

Targeting CDK9: a promising therapeutic opportunity in prostate cancer

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Abstract

Cyclin-dependent kinase 9 (CDK9) is a key transcriptional regulator and a lucrative target for cancer treatment. Targeting CDK9 can effectively confine the hyperactivity of androgen receptor and the constitutive expression of anti-apoptotic proteins; both being main causes of prostate cancer (PCa) development and progression. In castrate-resistant PCa, traditional therapies that only target androgen receptor (AR) have become obsolete due to reprogramming in AR activity to make the cells independent of androgen. CDK9 inhibitors may provide a new and better therapeutic opportunity over traditional treatment options by targeting both androgen receptor activity and anti-apoptotic proteins, improving the chances of positive outcomes, especially in patients with the advanced disease. This review focuses on biological functions of CDK9, its involvement with AR and the potential for therapeutic opportunities in PCa treatment.

Key Words

- ▶ CDK9
- ▶ prostate cancer
- ▶ RNAPII transcription
- ▶ anti-apoptotic proteins
- ▶ apoptosis
- ▶ therapeutics

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Introduction

Prostate cancer (PCa) is the most common cancer diagnosed in men and is considered as one of the major causes of death in developed countries (Siegel *et al.* 2015). It is highly prevalent in older men, with those over 65 years accounting for more than 65% of all reported cases, exposing a significant bias in high risk with advanced age. In addition, race and family history are also major risk factors in PCa. The risk is higher in African-Americans than Caucasians, and in individuals having a family history (Quinn & Babb 2002, Hsing & Chokkalingam 2006, Haas *et al.* 2008). Initially, PCa may develop as androgen dependent and respond to androgen deprivation therapy (ADT) or castration therapy. The treatment options are viable before the disease progresses and metastasises

(Loblaw *et al.* 2007). ADT was found effective in initial stages with significant clinical regression and remission in 80–90% of patients, but the cancer recurs inevitably, after 2–3 years. Subsequent progression of the cancer to a hormone-independent castrate-resistant state leads to castration-resistant PCa (CRPC), where cancer cells can survive ADT. Ultimately, CRPC metastasises to other organs such as bones, lungs, brain or liver. In this advanced stage, the disease progresses in a more serious and invasive manner with worse prognosis and lethal phenotype (Pienta & Bradley 2006). Due to prevailing medical conditions and shortage of bone marrow hematopoietic stem cell reserve, chemotherapy is not suitable for elderly PCa patients (Paller & Antonarakis 2011). Thus, treatment of CRPC remains a significant

clinical challenge and targeted therapies using small-molecule inhibitors are emerging as new options. In this respect, cyclin-dependent kinase 9 (CDK9) inhibitors, particularly, may offer exciting prospects.

CDK9 is a transcriptional regulator that controls the expression of anti-apoptotic proteins that institute immortality in cancer cells. It interacts with many transcription factors (TFs) and regulates their activities, particularly the androgen receptor (AR), a recognized molecular target in PCa treatment. As a consequence, targeting CDK9 may provide a unique opportunity to induce apoptosis of PCa cells by stopping their oncogenic driver, AR. Inhibiting CDK9 is known to cause minimal toxicity to normal cells (Lemke *et al.* 2014, Walsby *et al.* 2014, Li *et al.* 2015). *In vivo* mouse xenograft models and phase I clinical trials have also shown that CDK9 inhibition results in minimal toxicity while maintaining effective antitumor activity (Abdullah *et al.* 2011, Feldmann *et al.* 2011, Nemunaitis *et al.* 2013). Therefore, targeting CDK9 may offer safe and effective therapeutic strategy for patients afflicted with the advanced stages of PCa.

In this review, we assess the premise of CDK9 as a therapeutic target in PCa. We begin by providing an updated overview of the roles of CDK9 in cancer biology with the focus on PCa. This is followed by a thorough dissection into the CDK9–AR pathway, and the mechanisms underlying the implications of CDK9 in PCa. Finally, we explore critically the current landscape of CDK9 inhibitors available for PCa treatment, particularly those undergoing clinical trials.

Biology of CDK9

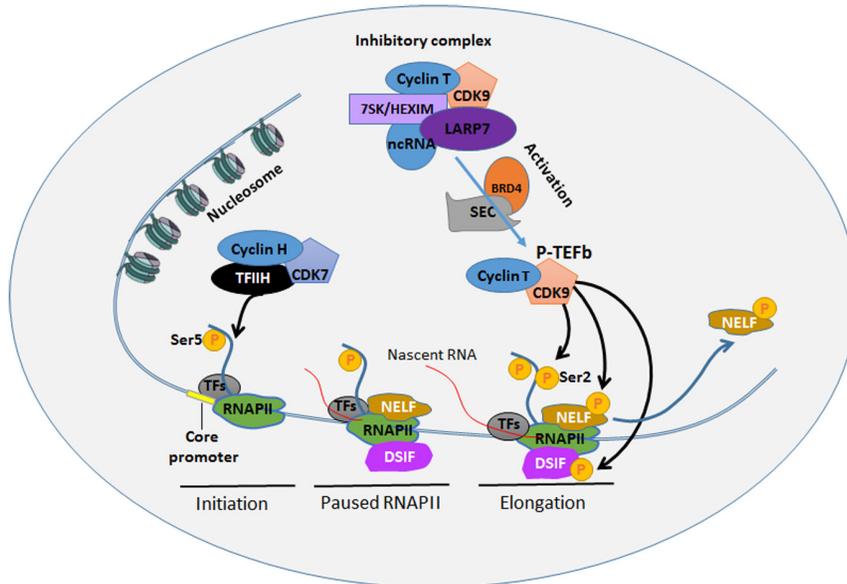
CDK9 is a serine/threonine kinase that was first identified in the early 1990s as a CDC2-related kinase named PITALRE for having the characteristic Pro-Ile-Thr-Ala-Leu-Arg-Glu motif (Grana *et al.* 1994). It is a member of the CDK family which plays critical roles in the regulation of cell cycle and transcription. Currently, at least 20 human CDKs and 30 partnering cyclins are known (Cao *et al.* 2014). CDK9 and its regulatory partner cyclin T or cyclin K (Fu *et al.* 1999) are components of the global transcription elongation factor known as the positive transcription elongation factor b (P-TEFb). It does not act in the regulation of cell cycle; but plays a crucial role in the regulation of transcription (de Falco & Giordano 1998). Two isoforms of CDK9 are known, CDK9-42 (372 aa, 42 kDa) and CDK9-55 (489 aa, 55 kDa), and they partner with four cyclins: cyclin T1, cyclin T2a,

cyclin T2b and cyclin K (Fu *et al.* 1999). About 80% of CDK9 binds to cyclin T1, and 10–20% to cyclin T2a and T2b (Peng *et al.* 1998). Although the catalytic activity of the two isoforms and their ability to partner with cyclin T1 are the same, different subcellular localization and expression patterns are observed in different tissues.

