Asian Journal of Urology (2020) 7, 271–283

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Review

Therapeutic targeting of the androgen receptor (AR) and AR variants in prostate cancer

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Received 29 March 2019; received in revised form 24 May 2019; accepted 20 June 2019
Available online 7 March 2020

KEYWORDS
Androgen receptor (AR);
AR variants;
Prostate cancer;
AR antagonists;
AR ligand binding domain;
AR activation function-1 domain;
Castration-resistant prostate cancer

Abstract Prostate cancer (PCa) accounted for over 300 000 deaths world-wide in 2018. Most of the PCa deaths occurred due to the aggressive castration-resistant PCa (CRPC). Since the androgen receptor (AR) and its ligands contribute to the continued growth of androgen-dependent PCa (ADPCa) and CRPC, AR has become a well-characterized and pivotal therapeutic-target. Although AR signaling was identified as therapeutic-target in PCa over five-decades ago, there remains several practical issues such as lack of antagonist-bound AR crystal structure, stabilization of the AR in the presence of agonists due to N-terminus and C-terminus interaction, unfavorable large-molecule accommodation of the ligand-binding domain (LBD), and generation of AR splice variants that lack the LBD that impede the discovery of highly potent fail-safe drugs. This review summarizes the AR-signaling pathway targeted therapeutics currently used in PCa and the approaches that could be used in future AR-targeted drug development of potent next-generation molecules. The review also outlines the discovery of molecules that bind to domains other than the LBD and those that inhibit both the full length and splice variant of ARs.

1. Introduction

The number of men living with prostate cancer (PCa) is increasing around the world with over 20 million men expected to live with PCa in 2024. Global statistics on cancer (GLOBOCAN 2018) indicate that 1 276 106 new PCa cases were diagnosed and 358 989 deaths due to PCa were reported in 2018 [1]. This accounts for 7.1% of the total cancer incidence and 3.8% of the total cancer-related deaths in the world [1]. Patients with PCa that receive an early diagnosis and are without metastases have a 100%

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Peer review under responsibility of Second Military Medical University.

https://doi.org/10.1016/j.ajur.2020.03.002

five-year survival rate, while those with PCa that has metastasized have a lower five-year survival rate [2]. The majority of patients are diagnosed at an early stage, attributed thanks to population-based screening with prostate-specific antigen (PSA) testing and digital-rectal examination, and only about 10%—30% of the patients present with high risk disease [3]. These high-risk disease patients have PCa with Gleason scores of greater than 7 and have higher proliferative indices. Surveillance and Epidemiology and End Results (SEER) database indicates that more than 80% of the PCa detected at the time of diagnosis are localized, while 12% are regional metastases, and only about 4%—5% are distant metastases [2]. PCa has several risk factors, which include age, race, genetics, and obesity. An increased risk in African American population compared to Caucasian American population, and in monozygotic twins compared to dizygotic twins has been frequently observed [2].

2. Androgens and androgen receptor (AR) in PCa

Huggins and Hodges [4] in 1941 discovered that the growth of the prostate and PCa is androgen-dependent and that PCa responds to a decrease in circulating testosterone by castration. This seminal finding has provided the indication to target the AR pathway to treat PCa for over 70 years. Although PCa is classified based on the Gleason score (the higher the score, the more aggressive the disease), it is also classified based on the expression of AR, the therapeutic target [5,6]. At the time of presentation, almost all cases of PCa are AR-positive [7,8]. However, persistent inhibition of the AR signaling pathway alters the course of the disease to an AR-negative phenotype in 10%—20% of the cases; these cases are referred to as more stem-like disease or neuroendocrine PCa [9]. One of the common features of the neuroendocrine disease is an absent or low expression of the AR [9]. Neuroendocrine PCa is an aggressive form of PCa with no targeted-therapeutics [10—12]. The readers are referred to other reviews for more information on the neuroendocrine PCa [11,13].

The fact that androgens increase the incidence of PCa has been established, and a prospective analysis of twenty studies was performed, where the risk of PCa was compared to levels of serum testosterone. Since serum testosterone has a broad-range (200—800 ng/dL), the patients were stratified into top and bottom deciles [14]. Men who had the lowest 10th percentile of serum testosterone had the lowest risk of developing PCa, while men who were at the highest 10th percentile of serum testosterone had the highest PCa risk. These and other clinical trials clearly suggested that androgens and AR are the drivers of PCa development. In addition, serum PSA, which is an AR target-gene, has been used as a PCa biomarker. Despite controversies surrounding serum PSA screening [15], it still remains to be the only available serum marker for detection of PCa and for determining therapeutic responses.

Before 2010, advanced castration-resistant prostate cancer (CRPC) was managed with docetaxel [16], however since then a few treatment options that target the AR signaling pathways have been approved, in the form of either androgen-synthesizing enzyme inhibitors or direct AR antagonist [17—23]. Persistent inhibition of the AR signaling-pathway to inhibit the growth of PCa results in a more aggressive disease, known as CRPC, which is responsible for majority of PCa-related deaths [24]. PCa is classified as CRPC when serum PSA levels rise despite low castrate-level serum testosterone (less than 50 ng/dL) [24,25]. Earlier, CRPC was considered to be androgen-independent PCa (AIPC) due to the evidence that the cancer grows in the absence of testosterone (castrate-level testosterone is considered in the range of extremely low to absent). However, recent evidence suggests that the AR adapts itself to the new environment and is still the driver of CRPC, despite castrate-level of serum testosterone.

3. AR dependence of CRPC

The majority of the approximately 360 000 deaths each year from PCa occur due to metastatic CRPC (mCRPC). CRPC is still dependent on androgen-signaling, and almost all the pathways that promote CRPC growth converge into the AR. Various mechanisms that contribute to continued CRPC growth include: AR over-expression where the AR becomes hypersensitive to the extremely low levels of androgens; AR promiscuity, where the AR is activated by a broad-spectrum of hormones such as progesterone and glucocorticoids; AR activation by non-canonical pathways such as growth factors and intracellular signaling pathways; AR variants (AR-Vs); intra-tumoral androgen-synthesis from adrenal-precursors; and AR coactivators that augment the function of the AR [26—29]. CRPC has a tendency to escape by one or the other alternate route and hence inhibiting the final convergence point, AR is efficient to address the continued progression of the CRPC. Several advanced therapeutics provide an efficient blockade of AR signaling, but still the therapeutic response in CRPC is short-lived and the disease escapes the treatment paradigm, resulting in the patient's death.

