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Complications of Treatment

Androgen receptor plasticity and its implications for prostate cancer therapy

Oliver Snow[a,b], Nada Lallous[a], Kriti Singh[a], Nathan Lack[a], Paul Rennie[a], Artem Cherkasov[a,*]

[a] Vancouver Prostate Centre, University of British Columbia, 2660 Oak St, Vancouver V6H 3Z6, BC, Canada
[b] School of Computing Science, Simon Fraser University, 8888 University Drive, Burnaby V5A 1S6, BC, Canada

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ABSTRACT

Acquired resistance to a drug treatment is a common problem across many cancers including prostate cancer (PCa) - one of the major factors for male mortality. The androgen receptor (AR) continues to be the main therapeutic PCa target and despite the success of modern targeted therapies such as enzalutamide, resistance to these drugs eventually develops. The AR has found many ways to adapt to treatments including overexpression and production of functional, constitutively active splice variants. However, of particular importance are point mutations in the ligand binding domain of the protein that convert anti-androgens into potent AR agonists. This mechanism appears to be especially prevalent with the AR in spite of some distant similarities to other hormone nuclear receptors. Despite the AR being one of the most studied and attended targets in cancer, those gain-of-function mutations in the receptor remain a significant challenge for the development of PCa therapies. This drives the need to fully characterize such mutations and to consistently screen PCa patients for their occurrence to prevent adverse reactions to anti-androgen drugs. Novel treatments should also be developed to overcome this resistance mechanism and more attention should be given to the possibility of similar occurrences in other cancers.

Introduction

Drug resistance is a common occurrence in many diseases, ranging from antibiotic resistance in bacteria and antiviral resistance by HIV strains to drug resistance in cancer, where it represents an especially pressing and unresolved issue. Resistance arises in nearly all cancer types and across numerous different drug classes with distinct mechanisms of action [1]. This is due to the inherent nature of cancer, where populations of tumour cells are put under selective pressure; such that the cells that survive are those which evolve ways to avoid the immune system and chemotherapies. Tumour heterogeneity plays a significant role as well, since cancer is often characterized by the compromise of DNA repair mechanisms resulting in high rates of unique mutations. This means that it is unlikely for a single targeted drug to work for all cells in a tumour, and thus the cells not killed by the drug are selected for and resistance develops.

It is important to distinguish here between innate resistance (also known as intrinsic resistance) and acquired resistance [2]. Innate resistance occurs when cancer cells already harbor mutations rendering the drug ineffective before its administration. In contrast, acquired resistance develops as a reaction to a drug, and represents a bigger issue as it is less dependent on the individual's underlying genetic profile, and is generally more common across patients.

In recent years, sequencing technology has improved to the point that researchers can easily interrogate hundreds of tumour profiles and discover the genetic alterations responsible for drug resistance. This has uncovered a number of different mechanisms through which resistance can arise including drug efflux, drug inactivation, drug target alteration, cell death inhibition, and DNA damage repair among others [3]. Recently, factors related to cancer stem cells and tumour microenvironments have also been implicated in drug resistance, further complicating the issue [4–6].

Not only does resistance develop through diverse molecular pathways, but also in response to many different drug types. In recent years, targeted therapies have shown promise as a more precise and effective alternative to traditional cytotoxic chemotherapies. However, the biggest barrier to the success of targeted therapies is the inevitable development of drug resistance, just as with past approaches [1]. The details of cancer drug resistance have been described in a number of comprehensive reviews [7–9] and it is not the subject of this paper. Here we only emphasize that the accumulated body of evidence outlines the complexity and ubiquity of cancer resistance and indicates the immense need to design innovative therapies to combat the resistance issue. More specifically, this review paper focuses on the androgen receptor (AR) and the multitude of ways it can become resistant to drugs. We highlight the distinct ways the AR can evade therapy, particularly

* Corresponding author.
E-mail address: artc@interchange.ubc.ca (A. Cherkasov).