CDK9 promoter has two different transcription initiation sites in humans (Shore *et al.* 2003, 2005). The promoter for CDK9-42 mRNA does not contain a functional TATA box, but a GC-rich transcriptional element similar to a housekeeping promoter at –352 to –1 that is required to sustain full promoter activity (Liu & Rice 2000). In contrast, CDK9-55 promoter contains a TATA box approximately 500 bp upstream of the transcription initiation site (Liu & Rice 2000, Shore *et al.* 2005). Although commitment and specification during differentiation and developmental stages determine the relative abundance of the two isoforms in tissues, CDK9 is expressed almost everywhere in the body, including the brain, lungs, spleen and thymus (Shore *et al.* 2005). At optimum level, endogenous CDK9 is a very stable protein with a half-life ($t_{1/2}$) of 4–7 h, depending on the cell type. In contrast, when CDK9 is overexpressed, it is not stable and degrades rapidly with a $t_{1/2}$ of <1 h, depending on the level of expression (Garriga *et al.* 2003).

The regulation of transcription by CDK9 is well established (Wang & Fischer 2008). CDK9, in association with cyclin T forms P-TEFb, phosphorylates the C terminal domain (CTD) of RNA polymerase II (RNAPII) and activates productive elongation of mRNA transcript. In human, RNAPII CTD consists of 52 heptad repeats of the YSPTSPS consensus sequence (Hsin & Manley 2012). Among them, Ser5 (YSPTSer5PS) by CDK7 and Ser2 (YSer2PTSPS) by CDK9 are the two main phosphorylation sites for transcription initiation and elongation, respectively. Although other CDKs are capable of phosphorylating CTD, only phosphorylation by CDK9 activates P-TEFb-dependent gene expression in a catalytic manner (Napolitano *et al.* 2000), and thus warrants a detailed look.

Initiation of transcription occurs with the general TF II (TFIIH) complex associated with CDK7 phosphorylating Ser5 residues of CTD heptad repeats (Fig. 1). Subsequently, RNAPII is paused by the negative elongation factor (NELF) and the DRB sensitivity-inducing factor (DSIF) at the promoter-proximal region, 20–30 nucleotides downstream of transcription start site (Rasmussen & Lis 1993, Diamant & Dikstein 2013). This stalling is a common regulatory process for nascent

**Figure 1**

Regulation of transcription by CDK9. Inhibitory complex consisting of LARP7, HEXIM, 7SK and ncRNA gets activated upon interaction with BRD4 and SEC, releasing P-TEFb. Activated P-TEFb is recruited to the initiation complex by interacting with TFs and phosphorylates CTD Ser2, NELF and DSIF for productive elongation.

transcript to protect it from degradation by 5'-capping. This initial pausing is also considered the general rate-limiting step in orchestrating the stages of 5' capping, intron removal and 3' end formation. CDK9 is then recruited again to the paused initiation complex as a component of P-TEFb complex. The pause is released when CDK9 phosphorylates CTD of RNAPII, now at Ser2, as well as NELF and DSIF. Upon phosphorylation by CDK9, NELF is evicted from RNAPII, and DSIF becomes a positive elongation factor. There is also evidence suggesting that CDK9 phosphorylates the elongation factor subunit Spt5 to relieve the early RNAPII pausing (Brès *et al.* 2008).

As a global transcriptional regulator, P-TEFb is under tight regulation (Fig. 1). It is negatively regulated or remains inactive by forming an inhibitory complex together with La ribonucleoprotein domain family member 7 (LARP7), hexamethylene bisacetamide inducible 1 or 2 (HEXIM1/2) and the small nuclear ribonucleoprotein 7SK, a non-coding RNA (ncRNA) (Zhou *et al.* 2012). Upon interaction with bromodomain-containing protein 4 (BRD4) (Jang *et al.* 2005) and the larger super elongation complex (SEC) (Smith *et al.* 2011), P-TEFb is activated by the removal of LARP7, HEXIM, 7SK and ncRNA, and recruited to the transcription initiation complex. For activation of P-TEFb, BRD4 competes with inhibitory complex HEXIM/7SK and is recruited to transcription start site via histone acetylation. SEC may co-localize with BRD4 at gene promoters and interact with coactivators such as the Mediator and polymerase-associated factor 1 (PAF1)

(Flajollet *et al.* 2013). Ultimately, the recruitment of these cofactors depends on TFs that bind to promoters or enhancers. The well-known DNA-binding TFs that interact with P-TEFb to release paused RNAPII are MYC and NF-κB (Rahl *et al.* 2010, Diamant & Dikstein 2013). MYC gene itself is regulated by the release of RNAPII into productive elongation. Activated NF-κB during immune response and stressed conditions interacts with P-TEFb either directly or indirectly through BRD4. Recent evidence suggests that the promoter-proximal rate-limiting pause also regulates the NF-κB pathway for NF-κB activation, modulation and fine-tuning.

Studies in human and murine systems showed that CDK9 is highly expressed in terminally differentiated cells, indicating its cellular function in differentiation and development. CDK9 contributes to T lymphocyte differentiation and malignant transformation (Leucci *et al.* 2007). Depending on tissue-specific signaling pathways, CDK9 responds to cytokines, including the tumor necrosis factor and interleukin-6 (MacLachlan *et al.* 1998, Bellan *et al.* 2004). Studies on different human cancers like lymphoma (Bettayeb *et al.* 2007, Gregory *et al.* 2015), neuroblastoma (De Falco *et al.* 2005), PCa (Lee *et al.* 2001) and several instances of hematopoietic malignancies (Huang *et al.* 2014) revealed that CDK9-related pathways are deregulated, indicating that CDK9 hyperactivity may promote the expression of anti-apoptotic factors and induce proliferation (Jin *et al.* 2015). Even though CDK9 has a crucial role in both normal and cancer cells, overexpression of short half-lived anti-apoptotic factors like MCL-1, BCL-2 and

XIAP is more important to cancer cell survival, which in part explains the selective toxicity of CDK9 inhibition against cancer cells (Liu *et al.* 2012).