4. Treatments targeting the AR signaling pathway for androgen-dependent PCa and CRPC

Several approaches have been developed to target the AR signaling pathways and reduce the growth of CRPC. The targets that are altered with drugs or molecules are summarized in Fig. 1. The approaches used thus far include blocking the ability of gonadotropins to induce the synthesis of testosterone in the testes, inhibiting the androgen synthesis in the testes by blocking one of the upstream enzymes, inhibiting the conversion of testosterone to dihydrotestosterone (DHT) by 5-α-reductase (5αR) inhibitors, or inhibition of the AR itself. Unfortunately, irrespective of the strategy used, a brief response is obtained followed by a cancer relapse.

4.1. Estrogens

Interestingly, Dr. Huggins' Nobel-prize winning work demonstrated that PCa growth can be controlled either by

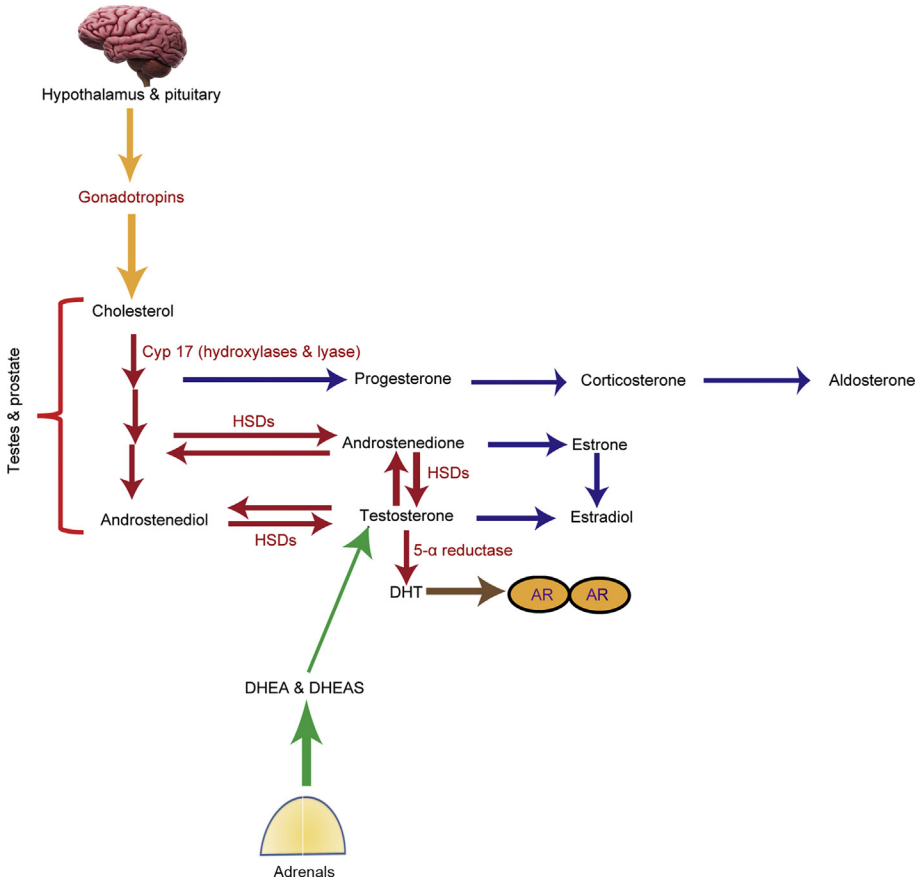

Figure 1 Hormone-synthesis pathway and various targets that are currently used or considered to inhibit prostate cancer growth. Clinically cyp-17 enzymes, 5-α reductase, and AR are validated as therapeutic targets and drugs targeting them are available. Preclinically HSDs have been validated, but yet to be extensively tested in the clinic. AR, androgen receptor; DHEA, dihydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; HSD, hydroxysteroid dehydrogenase.

androgen-deprivation therapy (ADT) or by using a high-dose of estrogen-therapy [4,30]. Based on this work, diethylstilbestrol (DES) became a standard of care for patients with PCa. DES and other estrogens function by two mechanisms, one by inhibiting the hypothalamus-pituitary-hypogonadal (HPG) axis, and the other by increasing sex-hormone binding globulin (SHBG) [31,32]. More than 90% of the serum testosterone is SHBG-bound with only a fraction being free testosterone and this free testosterone is the functional AR ligand [33,34]. Conversely, androgens inhibit the SHBG expression so that abundant free testosterone is available, while estrogens increase the SHBG expression. Taking advantage of this biology, estrogens were administered to increase the SHBG levels, resulting in more testosterone bound to SHBG and limited free testosterone left in circulation to be effective. Based on this hypothesis and historical results with estrogens, GTx, Inc. (Memphis, TN, USA) conducted a phase II clinical trial in 2012 with a non-steroidal estrogen receptor (ER) agonist, Capesaris (GTx-758). Administration of Capesaris to CRPC patients caused a dose-dependent increase in SHBG levels and a decrease in free-testosterone [35]. Despite the advantage of ER-targeted therapy for PCa, major adverse-effects such as venous thromboembolism and estrogenic proliferative actions on PCa cells contributed to the discontinuation of estrogen-based therapy for advanced PCa [31].

4.2. 5αR inhibitors

Although testosterone is the predominantly circulating hormone, it is converted to DHT in local tissues such as prostate and skin by the enzyme 5αR [36]. DHT is more potent than testosterone by at least ten-fold and it stably binds to and activates the AR LBD at lower concentrations than testosterone [37,38]. It was hypothesized that blocking the conversion of testosterone to DHT by inhibiting 5αR might inhibit the local conversion of testosterone to highly potent DHT and thereby reduce PCa growth. Preclinical studies to evaluate the role of 5αR in PCa were initiated in the 1970s. Several early compounds such as 6-methyl progesterone [39], 4-methyl-4-azasteroid [40,41], 4MA [42] were synthesized and evaluated. Administration of these compounds to Dunning and Noble rats and other PCa preclinical models resulted in a significant inhibition of tumor growth. These studies resulted in the conclusion that DHT, but not testosterone, is the primary promoter of PCa growth.