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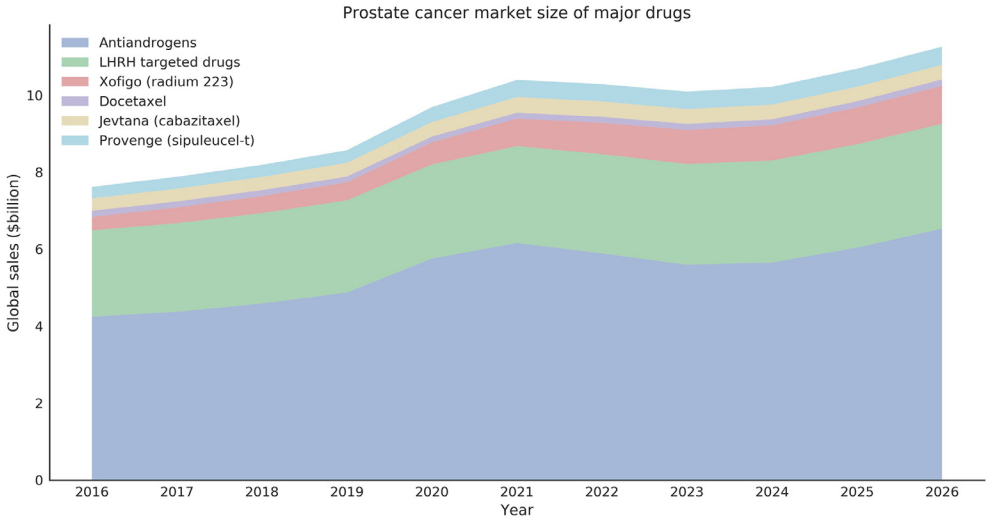


Fig. 1. Market share projected to 2026 for major prostate cancer drugs representing multiple drug types/classes.

through gain of function mutations and splice variants, mechanisms not seen to the same extent in other hormone nuclear receptors. The AR stands out as particularly difficult to target due to its adaptability but the body of research on AR targeted therapies provides some important lessons for other targets in cancer medicine.

Drug resistance in prostate cancer

It is hard to find a disease where the prevalence of drug resistance is more evident than in prostate cancer (PCa). PCa is one of the most commonly diagnosed malignancies in men and despite significant improvements in screening and treatment it remains the third leading cause of cancer death in Canada with similar prevalence around the world [10]. Patients initially respond well to surgery and medical castration by way of androgen deprivation therapy (ADT) which reduces androgen production, primarily dihydrotestosterone (DHT), through administration of luteinizing hormone releasing hormone (LHRH) targeted drugs [11]. DHT and other androgens bind the AR at its ligand binding domain (LBD), causing heat shock proteins to dissociate from the AR, allowing it to homodimerize and then translocate into the nucleus [12]. Reducing androgen production through ADT in turn reduces the activation of the AR, which is a transcription factor for genes related to cell cycle, sexual differentiation, and prostate development among others. Inhibiting the AR decreases cell proliferation and ultimately slows cancer growth [13]. This signalling cascade leading to transcription is known as the 'classical' or 'genomic' pathway but the AR, like other nuclear receptors, can also have effects through a non-genomic pathway, also known as rapid actions [14]. These rapid actions are still not fully understood but the emerging research on these non-canonical pathways suggests an even more complicated picture of the AR function and could lead to even more ways to effectively target the AR for cancer treatment. Additionally, Stoecklin et al., 1999 outline how hormone receptors can also effect transcription through interaction with signal transducers and activators of transcription (Stat) proteins, regulating different genes than through the classical pathway [15]. Interestingly, they found that the AR had no effect on Stat5 mediated transcription while other similar receptor either induced or inhibited transcription through this pathway, highlighting the variability of function between very similar proteins. Despite the complex and interesting picture of hormone receptor signalling, much of the current AR targeted therapy, including ADT, is focused on disrupting the canonical pathway and so this review will focus mainly on this pathway. Although ADT has been shown to prolong patient survival, almost all patients eventually progress to the castrate resistant (CRPC) form of the disease after only a couple years.

It was originally believed that this progression to CRPC signified that the cancer was no longer mediated by the AR, known as hormone refractory, because androgen levels were shown to be depleted. It is now well established, however, that CRPC continues to be mainly driven through AR signaling axes through a number of different adaptive mechanisms.