Inhibition of CDK9 kinase activity significantly affects the gene expression which could be used as a strategy for therapeutic intervention in cancer (Garriga & Graña 2014), despite early concerns over the safety of pharmacologically targeting it. This disagreement has been settled by evidence that the basal transcription does not require RNAPII CTD phosphorylation (Serizawa *et al.* 1993) and CDK9 is not necessary for transcription of some intronless mammalian genes (Medlin *et al.* 2005). Support in favor of targeting CDK9 is strengthened by the recent discovery revealing that both CDK12 and CDK13 also phosphorylate the RNAPII CTD (Dubbury & Sharp 2015). The functional overlap among CDK9, CDK12 and CDK13 reflects the fact that there might be redundancy in transcription elongation and regulation at least to a certain extent. This suggests that potential resistance mechanisms to CDK9 inhibition may exist. However, currently this remains uninvestigated and no evidence exists as such. On the other hand, CDK9 has been identified to facilitate resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a condition observed in 50% of all cancer cell lines (Lemke *et al.* 2014). CDK9 inhibition may circumvent TRAIL resistance and boost cancer cell apoptosis. Thus, the current consensus following recent accumulation of biochemical, genetic and pharmacological evidence is that CDK9 is a potent multilevel target that affects several factors of the complex transcriptional machinery in many cancers, including CRPC.

CDK9-mediated AR-dependent pathway in PCa

AR structure and variants

CDK9 phosphorylates and activates AR, which is a crucial TF for the survival and progression of PCa (Chen *et al.* 2012). AR is a 110kDa steroid hormone-activated TF, encoded from a ~180kb gene, and contains 919 amino acids (Gelman 2002). The human AR gene consists of eight exons that encode four main regions: the N-terminal domain (NTD), DNA-binding domain (DBD), ligand-binding domain (LBD) and a short hinge region (Fig. 2). NTD is poorly conserved, but is the largest functional region in AR and contains the activation function (AF1) motif. AF1 contains two transcriptional activation units (TAUs): TAU1 (amino acids 101–360) and TAU5 (amino acids 370–494) (Jenster *et al.* 1995). In contrast, DBD is highly conserved and contains two zinc fingers; one binds to a specific nucleotide, the androgen response element (ARE), and the other promotes homodimerization. LBD (amino acids 669–919) is moderately conserved and hosts the second transcriptional activation function (AF2) motif. LBD facilitates binding of the AR ligands, such as testosterone and dihydrotestosterone (DHT), whose binding is the primary control mechanism of the androgen signaling axis. Hinge region is a very short (~50 aa) and flexible sequence (amino acids 625–669) and is partly responsible for a bipartite ligand-dependent nuclear localization signal. It connects DBD and LBD, and plays roles in AR nuclear transport, DNA binding and coactivator recruitment (Gioeli *et al.* 2002).

AR is primarily localized in the cytoplasm in an inactive conformation, complexed with heat shock

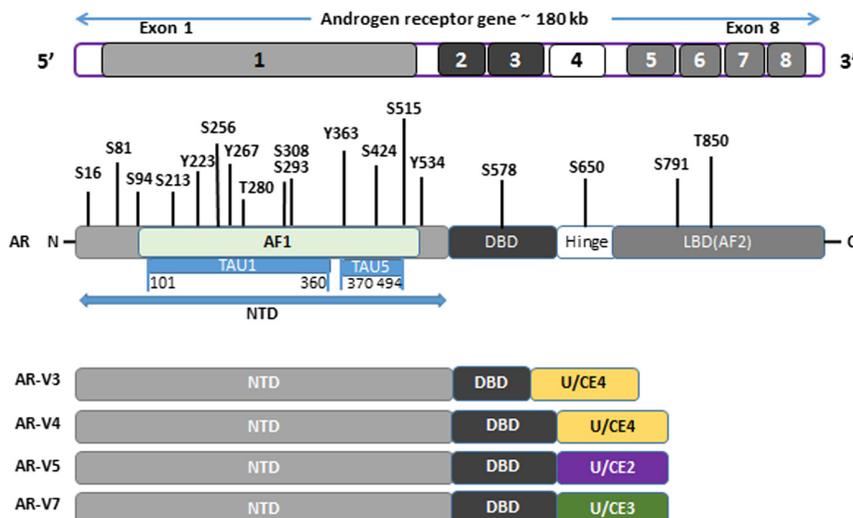
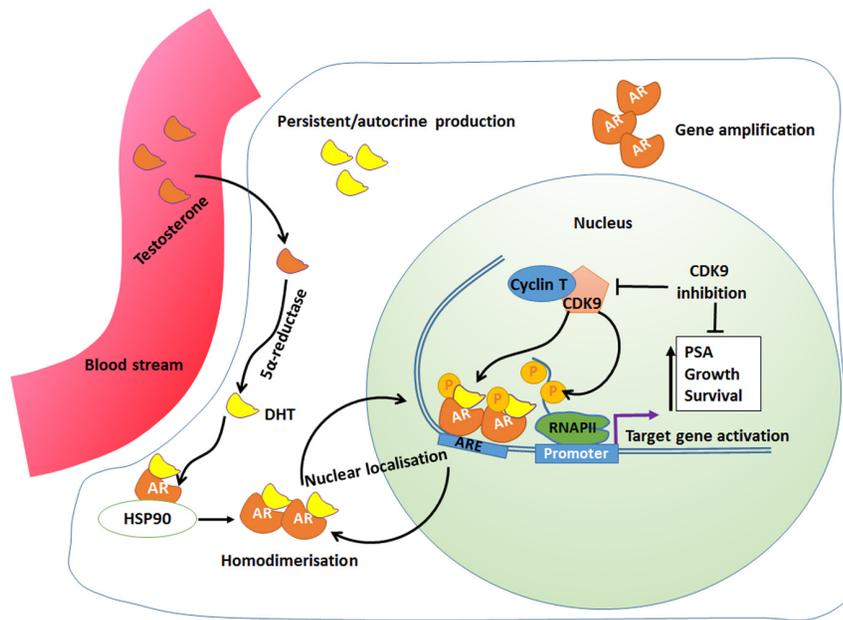


Figure 2

Schematic representation of the androgen receptor (gene and protein) major domains and phosphorylation sites. Exon1 codes for NTD; exons 2 and 3 code for DBD; exon 4 for hinge; and exons 5, 6, 7 and 8 for LBD. All the AR splice variants share similarity with the NTD and DBD. Introduction of cryptic exons due to exon skipping or alternative splicing results in unique regions.