Encouraged by the preclinical results, finasteride, a 5αR inhibitor that inhibits two out of the three isoforms (isoforms 2 and 3), was advanced to the clinic. However, it was advanced to treat benign prostate hyperplasia (BPH), but not to treat PCa. In a BPH trial, it reduced the serum DHT levels by 70% and was approved by the Food and Drug Administration (FDA) for the treatment of BPH in 1992

[43,44]. Subsequently, dutasteride, another 5αR inhibitor that inhibits all three isoforms of 5αR, was approved in 2001 for the treatment of BPH [45,46].

The 5αR inhibitors were also evaluated to potentially prevent PCa, and in PCa prevention trial (PCPT), the finasteride arm exhibited a 25% reduction in PCa incidence [47,48]. Comparable results were obtained with dutasteride in the reduction by dutasteride of PCa events (REDUCE) trial [49]. Despite these reasonably impressive results, and limited adverse effects in both the trials, 5αR inhibitors prevented only low grade PCa, *i.e.*, PCa that had Gleason score <6. Peculiarly, the incidence of high grade tumors was higher in the 5αR inhibitors arms in both trials. Although the mechanism for these confounding results was unclear, it was hypothesized that due to shrinkage in the prostate, the proportion of high-grade cells in the remaining prostate became easily detectable. Currently, there are no 5αR inhibitors that are approved for the treatment of PCa or CRPC.

4.3. Androgen-synthesizing enzyme inhibitors

Considering that several enzymes can catalyze the conversion of precursors to testosterone, it is difficult to inhibit a single downstream androgen-synthesizing enzyme [50]. One of the most prominent among the androgen-synthesizing enzymes that could be therapeutically targeted is the cyp17 class that contains lyase and hydroxylase [17]. In addition to their role in androgen synthesis, cyp17 enzymes have also been shown to be increased in tumor specimens compared to benign tissues, indicating their potential role in continued tumor growth [51].

The earliest compound that was shown to have an inhibitory effect on the cyp-17 enzymes and downstream testosterone levels was ketoconazole. Administration of ketoconazole, an anti-fungal drug, resulted in a dose-dependent inhibition of the serum testosterone and long-term administration resulted in a sustained inhibition of serum testosterone levels [52]. Experiments in rat testes determined that ketoconazole inhibited the steroidogenesis by inhibiting cyp17 enzymes (17-α hydroxylase and 17,20-desmolase), but not 17-β hydroxysteroid dehydrogenase (17-β HSD) activity [53]. Due to poor metabolic and pharmacokinetic (PK) properties, as well as unwanted side-effects, ketoconazole was not pursued as a treatment for PCa. However, these early studies were a good proof-of-concept, demonstrating that cyp17 class of enzymes was a promising therapeutic target to treat PCa and CRPC.

The ketoconazole results were a catalyst to screen for molecules that bind to and inhibit the cyp-17 enzyme class. In the 1990s, a Cancer Research UK team discovered a series of steroidal cyp-17 (17-α hydroxylase-C17,20-lyase) inhibitors that had nanomolar potency in binding and inhibiting the enzyme action [54]. The lead-molecule (abinraterone acetate) was patented and the initial phase I and II clinical trials were conducted by Cancer Research UK team with the support of Cougar pharmaceuticals. Late-stage phase III clinical trials, which were conducted by Johnson & Johnson pharmaceuticals, showed that mCRPC patients treated with abiraterone acetate lived about 4.5 months longer than those who were treated with placebo (15.8 months *vs.* 11.2 months). The LATITUDE (patients previously treated with chemotherapy) and STAMPEDE (patients not previously treated with hormonal therapy) trials treated over 3 000 mCRPC patients with 1 000 mg abiraterone acetate (after overnight fasting) and prednisone combined with the standard ADT. Since abiraterone acetate inhibited the entire steroidogenesis, the patients had to be co-administered with prednisone to reduce the corticosteroid excess due to the lack of negative feedback regulation of the hypothalamus-pituitary-adrenal (HPA) axis. The drug was approved in 2011 for use in men with mCRPC [18,55]. In addition to the patients previously treated with chemotherapy, abiraterone was evaluated in patients who were not previously treated with chemotherapy. In this trial, abiraterone arm did not reach the median survival, while prednisone group had a 27.2 months median survival [56].

Subsequently, abiraterone was evaluated in high-risk non-metastatic CRPC (nmCRPC) patients who have higher or rising PSA without any evidence of bone or visceral metastasis (IMAAGEN trial) [57]. Patients who received abiraterone acetate and prednisone demonstrated a greater than 50% reduction in serum PSA in a study that was recently completed in 2018.

4.4. HPG axis modulators

Another approach to inhibit androgen synthesis and subsequently PCa growth is to inhibit the HPG axis [58]. The hypothalamus synthesizes gonadotropin-releasing hormones (luteinizing-hormone releasing hormone [LHRH] and follicle-stimulating hormone releasing hormone [FSHRH]), which in turn stimulates the pituitary gland to release gonadotropins, leutinizing and follicle-stimulating hormones (LH and FSH) [59]. These gonadotropins stimulate the testes to synthesize testosterone. When abundant testosterone is synthesized, the testosterone through negative-feedback inhibits further release of gonadotropins. This concept was used to inhibit testosterone synthesis and reduce PCa growth and there are currently two drugs in the market that are being used as ADT. Leuprolide and Degarelix are gonadotropin-releasing hormone (GnRH) analogs that are approved to be used as ADT in androgen-sensitive PCa [60,61]. Although GnRH analogs are recommended as ADT in advanced androgen-dependent PCa they are still used in combination with other AR- and enzyme-targeting agents in advanced CRPC. Analysis of clinical evidences with the GnRH analog, leuprolide, indicates that greater than 90% of the patients achieved nadir levels of serum testosterone [62]. Despite the suppression in the testosterone synthesis, PCa escapes GnRH treatment and becomes CRPC at which point this line of treatment is ineffective.