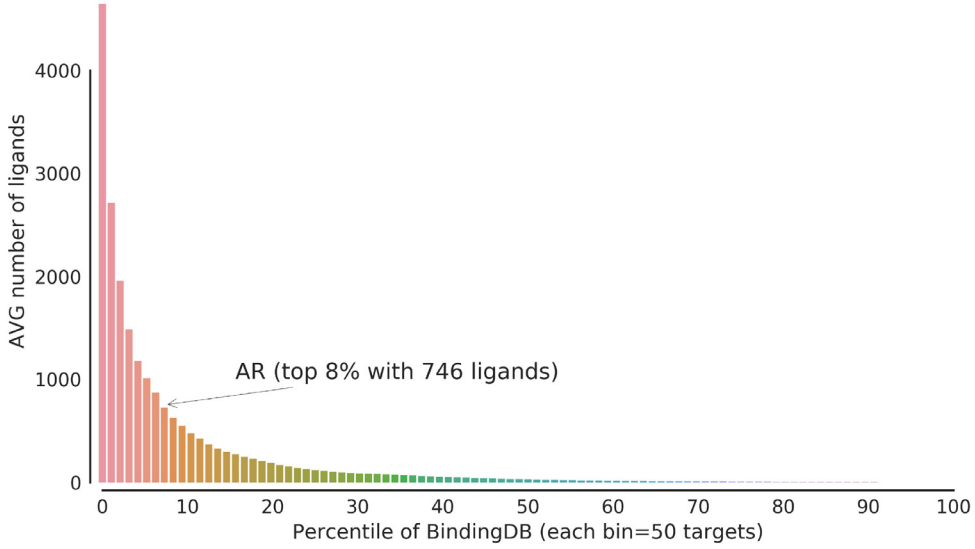
The first generation therapies - flutamide, nilutamide, and bicalutamide were developed to address this issue by out-competing endogenous steroids and inhibiting the AR. In combination with ADT these drugs provide only a modest and temporary increase in patient survival [16]. Furthermore, these drugs have also been shown to activate the AR in cells overexpressing the AR [17]. Research in recent years into the molecular details of the restoration of the AR signalling axis has led to the development of next generation targeted therapies - abiraterone [18] and enzalutamide [19]. Abiraterone is a potent inhibitor of the enzyme CYP17A1, which is crucial for androgen biosynthesis whereas enzalutamide is a pure antagonist of the AR, designed to overcome previous limitations of the first generation therapies [20]. However, despite the initial success of these treatments, acquired resistance to enzalutamide is well documented [21] and some preliminary works have suggested abiraterone is prone to resistance as well [22].

Mechanisms of AR resistance

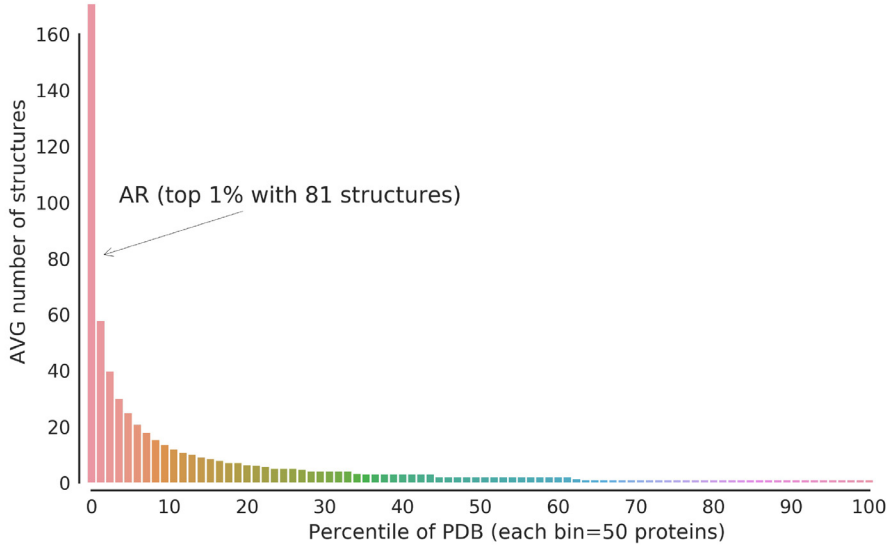
Although there are other drivers and therapeutic targets in PCa, the AR remains the main treatment focus for this disease. The PCa drug market is expected to grow from the current $7B dollars to well over $10B by 2026, where AR-directed drugs represent over 50% of the share, which keeps expanding (Fig. 1) [23].