**Figure 3**

CDK9-AR-mediated pathway in PCa. Under normal conditions, AR is activated and dimerizes upon androgen induction and mediates transcriptional activity by binding to the ARE. In androgen dependent CRPC, reactivation of AR occurs due to AR gene amplification, which makes it hypersensitive to low level of androgen or due to persistent/ectopic production of androgen by the prostate cells. Inhibition of CDK9 phosphorylating both the AR and RNAPII can put on a break on the AR overactivity and expression of prostate-specific genes.

proteins, predominantly HSP90 (Fig. 3). In the absence of steroid hormones, this conformation prevents it from entering the nucleus and binding DNA. Upon binding to androgenic hormones, AR undergoes conformational changes, leading to a detachment from heat shock proteins. The ligand-induced conformational change facilitates homodimerization of AR, as well as their nuclear transportation. Once in the nucleus, AR homodimer binds ARE in the promoter regions of target genes. This occurs both by direct binding to DNA and by association with other TFs, including E26 transformation-specific factors. Coactivators and chromatin remodeling complexes are recruited to facilitate the transcription of AR target genes. A well-known gene regulated by AR is the prostate-specific antigen (*PSA*), which is currently used as a biomarker for PCa (Crawford *et al.* 2014). Besides *PSA*, AR regulates many other genes that are involved in regulation of proliferation and apoptosis. Its localization, transport, activation and binding to the specific DNA in the nucleus are facilitated by several posttranslational modifications in the form of side-chain phosphorylations.

Aberrant AR activity has been linked with several pathological conditions including male infertility, androgen insensitivity syndrome, spinal and bulbar muscular atrophy, rheumatoid arthritis, hirsutism, baldness, acne, breast and PCa (Gao & Chen 2013, Xu *et al.* 2011). Huggins and Hodges (Huggins & Hodges 1941) first showed the correlation between androgen and prostate tumor growth and based on this pioneer study, ADT became the key treatment method. Disease progression to CRPC occurs through the adaption and

reprogramming of cancer cells through continuous activation of AR for survival at a castrate level of androgen, as well as alternative independent pathways (Harris *et al.* 2009). CRPC retains high level of AR expression and activation, despite low levels of androgen (Gelmann 2002, Decker *et al.* 2012, Urbanucci *et al.* 2012). As a result, *PSA* gene is constitutively expressed (Buchanan *et al.* 2001). Briefly, AR activation can be attained through hypersensitivity to androgen (Fujimoto *et al.* 2007) or agonist activity of alternative steroids or antiandrogens (Chan *et al.* 2015). AR can also be activated through ectopic expression of androgens by PCa cells (Fig. 3) (Minamiguchi *et al.* 2004).

By examining a number of human PCa model systems (Table 1), mechanisms of castration resistance can be broadly classified into three pathways; androgen dependent, androgen independent and bypass. Androgen dependent and independent pathways are mediated by a number of AR-centered aberrations, whereas bypass pathways are mediated by upregulation of anti-apoptotic proteins such as BCL-2 and MCL-1. Mechanistically, abnormal AR activity may originate from AR mutation, overexpression, posttranslational modification, altered expression of AR-associated coregulators or activation of signal transduction pathway to enhance the AR association with other TFs (Feldman & Feldman 2001, Tsao *et al.* 2012). Amplification of AR is the most common genetic mutation responsible for overexpression of AR in ~80% of advanced stage patients. However, AR amplification is very rare in early stages of PCa, indicating an escalation of AR role in PCa progression (Bubendorf *et al.* 1999, Chen *et al.* 2008). Chen and co-workers

Table 1 *In vitro* and *in vivo* models of human PCa.

Cell line	Tissue of origin	AR status	PSA	References
LNCAp	Lymph node metastasis (directly from patient)	Positive (T877A mutation)	Yes	(Tan <i>et al.</i> 1997)
22Rv1 or CWR22Rv1	Derived from CRW22 xenograft	Positive (H875Y mutation)	Yes	(Sramkoski <i>et al.</i> 1999)
CWR22Pc	CWR22 xenograft	Positive (H874Y mutation)	Yes	(Tan <i>et al.</i> 1997)
DU145	PCa brain metastasis (directly from patient)	Negative	No	(Scaccianoce <i>et al.</i> 2003)
LAPC-4	PCa lymph node metastasis	Positive (wild type)	Yes	(Garcia <i>et al.</i> 2014)
MDA PCa 2a	PCa bone metastasis (directly from patient)	Positive (L702H and T878A mutations)	Yes	(Navone <i>et al.</i> 1997)
PC-3	PCa vertebral metastasis (directly from patient)	Negative	No	(Tai <i>et al.</i> 2011)
VCaP	PCa bone metastasis (from mouse xenograft)	Positive (wild type)	Yes	(Korenchuk <i>et al.</i> 2001)
LuCaP	Metastatic human patient	Not reported	Yes	(Ellis <i>et al.</i> 1996)
C4-2	LNCAp tumor in nude mouse	Positive	Yes	(Pfitzenmaier <i>et al.</i> 2003)
C4-2B	LNCAp tumor in nude mouse	Positive	Yes	(Spans <i>et al.</i> 2014)

(Chen *et al.* 2004) found consistent overexpression of AR only in castrate-resistant xenografts. They also found that AR overexpression in androgen-sensitive human prostate adenocarcinoma (LNCAp) cells increases its sensitivity to very low level of androgen.

Constitutively active AR splice variants (AR-Vs) also play a crucial role in the development of CRPC (Qu *et al.* 2015). Expression of AR-Vs has been found in PCa cell lines, xenografts and human tumors, and, thus, are clinically highly relevant to CRPC (Sun *et al.* 2010, Hornberg *et al.* 2011). AR-Vs lead to androgen-independent PCa as they lack LBD (Fig. 2) and their activation does not require androgen (Dehm & Tindall 2006). They, however, retain the AF1 motif in NTD. The activity of AF1 of AR-Vs is similar to that of the full-length AR, but is androgen independent. This constitutive activity of AR has attracted it as a potential target for CRPC treatment. Dehm and Tindall (2011) have comprehensively reviewed alternative spliced variants of AR.

The pattern of AR variants' expression may vary in response to different intra- and extracellular stimuli. In CRPC, collective response of AR and AR-Vs raise a complex regulatory system of cellular responses to exert resistance to existing therapies. Still it remains a challenge to characterize these splice variants and how they are able to mediate catalytic activity even in the absence of some structural units. Although AR-Vs hints at the compensatory action of AR domains, the ability of these domains to takeover, identify and characterize the factors responsible for exon selection during alternative splicing of AR gene remains uncovered. Thus, further investigations are of utmost importance to better understand AR and AR-V regulation.