4.5. Other experimental androgen-synthesizing targets and agents

One of the promising therapeutic targets in the androgen-synthesizing machinery is aldoketo reductase 1C3 (AKR1C3) or also known as 17-β HSD5 [63]. AKR1C3 catalyzes the conversion of androstenedione (4'dione) to testosterone in extragonadal tissues such as the prostate. Elegant work by

Dr. Fernand Labrie [64] demonstrated that despite a complete reduction in serum testosterone levels after castration by over 90%, the total androgen pool was reduced by only 60%, indicating that adrenals continued to synthesize androgens. Adrenals synthesize dihydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS), which are converted to testosterone in the prostate by AKR1C3 and then to DHT by 5αR. In addition to this canonical pathway, AKR1C3 also uses a non-canonical pathway to synthesize DHT from 17β-diol. AKR1C3 has been shown to be highly expressed in both PCa cell lines and in aggressive forms of CRPC, which is considered to be an adaptive response to low levels of androgens, and to intra-tumorally promote active androgen synthesis from precursors. In addition, AKR1C3 might also have an AR coactivator function, which would make it one of the first pharmacologically-targetable coactivators [50]. Several AKR1C3 inhibitors have been developed, all of which show promising results in preclinical CRPC models [50,65,66]. However, a clinical trial with one of the inhibitors failed to demonstrate any efficacy [67]. There could be several reasons for this lack of efficacy, one of which could be the use of bypass pathways by the cancer cells to synthesize DHT from precursors.

5. AR

Other than the cyp17 enzymes, the only reliable target in the androgen signaling pathway is the AR itself. AR is a member of the nuclear hormone receptor family of ligand-activated transcription factors. AR has been the most reliable therapeutic target in treating PCa and CRPC, which is not surprising considering that almost all cases of PCa at origin and most cases of the CRPCs are androgen-dependent.

5.1. AR ligand binding domain (LBD)

The AR consists of an N-terminus domain (NTD) that is comprised of the activation function-1 (AF-1) domain that mediates the majority of the AR transcriptional activity, a DNA binding domain (DBD) that is responsible for binding to the DNA, a hinge region important for nuclear localization, and lastly a LBD to which all natural and synthetic ligands bind. The AR-LBD consists of AF-2 and 11 helices that are important for the conformation of the LBD and for the binding of various cofactor proteins [68,69].

In order to discover molecules that target the AR or any other protein, the crystal structure of the protein needs to be resolved so that chemical structures can be modeled in the relevant binding pocket for appropriate fit. The AR-LBD has been crystalized in the presence of agonists and a non-steroidal tissue-selective AR modulator (agonist) [70,71]. The crystal structures of AR-LBD bound to antagonists namely bicalutamide-bound AR-LBD and cyproterone-bound AR-LBD have been resolved with W741L or T877A AR-LBD, respectively wherein bicalutamide and cyproterone are agonists and not antagonists [72,73]. Unfortunately, the AR-LBD crystal structure in the presence of an antagonist has not yet been resolved [69].

The AR protein is atypical compared to other steroid receptors as it gets stabilized in the presence of its agonists, while other receptors such as estrogen receptor (ER) and progesterone receptor (PR) get ubiquitinated and proteasomally-degraded in the presence of their agonists [37,74—76]. Also, unlike ER or other hormone receptors, full-length AR protein or the AR-LBD forms tight complexes with chaperones in the absence of agonists, making it difficult to purify. Lack of purified unliganded AR-LBD hinders the development of reliable competitive AR binding assays for ligand screening. While ER-LBD has been crystalized with both agonists (estradiol) and antagonists (4-[OH]-tamoxifen and fulvestrant), AR-LBD has been crystalized only with agonists such as testosterone, DHT, and R1881 (synthetic steroidal androgen) and with non-steroidal tissue-selective AR agonists [77,78]. The reason why the AR-LBD crystal structure could not be resolved and what is unique about the AR-LBD that prevents its structural elucidation, remains important unanswered questions. The only information that is currently available comes from limited trypsinization studies and cofactor-interaction profiling that indicate that the AR conformation in the presence of agonists and antagonists is distinct [79,80].

Similar to the other hormone receptor LBD domains, AR-LBD is also comprised of an α-helical structure. While other steroid receptor LBDs contain twelve α-helices, AR-LBD has only eleven α-helices as it lacks helix 2 [69]. They are stacked as a sandwich with helix 12 forming the core of the ligand-binding pocket (LBP). From the crystal structures of ER-LBD, it has been determined that the helix 12 functions as a lid which tightly closes in the presence of agonists to hold the ligand tightly in its pocket, facilitating coactivators to bind, while it opens up in the presence of antagonists due to bulky side-chains. From the agonist-bound AR crystal structures, it was determined that the steroidal ligands interact with four key amino acids, R752, Q711, N705, and T877 [69]. Interestingly, the agonists, both natural and synthetic non-steroidal, bind to the AR in picomolar to nanomolar range similar to other hormone receptor agonists, while the antagonists bind and modulate the activity of the AR in the sub-micromolar range, a range that is higher than that of the other hormone receptor antagonists. Discovering more potent antagonists requires a knowledge of the structural information of the AR-LBD, resolving which seems to be an arduous task with the currently available technologies. Collective observations from previous studies suggest that the AR-LBD is structurally unique compared to other hormone receptor LBDs.