This importance of the AR as a drug target is also reflected in the impressive amount of compounds that are known to bind to the AR. When compared to other proteins in the Binding Database the AR is in the top 8% of targets with 746 ligands known to bind to it (Fig. 2a). Further indication that the AR is one of the most studied drug targets, is the large number of published crystal structures for the AR in the Protein Data Bank (PDB). An analysis of the content of PDB puts the AR in the top 1% of proteins with 81 structures resolved for the human receptor, remarkably in the same league as other simple and highly studied proteins such as lysozymes (Fig. 2b) [24].

The pressure put on the AR by various targeted therapies has in turn given rise to a number of defense mechanisms by which the tumour can evade treatment that can be categorized as non-AR mechanisms



(a) Targets ranked by number of ligands known to bind them with the AR in top 8%



(b) Human proteins ranked by number of published crystal structures; androgen receptor in top 1%

Fig. 2. Ranking of androgen receptor with other proteins in BindingDB and PDB.

occurring either through bypass signalling or through complete AR independence, and restoration of AR signalling, occurring through gain of function mutations and active splice variants [25]. While these concepts have been explained in detail elsewhere [26], we will reiterate some of their main characteristics here and expand on the AR dependent mechanisms that are particularly troubling in PCa treatment.

AR independent resistance

Bypass signalling, originally discovered with respect to kinases, is now understood to occur with hormone receptors as well, including the AR. This happens when downstream signalling of the target protein is restored by the activity of a different protein. In the case of the AR, resistance to enzalutamide has been shown to be caused in some cases by increased activity of the glucocorticoid receptor (GR), which is able to drive the transcription of AR related genes without activation of the AR [27,28].Bypass signalling can also occur through non-canonical signalling pathways as mentioned previously either dependent on ligand binding. Davey and Grossman, 2016 describe some of these alternate pathways in detail, and although currently limited mostly to *in vitro* studies it will be important to better understand these pathways and how they might be involved in PCa drug resistance by circumventing classical AR signalling [29].

While in such AR signalling bypass states tumour cells continue expressing the AR, cells can also express low or no AR protein at all and still progress to CRPC with complete independence of AR signalling. These cells have been shown to display markers of neuroendocrine differentiation and even signs of small-cell carcinoma, both representing relatively rare variants of PCa [30,31]. The implications of these findings is that the pressure of prolonged treatment with the second generation anti-androgens can cause the cancer to change to a different sub-type, rendering treatments ineffective.

AR dependent resistance

The third major category of AR drug resistance is the restoration of AR signalling, which can occur via multiple mechanisms and is perhaps the most complicated of the three. The first way of such restoration is through adrenal production of androgens that can replenish their depleted levels in the prostate after ADT [32]. Hence, dehydroepiandrosterone (DHEA) and androstenedione (AD) are produced in the adrenal gland and can then be converted into testosterone and DHT in the prostate, activating the AR despite castration and competition from anti-androgens [33,34]. Abiraterone targets this conversion process and leads to the reduction of both DHEA and AD but residual androgens in other forms still remain and may reactivate the receptor [35]. Targeting other enzymes responsible for this conversion of adrenal androgens to DHT is a valuable avenue for future drug development in combination with the current CRPC treatments.

Another way that AR signalling can be restored is through alternative splicing of the AR mRNA or through truncated forms of the AR resulting from mutations [36]. A number of AR variants (ARVs) have been discovered both in the clinic and in cell lines, and have been significantly implicated in CRPC and resistance to enzalutamide and abiraterone (Fig. 3) [37,38].

Interestingly, most of these ARVs have intact DNA-binding and amino-terminal transactivation domains but have completely truncated LBDs [39]. In some cases, the truncation can cause the AR to be constitutively activated and localized to the nucleus, allowing it to drive gene transcription independent of any hormones or anti-androgens [22]. The most well documented variant is AR-V7, which is missing the LBD but remarkably is still active and over-expressed in resistant tumours [40]. Some evidence has shown that AR-V7 and other splice variants may still require binding to the full length AR to become active and that anti-androgens remain effective [41]. However, clinical studies of patients with advanced PCa demonstrated that AR-V7 expression is associated with resistance to enzalutamide and abiraterone, suggesting that AR variants provide yet another way for the AR to evade treatments [42]. As the evidence on ARVs continues to grow, it is becoming clear that the expression of different variants such as AR-V7 is not static and that the function of these altered proteins in PCa remains to be uncovered [43]. Overall, the, current understanding of ARV biology suggests that future treatments for the AR should target domains other than the LBD to avoid the rise of resistance due to gain of function splice variants.