Significance of AR phosphorylation by CDK9

Phosphorylation of AR modulates its activation and transcriptional activity. AR-dependent gene regulation involves interactions between NTD and CTD, AR and ARE, and the coregulatory proteins that facilitate recruitment to the transcriptional machinery (He *et al.* 2002). Phosphorylation events can be androgen induced or independent, depending on the nature of the phosphorylation site. Other than from androgen, AR is capable of receiving signals from cell-derived factors and chemical compounds that trigger transcriptional activation of specific genes such as *PSA*. This capacity of AR to respond to different extra- and intracellular signaling makes it a central node interconnecting the pathways and is highly relevant to both normal development and cancer.

Posttranslational modification via phosphorylation also regulates AR stability, nuclear retention and cell growth (Table 2). AR contains at least 18 phosphorylation sites (Fig. 2 and Table 2). Although, each AR domain contains at least one phosphorylation site, NTD houses the majority of sites reflecting its importance in regulatory functions and activity (Fig. 2 and Table 2) (Gioeli 2005). Previous studies have shown that among the 18 sites, Ser81 is the most frequently phosphorylated, and upon androgen stimulation, Ser81 phosphorylated AR accumulates gradually over ~8h in PCa cells (Gioeli *et al.* 2002, Chen *et al.* 2006). Gordon and his colleagues (Gordon *et al.* 2010) found that in *in vitro* growth conditions, Ser81Ala mutated LHS cells had a ~15% delayed growth rate and concluded that the loss of Ser81 phosphorylation results in growth inhibition (Gioeli *et al.* 2002). Several investigations attribute the role of Ser81 phosphorylation to the regulation of AR

Table 2 Phosphorylation sites of AR with their cognate kinase(s) and their functions.

	Phosphorylation site	Androgen dependency	Kinase	Function			
				Transcriptional activity	Stability	Localization	Growth
NTD	Ser16	Yes	–	–	–	–	–
	Ser81	Yes	CDK1, CDK5, CDK9	Yes	Yes	Yes	Yes
	Ser94	No	–	–	–	–	–
	Ser213	Yes	Akt, PI3K, Pim1	Yes	–	–	–
	Tyr223	–	Fer	Yes	–	–	–
	Ser256	Yes	–	–	–	–	–
	Tyr267	–	Ack1	Yes	–	–	Yes
	Thr282	No	Aurora-A	Yes	–	–	–
	Ser293	No	Aurora-A	Yes	–	–	–
	Ser308	Yes	CDK11 ^{p58}	Yes	–	–	–
	Tyr363	–	Ack	Yes	–	–	Yes
	Ser424	Yes	–	–	–	–	–
	Ser515	–	CDK7, MAPK	Yes	Yes	–	–
	Tyr534	Yes	Src	Yes	Yes	–	Yes
	DBD	Ser578	–	PAK6, PKC	Yes	–	Yes
Hinge	Ser650	Yes	MKK4/JNK, MKK6/P ³⁸	Yes	–	Yes	–
LBD	Ser791	–	Akt / PI3K	Yes	–	–	–
	Thr850	–	PIM-1L	Yes	Yes	–	Yes

stability, nuclear retention and translocation (Hsu *et al.* 2011, Chen *et al.* 2012, Wu *et al.* 2014). Therefore, Ser81 phosphorylation is a core event in the modulation of AR activity. Ser81 can be phosphorylated by CDK1, CDK5 and CDK9. The involvement of CDK9 here is particularly of interest as it does not appear to phosphorylate any other AR site (Gordon *et al.* 2010, Chen *et al.* 2012). However, an exhaustive investigation into all plausible AR phosphorylation sites by CDK9 is yet to be carried out. CDK9's importance is also highlighted by the facts that phosphorylation of Ser81 by CDK9 regulates the nuclear retention, chromatin binding, transactivation of specific gene expression, transcriptional activity of AR, as well as cell proliferation (Chen *et al.* 2006, Gordon *et al.* 2010). According to Gordon *et al.* (2010), overexpression of CDK9 along with its partner cyclin T results in an increased Ser81 phosphorylation in the cells. Using siRNA to knock-down CDK9 protein level in LNCaP cells caused a significant reduction in Ser81 phosphorylation even after androgen induction. In addition, inhibition of CDK9 using pharmacological inhibitors of CDK9, that is DRB and flavopiridol reduced Ser81 phosphorylation in LNCaP cells, indicating CDK9 regulation of Ser81 phosphorylation in PCa cells. Certainly, further studies are required to fully establish the biological consequences of CDK9-mediated AR phosphorylation.

Although CDK1 also phosphorylates AR Ser81, its function in regulation of AR transcriptional activity is questionable. This is primarily due to the fact that CDK1 is active and phosphorylates Ser81 during

G2/M phase of the cell cycle, when the transcription of most genes is silenced. It is suggested that Ser81 phosphorylation by CDK1 may regulate AR activity only in a subset of genes (Gordon *et al.* 2010). On the other hand, CDK9 activity does not fluctuate but maintains a consistent rate of Ser81 phosphorylation throughout the cell cycle. In addition, CDK5 may phosphorylate Ser81 as well (Hsu *et al.* 2011). A Ser81Ala AR mutant was shown to have diminished interactions with CDK5, reducing its nuclear retention and stabilization, and indicating an overlap of function with CDK9. Thus, AR shuttling between the cytoplasm and nucleus is dependent on its phosphorylation state, and is delicately balanced on a complex interplay of signals (Saporita *et al.* 2003, Gioeli *et al.* 2006, Kesler *et al.* 2007, Gong *et al.* 2012). A detailed mechanism of this event is yet to be unraveled.

Targeting CDK9 in CRPC

Targeting AR axis for the treatment of PCa has been investigated for a long time. However, the changing paradigm in AR activity has so far proven highly challenging. Nonetheless, exploitation of the role of CDK9-mediated Ser81 phosphorylation in regulating the catalytic properties of AR may provide a decisive opportunity for therapeutic intervention encompassing all three major mechanisms of CRPC. Therapeutic advantages of selectively targeting CDK9 have been noticed in several human cancers. Its therapeutic scope in PC, however, has not yet been fully explored, although

studies supporting the notion have begun appearing in literature steadily.