5.2. Outline of techniques used in the discovery of AR antagonists

With the absence of an antagonist-bound AR-LBD structure, targeting the AR needs to go through a systematic discovery paradigm to identify next-generation AR antagonists to treat CRPC (Fig. 2). Structures of the currently known AR antagonists are provided in Fig. 3. Currently, improvements in existing molecules' backbones are the more logical approaches as starting points. The molecules synthesized by the chemists undergo a series of tests starting with the competitive LBD-binding assay; this assay is conducted using either purified protein from constructs grown in *E. coli* or baculovirus or in cell-based system using one of the ^{3}H androgens (mibolerone or R881). After the discovery

of EPI-001 and other molecules that bind to domains different from the LBD [81—83], the competitive LBD-binding assay has become less relevant and the AR functional assay has become more critical. Whether a molecules binds to the AR-LBD or not, an AR transactivation assay in an antagonist mode in the presence of 0.1 or 1 nmol/L androgen (R1881 or DHT) will determine whether the synthesized molecule(s) is/are antagonist(s). While conducting these studies, it is important to compare against standard AR antagonists such as enzalutamide or EPI-001 or others that could differ between laboratories according to their internal practices. If a molecule is an AR antagonist, further studies in PCa cells such as gene expression and cell proliferation will provide additional information on the potency and efficacy of the compounds. In addition, after the recent discovery of PROTACs (proteolysis targeting chimeras) and other degraders (selective androgen receptor degraders [SARDs]) [82,84], performing Western blots using protein extracts from PCa cells treated with the compounds might provide information on whether a particular molecule is a degrader.

After the *in vitro* screening, the optimum molecules would be evaluated for metabolism properties in liver microsomes obtained from mice (MLM), rat (RLM), and humans (HLM). Molecules with a long half-life and less clearance are appropriate for *in vivo* screening. The Hershberger assay is a quick *in vivo* screening assay used to screen AR antagonists, where rats or mice will be administered with AR antagonists for 14 days and the effect of the compounds on AR-target tissues (such as prostate and seminal vesicles) will be determined. Molecules that are effective in Hershberger assay can be further tested in xenograft studies in

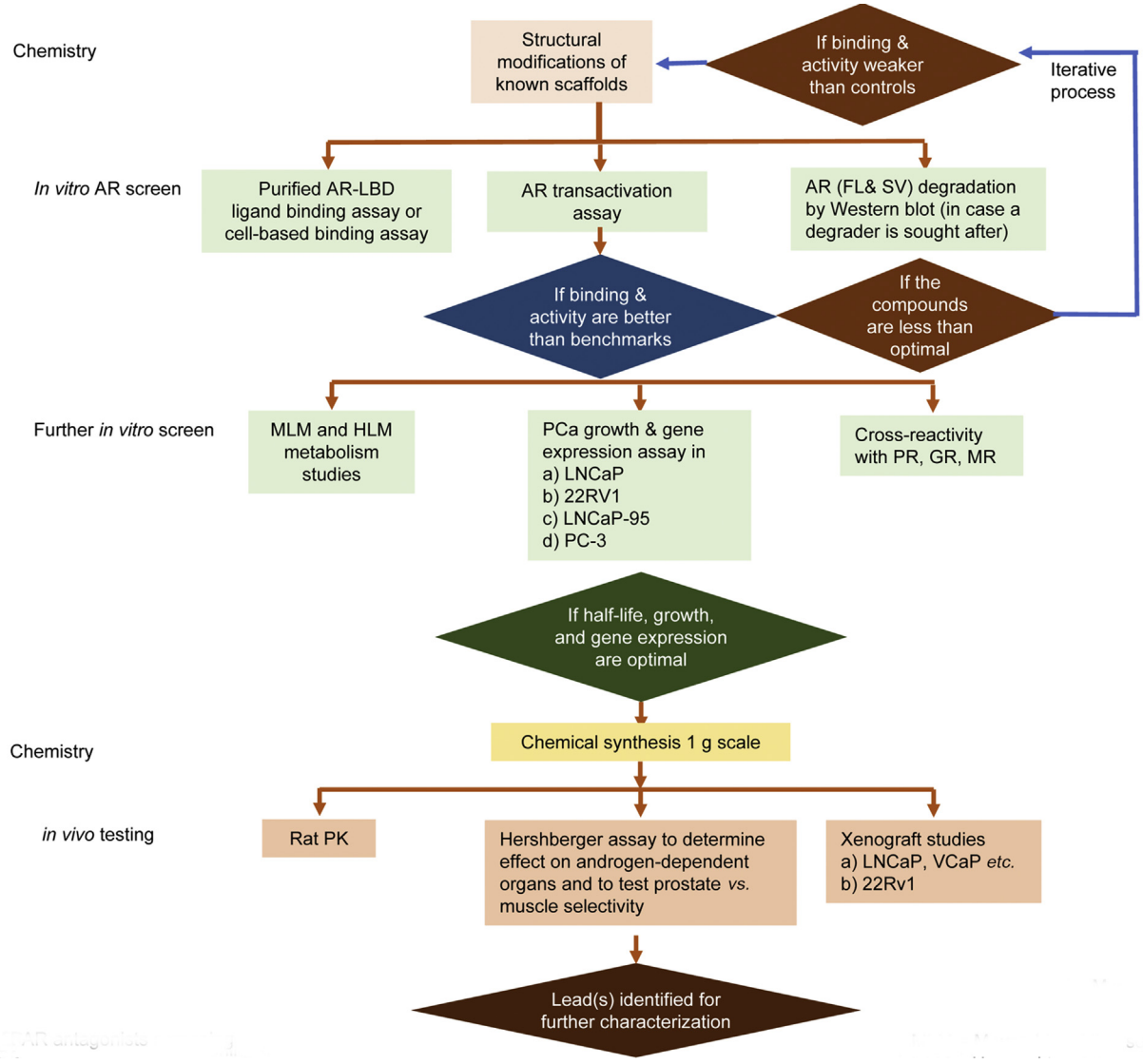

Figure 2 AR antagonists screening paradigm. AR, androgen receptor; GR, glucocorticoid receptor; HLM, human liver microsome; LBD, ligand binding domain; MLM, mouse liver microsome; PCa, prostate cancer; MR, mineralocorticoid receptor; PR, progesterone receptor.