Another mechanism by which the AR can be restored after exposure to current anti-androgens is through the occurrence of the gain-of-function mutations in the LBD. Several of such AR mutations have been documented to date [44,45]. However, only in recent years have researchers begun to reveal the greater extent of these mutations and their causal role in antiandrogen resistance and cancer progression. Next generation sequencing studies have identified quite a significant number of AR mutations, with the majority of them found in the LBD. There are four such recurrent mutations that present in 20% of patients (L702H, T878A, W742C, H875Y) [46]. The frequency of these mutations found in patients is demonstrated in Fig. 4, where there are noticeable spikes in the LBD, also reflected in the 3D structure where many mutations cluster around the androgen binding site (ABS). The list of gain-of-function AR mutations continues to grow and in combination with upregulation of the AR the prevalence of these mutations can be found in up to 60% of patients treated with anti-androgens. The most concerning aspect of these point mutations is that they do not simply render current drugs ineffective but can convert them from an antagonist into an agonist, turning the treatment into disease promoter.

In 2016, Lallous et al. [47] utilized circulating cell free DNA from

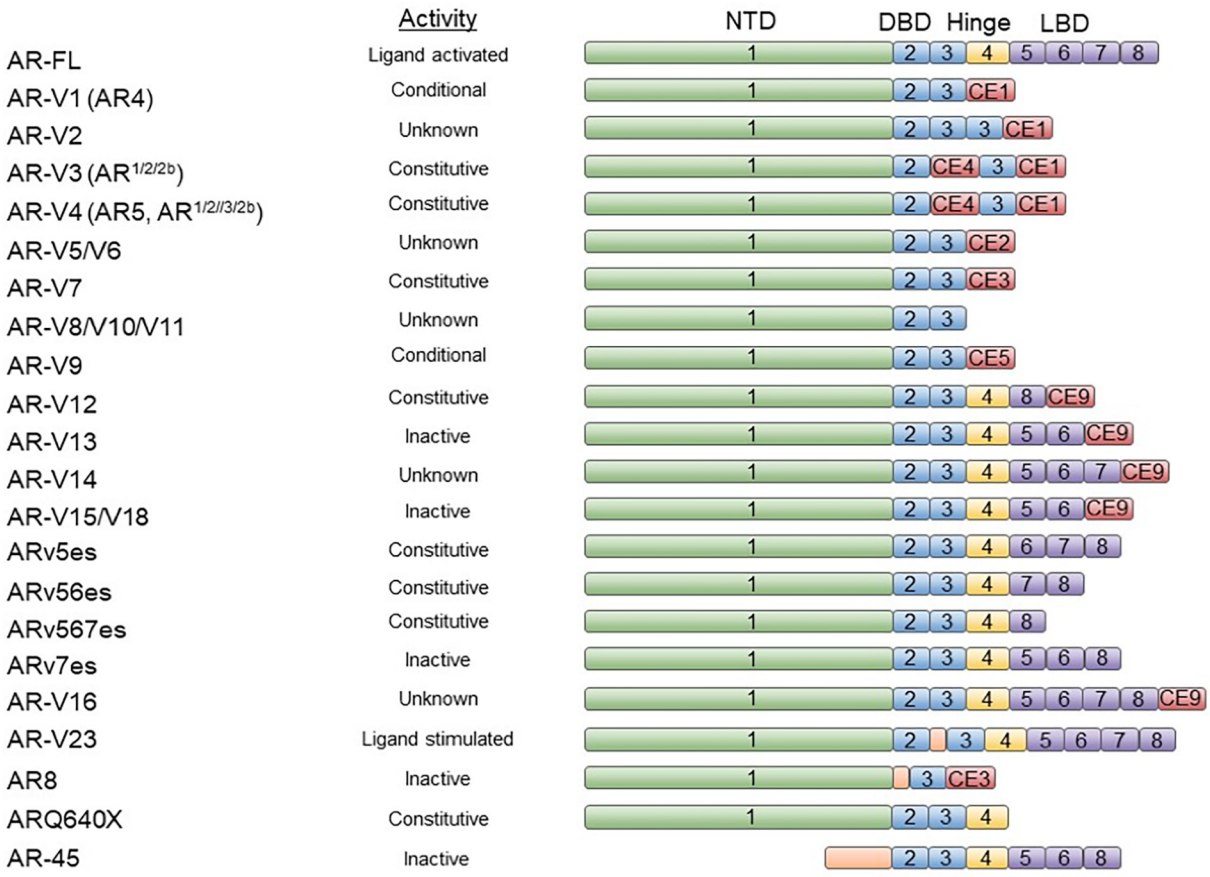


Fig. 3. Androgen receptor variants discovered in prostate cancer and their corresponding activities and exon sequences.