CDK9 inhibition can restrict nuclear retention and activation of AR, and inhibit the disease progression to CRPC stage. [Chen et al. \(2012\)](#) used double-mutant LNCaP cells to elucidate that these cells were able to maintain stable AR expression, but failed to transactivate *PSA* and *TMPRSS2* transcription. They also showed that AR nuclear retention required for proper chromatin association was dependent on Ser81 phosphorylation. Moreover, chromatin immunoprecipitation assays showed Ser81 phosphorylation by CDK9 transcriptionally regulates *PSA* and *TMPRSS2* genes, as Ser81Ala mutant failed to induce chromatin binding in response to hormones ([Chen et al. 2012](#)). [Gordon et al. \(2010\)](#) also tested the effects of the Ser81 phosphorylation on growth, using wild type (WT) and Ser81Ala mutant AR in LHS and LAPC4 PCa cells. They found that both cells expressing WT AR grew faster than cells expressing the Ser81Ala mutant, indicating that the loss of Ser81 phosphorylation restricts cell growth. Thus, CDK9 inhibition can decrease the nuclear retention, restricting chromatin binding and reducing AR transcriptional activity.

Constitutively active AR-Vs have been found in CRPC and they have the capability to regulate AR target gene activity in the absence of ligands ([Chan et al. 2012](#)). As AR-Vs lack the LBD, inhibitors targeting androgen and LBD are not effective against CRPC. Therefore, there is a newfound interest at targeting NTD, as well as coregulators of AR activity. [Myung et al. \(2013\)](#) have shown that a small-molecule antagonist of AR NTD has the ability to block transcriptional activity of AR-Vs. Similarly, kinases that modulate NTD protein–protein interactions could provide an opportunity to target AR-Vs. Assuming the

role of Ser81 phosphorylation remains unchanged in AR-Vs, targeting NTD may be an attractive proposition for CRPC therapeutic intervention. However, further studies are required to confirm the role of phosphorylation in AR-V function.

AR mutations modify their interactions with coregulators or cross-talk with activated signal transduction pathways to elicit androgen independent activation of AR. Greater association between AR and other TFs such as Forkhead box protein A, Myc, NF- κ B and STAT family proteins provide clues for targeting this complex network through transcriptional regulatory pathway of CDK9 ([Fig. 4](#)). The role of CDK9 in regulation of these TFs has been reviewed ([Kryštof et al. 2012](#), [Schmitz & Kracht 2016](#)): Myc and NF- κ B are overexpressed in CRPC and their simultaneous overexpression is a signature in the progression of PCa ([Hawksworth et al. 2010](#), [Jin et al. 2014](#)). Moreover, in relapsed PCa that occurs via bypass pathway the underlying molecular defects are characterized by the upregulation of anti-apoptotic proteins like BCL-2, MCL-1 and XIAP ([Krajewska et al. 1996](#), [Chaudhary et al. 1999](#), [Devi 2004](#)). [Li et al. \(2000\)](#) showed that flavopiridol induces apoptosis in PC3 cells by downregulating BCL-2 and significantly restricting PCa cell growth. Particularly, MCL-1 is highly expressed in CRPC ([Reiner et al. 2015](#)). MCL-1 is upregulated during ADT and mediates the resistance in cellular response to androgen ablation. Thus, [Santer et al. \(2015\)](#) reasoned targeting MCL-1 as a strategy in CRPC treatment. On the other hand, these anti-apoptotic proteins are dependent on continuous RNAPII activity mediated by overexpression of CDK9. CDK9 inhibition using small-molecule inhibitors ([Table 3](#)) downregulates these short-lived anti-apoptotic proteins and triggers cell death ([Lam et al. 2001](#), [Chen et al. 2009](#)). In 2014,

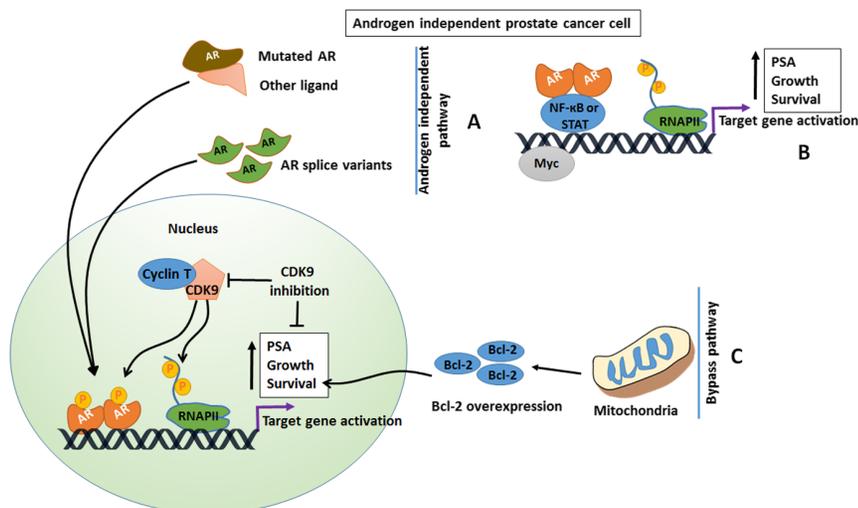


Figure 4

Proposed mechanism of androgen independency in CRPC. (A) Mutated AR binding to alternative steroids or antiandrogen and AR-Vs act through androgen independent pathway of CRPC. (B) Higher association with other TFs like MYC, NF- κ B and STAT family members affects AR recruitment to DNA and/or regulates AR target gene expression. (C) In bypass pathway, overexpression of anti-apoptotic Bcl-2 family of proteins prevents prostate cells from death despite of ADT.

Booher *et al.* showed that CDK9 inhibitor dinaciclib induces apoptosis by downregulating MCL-1 and BCL-XL in 22rv1 and PC3 PCa cells, as well as other 11 solid tumor cells. In another study, Arisan *et al.* (2014) determined the apoptotic efficacy of CDK inhibitor roscovitine, which has greater selectivity than flavopiridol when complete kinase inhibition profiles are considered, in LNCaP, Du145 and PC3 cell lines. They showed that apoptotic effect of this inhibitor was due to the modulation in the ratio of BCL-2 of pro- and anti-apoptotic proteins of BCL-2 family. Moreover, it is well-established that CDK9 inhibition can target multiple facets of the apoptotic regulatory machinery. Consequently, CDK9 inhibition may create a new therapeutic opportunity in CRPC by simultaneous suppression of these anti-apoptotic proteins.

