav-1 is associated with multiple human malignancies, including prostate cancer.<sup>146,147</sup> Serum Cav-1 levels are higher in men with prostate cancer than in those with benign prostate hyperplasia,<sup>148</sup> and high serum levels have been associated with elevated risk of cancer recurrence after radical prostatectomy.<sup>149</sup> Moreover, Cav-1 levels were found to be elevated in metastatic mouse and human prostate cancer,<sup>150</sup> and this molecule was found to be a downstream effector of testosterone-mediated prostate cancer cell survival and clonal growth.<sup>151</sup> On the other hand, Cav-1 is known to be highly expressed and secreted by AR-negative cells, and increased levels of Cav-1 expression have been observed after ADT.<sup>152</sup> Further, downregulation of Cav-1 in cell and animal models converted androgen-insensitive metastatic mouse prostate cancer cells to an androgen-sensitive phenotype.<sup>153</sup> Finally, recombinant Cav-1 is taken up by prostate cancer cells and endothelial cells *in vitro* and increases angiogenic activities, suggesting a significant role of Cav-1 not only in the tumor cell but also in the tumor microenvironment.<sup>154</sup> These data suggest that Cav-1 expression provides a significant survival benefit for prostate cancer cells, especially on ADT.

Mechanistic studies show that Cav-1 induces the PI3K/Akt pathway by inhibiting the phosphatases PP1 and PP2, leading to increased phosphorylation and activity of PDK1, Akt and Erk1/2.<sup>155</sup>



**Figure 2.** Stimulation of aerobic glycolysis in the development of CRPC. Cav-1 interacts with growth factor receptors and low density lipoprotein receptor-related protein 6 (LRP6). These activities stimulate PI3K/Akt signaling, leading to increased phosphorylation of Akt (pAKT) and increased expression of glycolytic enzymes, that is, glucose transporter 3 (Glut3) and hexokinase II (HK II), promoting aerobic glycolysis. Induction of aerobic glycolysis may contribute to prostate cancer cell survival, proliferation and drug resistance. Cav-1 is secreted by prostate cancer cells and establishes a positive autocrine and paracrine loop within the tumor microenvironment. Secreted, soluble Cav-1 is taken up by prostate cancer cells and can interact with specific growth factor receptors. Cav-1 is also taken up by endothelial cells, and can stimulate angiogenesis, further supporting tumor growth. ADT leads to de-repression of alternative survival pathways, that is, PI3K/Akt and increases Cav-1 expression. These activities may lead to metabolic reprogramming, and potentially contribute to prostate cancer progression.



As PI3K/Akt is a critical survival pathway for prostate cancer cells, especially during ADT, this explains the significance of Cav-1 expression in AR-negative cells. In a subsequent study, FGF, vascular endothelial growth factor and TGF- $\beta$ 1 downstream pathways induced the secretion of Cav-1 in AR-negative cells. Cav-1 upregulates multiple oncogenic pathways through PI3K/Akt and mRNA stabilization, suggesting the presence of a positive feedback loop between Cav-1 and these oncogenic pathways.<sup>156</sup> In accord with these findings, Tahir *et al.*<sup>157</sup> showed that sunitinib and dasatinib treatment of PC-3 and DU145 cells and xenografts decreased Cav-1 expression in cells and serum Cav-1 levels, respectively, in mice and serum Cav-1 levels were positively associated with tumor growth. Finally, the co-administration of anti-Cav-1 antibody with sunitinib and dasatinib led to greater tumor regression than did either treatment alone, further supporting the concept that Cav-1 mediates activation of multiple oncogenic pathways in the prostate cancer cell. Recently, Tahir *et al.*<sup>158</sup> demonstrated an interaction between Cav-1 and LRP6 in the cellular membrane, which leads to the activation of IGF-1R/IR and results in stimulation of Akt-mTORC1-mediated activation of aerobic glycolysis that includes upregulation of HK2 and GLUT3. These data, combined with recent results that show a contribution of aerobic glycolysis to prostate cancer progression, suggest a possible role of Cav-1 as an inducer of multiple oncogenic pathways, with aerobic glycolysis being the end result (Figure 2).

## CONCLUSION

Metastatic prostate cancer remains an incurable disease and there are few reliable biomarkers to monitor disease progression or guide therapeutic decisions. The evolution of CRPC involves both androgen-stimulated and -repressed genes related to numerous alternative growth and survival pathways activated during the development of this disease state. Interactions between AR signaling and alternative survival pathways are cell-type and context-dependent and are currently under extensive investigation. Specific oncogenic pathways, for example, PI3K/Akt, are activated in prostate cancer cells and ADT can provide selective pressure in favor of their expression, potentially promoting castrate-resistant growth. Extrapolation of this complex information will hopefully lead to the identification of new predictive and surveillance biomarkers, and novel and combined therapies targeting critical pathways which provide a survival advantage to prostate cancer cells and promote CRPC.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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