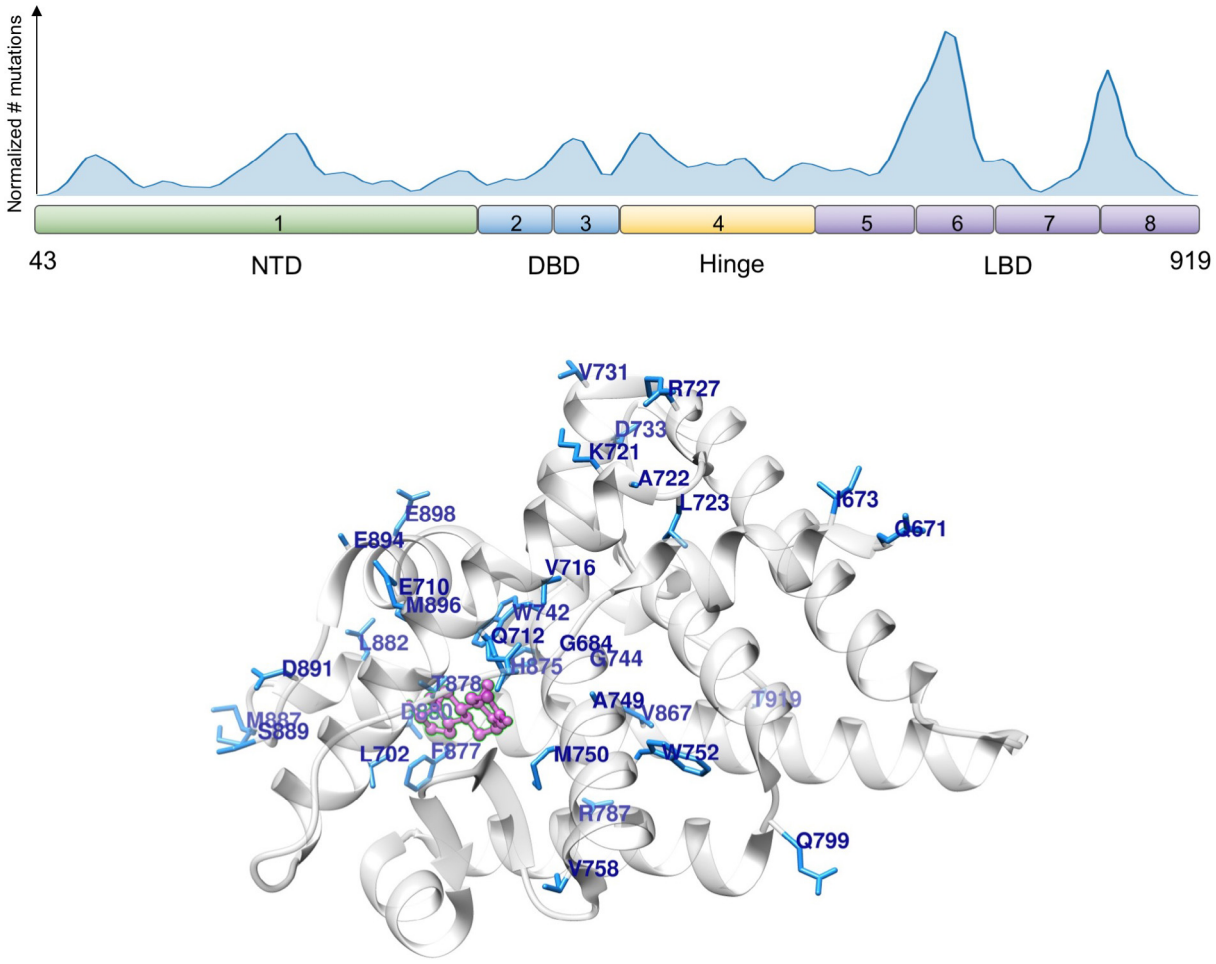


Fig. 4. Normalized number of unique AR mutations found in prostate cancer patients and their location on the androgen receptor (AR) gene. Mutations mapped to 3D structure of AR (PDBID: 2AM9) with testosterone bound.