Development of pharmacological inhibitors of CDK9 for PCa

Rapidly developing resistance to existing treatment options and bypass signaling mechanisms in PCa progression are restricting the therapeutic scope of CRPC. As CDK9 is involved in phosphorylating AR and RNAPII, evidences are accumulating supporting the re-establishment of transcriptional regulation via CDK9 inhibitors, not only in PCa, but in many other types of cancers. Inhibition of CDK9 expression using shRNA, triggered apoptosis of chronic lymphocytic leukemia cell and ovarian cancer, further supporting CDK9 as a potential anticancer therapeutic target (Lam *et al.* 2014, Walsby *et al.* 2014). There have been many efforts to develop CDK9 inhibitors and as a result several compounds have been shown to possess promising antitumor activities via inhibition of CDK9 in many human cancer models *in vitro* as well as *in vivo* (Table 3). The earliest compounds were multikinase inhibitors, while the recent developments focus on selectively targeting CDK9 (Wang *et al.* 2010, Shao *et al.* 2013, Walsby *et al.* 2014, Scholz *et al.* 2015).

In vitro and *in vivo* studies of flavopiridol on multiple human cancer models revealed that it has a mean IC_{70} of 8 ng/mL, which indicates the cytotoxic potency of the compound. Specifically, a study by Drees *et al.* (1997) showed that flavopiridol has high selective toxicity to PCa cells which was further shown by *in vivo* xenograft models of two PCa cell lines (PRXF1337 and PRXF1369). At the maximum tolerated dose (10 mg/kg/day), flavopiridol produced an optimal relative tumor growth inhibition of 33% and a growth delay of 30 days. Though flavopiridol proved to be active in both PCa xenografts, its therapeutic window was narrow and

it produced its antitumor activity only at the maximum tolerated dose.

Following the demonstration of promising cytotoxic activities in preclinical models (Drees *et al.* 1997, Soner *et al.* 2014), flavopiridol was introduced to several clinical trials to various cancers such as gastric (Schwartz *et al.* 2001), lung (Shapiro *et al.* 2001), colon (Aklilu *et al.* 2003), renal cell (Stadler *et al.* 2000) and prostate (Liu *et al.* 2004) cancers. Particularly, a multicenter phase II clinical trial was conducted using flavopiridol against hormone-independent PCa and the results showed that flavopiridol was unsatisfactory as a stand-alone therapeutic agent (Liu *et al.* 2004), due to its narrow therapeutic index which made drug scheduling and administration challenging. Its multikinase inhibitory nature was likely to be responsible for the observed clinical toxicity (Hofmeister *et al.* 2014). Flavopiridol failed to achieve overall objective responses in the clinical trials despite its promising preclinical outcomes (Liu *et al.* 2004).

Nevertheless, flavopiridol or other CDK9 inhibitors can be considered for combination therapies with other approved agents. Accordingly, a phase I trial of flavopiridol with paclitaxel has already been undertaken and the results showed that the combination produces promising outcomes (Schwartz *et al.* 2002). Mohapatra *et al.* (2009) reported that the combination of roscovitine with LY294002, an AKT inhibitor, produced significant apoptosis in PC3 PCa cells. Another CDK9 inhibitor, CDKI-73, when combined with fludarabine against chronic lymphocytic leukemia, resulted in synergistic cytotoxic activity (Walsby *et al.* 2014). As such, CDK9 inhibitor can provide significant benefit when combined with a chemotherapeutic or targeted therapeutic agent.

Despite showing promising cellular antitumor activity, therapeutic benefits of CDK9 inhibitors have experienced challenges translating into the clinic. Low selectivity and narrow therapeutic index are cruxes faced by CDK9 inhibitor development programs. For example, first generation inhibitors like flavopiridol have very narrow therapeutic indices, challenging the discrimination between normal and cancer cells. In addition, adverse effects of pan-CDK nature of CDK9 inhibitors may lead to dose limitations as evidenced in the phase I trial of TG02 (Hofmeister *et al.* 2015). While, highly potent CDK9 inhibitors are currently available (Table 3), they are seldom selective, especially within the CDK family. Structurally, this could be attributed to the large amount of shared features among the CDK family, particularly at the ATP-binding pocket, where current inhibitor discovery efforts are focused on. These structural similarities cause CDK9

Table 3 List of CDK9 inhibitors that reached preclinical or clinical development.

Compound	CDK inhibition (IC ₅₀ or K _i , μM)	Cellular activity (IC ₅₀ or LD ₅₀ , μM)	Cellular mode of action	Developmental stage	References
CDK1-73	CDK9 (0.004) CDK1 (0.004) CDK2 (0.003) CDK4 (0.009) CDK6 (0.052) CDK7 (0.091)	Primary CLL cells (0.08) Normal B cells (40.5) Normal CD34+ bone marrow (23) Therapeutic index >280 Multiple cancer cell lines Ovarian cancer (0.007) Epithelial cancer (0.057) Colon cancer* (0.044) Prostate cancer* (0.05) Breast cancer* (0.065) Pancreatic cancer (0.573) Leukemia* (0.164)	Reduces phosphorylation of RNAPII Ser2 Downregulates MCL-1, BCL-2, XIAP, MDM2, C-MYC Induces caspase3/7 activity and apoptosis Synergises with fludarabine	Preclinical	(Walsby et al. 2014, Lam et al. 2014)
Dinaciclib*	CDK9 (0.004) CDK1 (0.001) CDK2 (0.001) CDK4 (0.1) CDK5 (0.004)	Breast tumors* (0.008) Colon tumors* (0.017) Leukemia* (0.006) Mantle cell lymphoma* (0.007) Melanoma* (0.009) Ovarian tumors* (0.014) Prostate tumors* (0.012)	Reduces phosphorylation of RNAPII Ser2 Reduces Rb phosphorylation Downregulates MCL-1 and BCL-2 Induces apoptosis	Multiple clinical trials for hematologic and solid tumors	(Parry et al. 2010, Desai et al. 2013, Flynn et al. 2013, Nemanitis et al. 2013, Kumar et al. 2015, Baker et al. 2016) www.clinicaltrials.gov
Flavopiridol	CDK9 (0.02) CDK1 (0.03) CDK2 (0.07) CDK4 (0.1) CDK5 (0.17) CDK6 (0.08) CDK7 (0.88) CDK9 (0.011) CDK8 (0.016)	Primary CLL cells (0.35) Normal B cells (0.59) Normal CD34+ bone marrow (0.52) Therapeutic index ~1 A2780 (0.015) HCT116 (0.013) PC3 (0.01) Du145 (1.0) MV4-11 (0.049) OCIAML2 (0.063) PC3M (0.5)	Arrests G ₀ /G ₁ and G ₂ /M phase Increases expression of caspase3, 8, 9, P53 and BAX Induces apoptosis	Phase II clinical study against different solid tumors and leukemia (the only CDK9 inhibitor in clinical trial in prostate cancer)	(Kim et al. 2000, Soner et al. 2014, Walsby et al. 2014)
LY2857785	CDK9 (0.079) CDK1 (0.079) CDK2 (0.224) CDK4 (0.063) CDK6 (0.396) CDK7 (2.87)	PL21 (0.072) Colo25 (0.65) FaDu (0.8) H-460 (0.8) HCT116 (0.31) HT-29 (0.6) MCF-7 (0.52) PC3 (0.56) RPMI-8226 (0.9) SCC-25 (1.7) SiHa (0.42) T24 (0.39) U266B1 (0.5) Therapeutic index >14	Inhibits RNAPII Ser2 phosphorylation Inhibits XIAP and BCL-2, expression	Preclinical	(Yin et al. 2014)
P276-00	CDK9 (0.079) CDK1 (0.079) CDK2 (0.224) CDK4 (0.063) CDK6 (0.396) CDK7 (2.87)	PL21 (0.072) Colo25 (0.65) FaDu (0.8) H-460 (0.8) HCT116 (0.31) HT-29 (0.6) MCF-7 (0.52) PC3 (0.56) RPMI-8226 (0.9) SCC-25 (1.7) SiHa (0.42) T24 (0.39) U266B1 (0.5) Therapeutic index >14	Inhibits RNAPII Ser2 phosphorylation Downregulates Cyclin D1, MCL-1 and BCL-2 expression Increases caspase 3 activity Decreases Rb phosphorylation at Ser780 Arrests G ₁ phase	Phase I/II clinical trials for pancreatic cancer, multiple myeloma, mantle cell myeloma, breast cancer and melanoma	(Joshi et al. 2007, Manohar et al. 2011, Mishra et al. 2013) www.clinicaltrials.gov