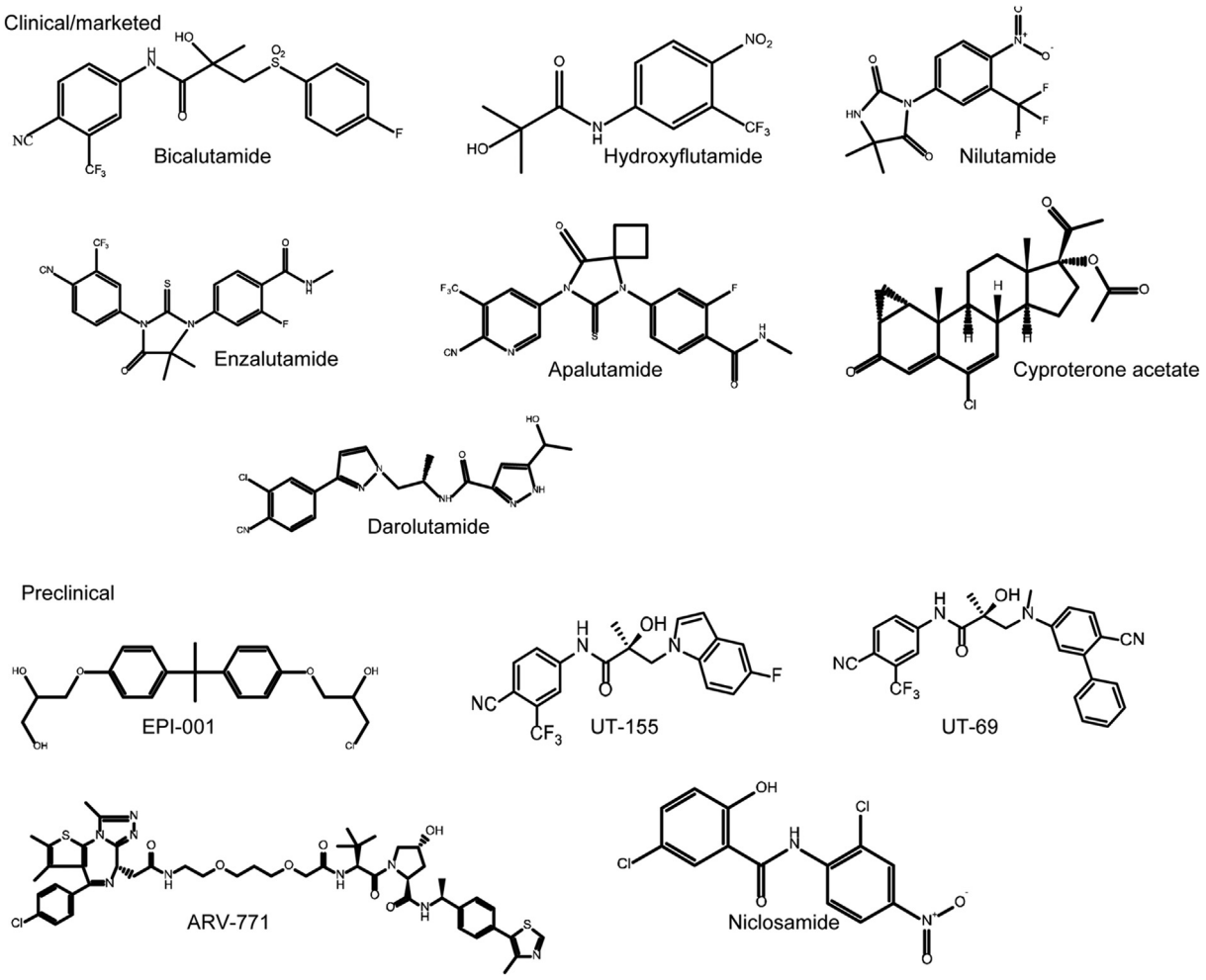

Figure 3 Structures of known AR antagonists that are in the clinical and preclinical stages. AR, androgen receptor.

immunocompromised mice using standard PCa cell lines or patient-derived tissues (PDXs). Molecules that demonstrate significant inhibition in a xenograft model can further be tested for pharmacokinetic (PK) properties, additional xenograft efficacy, and safety before a decision is made to proceed with clinical evaluation.

5.3. First-generation AR antagonists

Flutamide was one of the first-generation AR antagonists discovered by Schering Plough Corporation (New Jersey, USA) in the 1960s. It entered the clinic in the 1970s and became available to the patients around 1985 [85,86]. It was approved for the treatment of metastatic PCa in combination with GnRH analogs. Flutamide is metabolized into hydroxyflutamide, which is the active metabolite of flutamide with over 10-fold higher affinity to the AR than the parent compound, flutamide [87]. Flutamide has moderately inferior PK properties compared to other AR antagonists such as bicalutamide or enzalutamide. The recommended flutamide dose for PCa is 250 mg thrice daily that equals to 750 mg/day. Despite this high dose, flutamide does not completely inhibit the serum PSA levels, unlike other AR antagonists. In a controlled trial in PCa patients with metastatic disease, the GnRH analog leuprolide alone was compared to patients who received leuprolide in combination with flutamide. The combination treatment increased the progression-free survival (PFS) and overall survival (although the *p* values were not impressive [$\sim$0.035]), indicating that the combination treatment may be more effective than using GnRH alone. Due to the discovery of next-generation AR antagonists, flutamide is not currently widely used. Even in preclinical comparative studies, flutamide is not used as a standard comparator.

Bicalutamide (Casodex) is another first-generation competitive AR antagonist that was approved for the treatment of advanced PCa in combination with GnRH analogs in 1995. Bicalutamide was discovered by the Imperial Chemical Institute (ICI), which later became Astra Zeneca. Although bicalutamide was derived from flutamide, it possesses a diaryl propionamide structure. Bicalutamide has a chiral center and it is sold as a racemic mixture, and R-bicalutamide is more potent than S-bicalutamide. Bicalutamide antagonizes the AR in transient transactivation assays at about 4—5-fold lower the half maximal inhibitory concentration (IC50) than flutamide (stronger) and 8—10 times higher IC50 than enzalutamide (weaker), the second-generation AR antagonist [88,89]. While flutamide inhibits

AR both peripherally and centrally causing an increase in the LH and testosterone levels, bicalutamide inhibits the AR only peripherally, inhibiting the AR function only in target tissues, without causing any increase in serum LH or testosterone [88]. Bicalutamide is orally bioavailable and is well absorbed. Bicalutamide (50 mg/day) was compared to flutamide (750 mg/day) in a head-to-head study conducted in patients with metastatic PCa. Both drugs were administered in combination with GnRH analogs [90]. Bicalutamide combined with a GnRH analog was significantly superior to flutamide combined with a GnRH analog ($p < 0.001$). Also, the study suggested that men with metastatic PCa treated with flutamide were more likely to relapse compared to bicalutamide. Despite these advantages in favor of bicalutamide over flutamide, persistent exposure to bicalutamide causes a mutation in the LBD at W741 that converts bicalutamide into an agonist. Crystal structure of bicalutamide in W741L mutated AR suggests that the B-ring of bicalutamide interaction with the AR was comparable to that of the DHT-bound AR [73].