Roscovitine	CDK9 (0.6) CDK1 (2.6) CDK2 (0.1) CDK5 (0.2) CDK7 (0.49)	DU145 (18.8) HCT116 (10.7) MCF (10.8) MES-SA/Dx5 (7.9)	Arrest G ₁ and G ₂ /M phase Induces apoptosis Increases P ⁵³ and reduces XIAP	Phase I for advanced solid tumors Phase II studies for Cushing's disease and cystic fibrosis	(Wang et al. 2001, McClue et al. 2002, Mohapatra et al. 2005) www.clinicaltrials.gov
SNS-032	CDK9 (0.004) CDK1 (0.48) CDK2 (0.038) CDK4 (0.925) CDK7 (0.062)	A2780 (0.039) A549 (0.043) HCT116 (0.07) MCF-7 (0.184) MDA-MB-435 (0.134) WM2664 (0.045)	Reduces RNAPII Ser2 and Ser5 phosphorylation Downregulates MCL-1 and XIAP Induces apoptosis	Phase I clinical trials in advanced solid tumors and advanced B-lymphoid malignancies	(Nuwayhid et al. 2006, Ali et al. 2007, Chen et al. 2009, Xie et al. 2014) www.clinicaltrials.gov
TG02	CDK9 (0.003) CDK1 (0.009) CDK2 (0.005) CDK3 (0.008) CDK5 (0.004) CDK6 (0.113) CDK7 (0.037)	MOLM-13 (0.15) MV4-11 (0.16) U937 (0.18) HL-60 (0.19) KG1-a (0.21) TF-1a (0.29)	Reduces RNAPII Ser2 phosphory- lation Downregulates MCL-1 and XIAP Induces apoptosis	Phase 1 clinical trials in advanced hematological malignancies	(Goh et al. 2012, Pallis et al. 2012) www.clinicaltrials.gov

*LD₅₀ or IC₅₀ values were reported as mean values of different cell lines of the same tumor type.

inhibitors to display residual, but significant, off-target activities. Increasing selectivity will promote the safety profile both *in vitro* and *in vivo* (Walsby et al. 2014, Scholz et al. 2015). A rare example is BAY1143572, a P-TEFb/CDK9 inhibitor with at least 50-fold selectivity against other CDKs, and was reported to show *in vivo* efficacy at tolerated doses in tumor models. As a result of promising preclinical results, this compound is currently undergoing phase I clinical trial as first in class P-TEFb/CDK9 inhibitor (Scholz et al. 2014). The recent discovery of i-CDK9, which showcases >600-fold selectivity *in vitro* against other tested CDKs, demonstrates that CDK9 selectivity may be achievable. However, the compound also inhibits other kinases potentially including DYRKs and PCTKs (Lu et al. 2015). Access to highly selective inhibitors undoubtedly will unveil the real clinical value of CDK9 inhibition. With the entry of Dinaciclib into phase III clinical trials, the likelihood of CDK9 inhibitors becoming a therapeutic option in PCa is higher than ever.

While increasing selectivity is important, it must be mentioned that cross activity toward other known targets may not always be harmful, as this may translate into higher efficacy against cancers, particularly relapsed diseases: a strategy often explored by combination therapeutical approaches. A broader target profile may also provide better opportunities at circumventing resistances. In addition, the fact that the fate of CDK9 inhibitors seems to depend on late-stage experiments, despite potent antitumor activities *in vitro*, illustrates an existing disparity in the potential to translate *in vitro* data to real therapeutic outcomes. As such, further detailed investigations into their modes of action and the roles of CDK9-involved normal and aberrant biological pathways in prostate and other cancers are particularly warranted.

Conclusion

CDK9 is involved with key events that promote early and late-stage PCa development and progression. It phosphorylates Ser81 of AR, which is required for receptor and downstream activation; plays a role in the regulation of MYC, NF-κB and STAT family; and activates RNAPII transcription which is associated with the overexpression of anti-apoptotic proteins MCL-1, BCL-2 and XIAP. These render CDK9 to be a highly attractive target for the treatment of PCa. Significant evidence also exists that inhibiting CDK9 by siRNA and pharmacological inhibitors downregulates anti-apoptotic proteins and induces apoptosis in CRPC cells. Although unsatisfactory outcomes halted the first generation of CDK9 inhibitors

regrettably during their clinical development, searching for CDK9 inhibitors with high efficacy and low toxicity and advancing them hastily through clinical evaluation is of paramount importance, as CDK9 presents potentially a vital opportunity for treating CRPC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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