Two other competitive first-generation antagonists, nilutamide and cyproterone acetate (steroidal) were discovered and developed. However, they were not extensively used, due to either the steroidal structure of the cyproterone or weaker properties of both the compounds.

5.4. Second-generation AR antagonists

Dr. Charles Sawyers at Memorial Sloan Kettering and Dr. Michael Jung at University of California Los Angeles co-discovered a potent competitive AR antagonist, enzalutamide (MDV-3100 or Xtandi) in 2006, which binds to the AR at least 8—10 folds better than bicalutamide and inhibits AR by blocking the AR nuclear translocation [89]. Mechanistically, enzalutamide was superior to the first-generation antagonists due to its ability to prevent nuclear localization. The clinical trials that were conducted with enzalutamide were pivotal as they proved that patients who were thought to be androgen-independent because their relapse from AR-targeted agents were after all castration-resistant and continue to depend on the AR signaling axis. As AR is a transcription factor, it translocates into the nucleus to bind to the DNA and alters the expression of target genes. The hypothesis behind the discovery of enzalutamide was that if the AR is prevented from translocating into the nucleus, then it cannot be activated by either androgens or by growth factors. Although enzalutamide was not potent *in vitro* when compared to picomolar or nanomolar antagonists of other nuclear receptors such as ER, PR, or GR, *in vivo* performance was excellent due to its superior PK properties. Enzalutamide belongs to the hydantoin structural series that is distinct from the diarylpropionamide backbone of bicalutamide and the anilide backbone of hydroxyflutamide. A phase III clinical trial (AFFIRM) in 1 199 CRPC patients who have relapsed while on other chemotherapy was conducted with 160 mg/day enzalutamide and placebo at 2:1 ratio [19]. The median overall survival in enzalutamide arm was 18.4 months compared to 13.6 months in the placebo arm with a p-value of <0.001. The favorable response was not only observed in overall survival, but also in PSA reduction, soft-tissue response, the time for PSA progression and other endpoints. The major side-effect observed with enzalutamide in a subset of patients was seizure. Enzalutamide was developed by Medivation in partnership with Astellas. Currently, Pfizer markets enzalutamide (Xtandi) in the US and Astellas markets globally.

Prolonged treatment with enzalutamide results in a mutation at the F876 (F876L) in the AR-LBD, switching enzalutamide to an agonist [91]. Missense AR mutations, including this mutation (although rarely or infrequently detected in the clinic), are observed in 20% of the patient population treated with enzalutamide for a sustained period [92]. CRPC responds to enzalutamide for 12—24 months before relapsing again due to various reasons, including the F876L mutation, and AR-Vs, particularly AR-V7 [93—95].

Another second-generation AR-LBD competitive-antagonist co-discovered by Drs. Sawyers and Jung group, apalutamide (ARN-509) that has an almost identical structure to enzalutamide, and was developed by Janssen pharmaceuticals [96]. Due to the structural similarity between enzalutamide and apalutamide, it was expected that patients treated with apalutamide would also exhibit F876L mutated AR and the AR-V7 [91]. Since apalutamide was approved only recently in 2018, the duration of response is unknown, although phase I trials have detected F876L mutation in treated patient population [93]. Apalutamide is thought to have marginally better activity than enzalutamide, but with less seizurogenic effects than enzalutamide [97].

Another competitive ligand-binding AR antagonist that was recently approved was darolutamide [98]. It was developed by Orion pharmaceuticals as ODM-201 and further developed by Bayer [99]. Darolutamide does not cross the blood-brain barrier and hence will not likely have any seizurogenic effects. *In vitro* studies have shown that darolutamide is susceptible to the same mutations that inactivate hydroxyflutamide and bicalutamide. Darolutamide phase III clinical trial was recently completed in 2018 for the treatment of metastatic CRPC.

5.5. Molecules in preclinical testing that binds to domains other than the LBD

Considering that approximately 30% of the patients treated with abiraterone or enzalutamide exhibit AR-Vs, variants that lack the LBD, it has been anticipated that targeting domains other than the LBD might be beneficial. Recent blinded-prospective clinical trial in patients with CRPC demonstrated that those patients who express AR-V7 have shorter PFS and overall survival (OS), and fail to respond to enzalutamide and abiraterone [100]. This and other clinical studies are clear indication that molecules that target the AR-Vs through the NTD are important. However, due to the lack of structural information and knowledge of chemical moieties that might interact with other domains, such a pursuit is extremely complicated and cumbersome.

One of the essential steps in drug discovery is the development of an assay to screen for direct binding of molecules to the target protein. Due to the lack of a

competitive-binding assay to domains other than the AR-LBD, it is not possible to demonstrate a direct binding of newly discovered molecules. Currently used methods are biophysical in nature and can only measure indirect interactions. The most widely used method to determine binding to domains other than the LBD is fluorescence polarization (FP) with purified AR AF-1 [81,101]. This technique measures the shift in fluorescence emission of the tyrosine and tryptophan amino acid residues in the AF-1 as a measure to demonstrate the interaction of the compounds with the domain. Using FP and few indirect biochemical methods, Dr. Sadar's group [81] discovered the first-in-class AF-1-interacting molecule (EPI-001). Subsequently, a structural analog of EPI-001, EPI-506, was tested in a clinical trial in CRPC patients. Unfortunately, the molecule was ineffective even at doses as high as 2.5 g. Essa biotech that develops the EPI series has gone back to the drawing board to identify more potent AF-1-interacting molecules, and they have recently identified another class of molecules called sinkotamides [102].

Another group at the University of Tennessee Health Science Center (Memphis, TN, USA) discovered a series of molecules that interact with the AR AF-1 and degrade the AR and AR-V. Several biophysical methods such as FP, nuclear magnetic resonance (NMR), and surface plasmon resonance (SPR) were used to demonstrate the interaction between the small molecules and the AR AF-1 [82,83]. Although the biophysical and biochemical methods are compelling, they are not confirmatory evidence of direct binding.

Discovering molecules that bind to domains other than the LBD requires reliable screening methods. Biochemical methods such as transactivation, mammalian two hybrid, or Western blots determine the effect of small molecules on the function of either individual domains or the full length AR. Biophysical methods such as FP, NMR, SPR, and Raman spectroscopy are used to determine the interaction of a molecule with full length AR or an individual domain. Most biophysical techniques are not confirmatory and can only serve as a screening method. The results from single biophysical method require further validation using different approaches or multiple biophysical techniques. Radioligand competitive-binding assay or determination of crystal structure using X-ray diffraction or cryo-electron microscopy (Cryo-EM) are necessary to confirm the binding of molecules to domains other than the LBD. Absence of these confirmatory assays disadvantage the discovery of molecules binding to domains other than the LBD. Fig. 4 shows the potential steps that could be adopted to discover molecules targeting domains other than the LBD. If a molecule binds to the LBD, then it could be potentially evaluated as an AR-LBD-binding-antagonist and traditional approaches need to be adopted. However, if a molecule fails to bind to the AR-LBD, but inhibits the AR activity in a transient-transactivation assay, then it must be tested in various biophysical methods to determine if an interaction with a domain other than the LBD could be detected. If such interaction can be observed using a biophysical method, then lead optimization using an appropriate biophysical method should be pursued. One of the reliable methods, thermal-shift assay, provides information not only on the interaction of a small molecule with a protein, but also on the ability of the molecule to alter the protein's conformation. From our experience, this method produces consistent results [103].

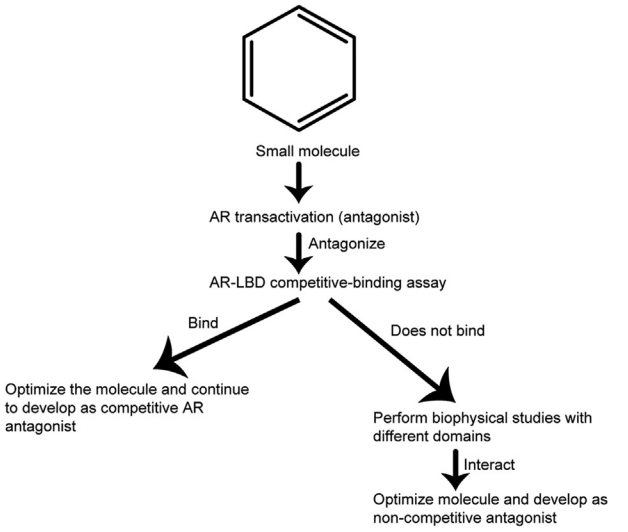

Figure 4 Screening assays to discover molecules to domains other than the LBD. AR, androgen receptor; LBD, ligand binding domain.

Although AF-1 has become the domain of interest for discovering drugs targeting AR and AR-Vs, several groups have also tried to develop drugs targeting DBD, and an alternate region on the LBD, BF-3 [104—106]. Many of these preclinical molecules in development are promising and interesting, however, recent failures of galetrone and EPI-506 have raised questions regarding the efficacy of molecules other than conventional competitive antagonists targeting the AR to treat CRPC or PCa. It could also be possible that extensive treatment of these cancers with enzalutamide and abiraterone has resulted in disease that has acquired a completely different pathological characteristics that are no longer susceptible to next-generation targeted therapeutics. Galeterone and EPI-506 also have other potential problems such as poor PK properties requiring the use of extremely high doses to observe efficacy, if any. In addition, EPI-001 (the EPI-506 analog) has weak *in vitro* activity, requiring over 30 μmol/L to achieve any effect in transactivation or gene-expression assays. Poor efficacy combined with inferior PK properties have resulted in their failure.

The recent discovery of PROTACs by a Yale group has triggered interest in chimera molecules. Arvinus has initiated a phase I clinical trial with a PROTAC targeting the AR [84]. PROTACs are novel set of compounds that are chimeric molecules that combine known binders of the AR or any protein of interest with an E3 ubiquitin ligase binder using a linker. The concept is to bind AR with the known binder and attract the E3 ubiquitin ligase to the AR for degradation. Based on this concept several chimeric complex molecules have been discovered and a lead has entered phase I clinical trial for advanced CRPC. Considering that the chimeric molecules are greater than 1 kDa, it will be interesting to see how the oral delivery of such large molecules will be possible. Moreover, these PROTACs and chimeric molecules bind to the LBD and do not affect the AR-Vs.

6. Conclusion

CRPC is an evolving disease that is challenging to treat. Several new-generation drugs with distinct mechanisms of action and large windows of safety are required to provide sustained benefit to patients. Until one of the new molecules with a distinct mechanism shows success in the clinic, there will be skepticism whether such molecules can provide therapeutic benefits. Considering the success of fulvestrant, an ER degrader, in breast cancer, it could be expected that degraders of the AR could be successful to treat CRPC. However, it is still unclear whether the unique properties of the AR within its susceptibility to ubiquitin proteasome pathway might play a role in the success or failure of SARDs. Until reliable structural information or assays are developed, AR antagonists are likely to lack potency. In order to be more successful in discovering highly potent AR antagonists or degraders, it is not only relevant to discover molecules with similar chemical structures or backbones, but also crucial to resolve the crystal structure of the AR-LBD or the full-length AR to enable improvements in structure-aided drug discovery.

Conflicts of interest

The author is a consultant to Oncternal Inc (San Diego, CA, USA).

Acknowledgements

This work was supported by a grant from National Cancer Institute (NCI) 1R01CA229164-01A1.

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