CRPC patients to sequence and characterize the mutational landscape of the AR, adding 8 new LBD mutants to the list of the previously reported ones. Importantly, they tested the *in vitro* activity of these variants in the presence of current and prospective anti-androgens (enzalutamide, hydroxyflutamide, bicalutamide, darolutamide and apolutamide). Strikingly, all of the mutants had an agonist reaction to at least one of the studied anti-androgens, strongly indicating the need to find and characterize all the resistant AR mutations in order to screen patients and alter treatments before adverse reactions can occur.

Similar examples to AR agonist switch

This above-mentioned mechanism of resistance, in which point mutations in AR LBD can convert antagonist drugs into receptor agonists is particularly troubling from therapeutic perspective, but is also very intriguing from the standpoint that it appears to be mostly specific for the AR (and with similar, but partial evidence for occurrence in the estrogen receptor (ER)).

To our knowledge, there are no other examples of cancer drug resistance where this similar drug conversion occurs to such extent as with the AR. There are many documented cases in which target proteins become insensitive or less sensitive to the corresponding drugs, such as the receptor kinases BCR-ABL, KIT and EGFR, for example [48], where a point mutation in an active site can drastically reduce drug binding affinity [49]. While many of these mutations can reduce or even eliminate drug efficacy and can be very prevalent (thus, KIT mutations have been observed in up to 50% of patients) [50], there are still no reports of those drugs being switched into agonists.

There is also significant research demonstrating that cancer can become resistant to treatment when point mutations cause receptors to become constitutively active. The ER, an important driver of breast cancer and very closely related to the AR both structurally and functionally, and is an additional example of such constitutive activation by mutations. Both the AR and ER are ligand-activated transcription factors for a number of genes that drive cell growth and proliferation and therby are prime targets in cancer therapy [51]. Beato et al., 1996 provides an extensive review of the common transcriptional regulation mechanisms among steroid hormone receptors including the AR and ER [52]. In fact, much of our knowledge of the ligand binding mechanisms for the AR comes from research on the ER as they are so closely related both in structure and function. Both receptors contain the same three principle domains of the N-terminal domain (NTD), DNA binding domain (DBD) and LBD, and both require the recruitment of coactivators. Although many details remain to be discovered, there is strong evidence indicating that a small inward movement of helix 12 is crucial to activation and it is through helix 12 and its interaction with coactivators that ligands have their effect [53].

A number of studies, reviewed by Alluri et al., 2014, have reported acquired mutations in the ER caused by the treatment with tamoxifen, raloxifene and other anti-estrogens and that those mutations increased ER activity and led to progression of the disease [54]. This increase in activity, however, was independent of ligand binding, with the mutations locking the receptor into its active state without any effect from the drug [55].

Despite the growing number of documented resistant ER mutations, only a few of them exhibited some antagonist-to-agonist drug conversion. One early *in vitro* study by Mahfoudi et al. (1995) demonstrated that mutations in the ER LBD can cause tamoxifen to act as a strong agonist [56]. Furthermore, Zhao et al. (2003) showed that Leu-536 in the ER LBD is important for mediating the ligand effects and that its mutation could mediate ligand-independent receptor behavior and alter partial agonist effects of tamoxifen depending on the assay and receptor sub-type [57]. A more recent study by Arao et al. (2013) demonstrated *in vitro* and in a knock-in mouse model that point mutations in the same area of the LBD could change antagonists to agonists [58]. Specifically, L543A and L544A could cause ER antagonists to activate the ER and promote downstream effects of the receptor. Despite these exciting works, the presence of these ER mutations in patients and thus the clinical relevance of these mutations has not been well documented and their role in cancer resistance in humans remains to be uncovered.

Another hormone nuclear receptor similar to the AR and ER is the progesterone receptor (PR) - a crucial drug target in the treatment of breast cancer. The PR could represent yet another close example of an agonist switch. In 1993 it was demonstrated that elevated levels of 8-Br-cAMP PR can allosterically activate the PR promoting its DNA binding and transcriptional activity [59]. The same authors went on to show that it is only the full-length hPRB isoform that is prone to cAMP activation [60]. Other research further described the potential for certain PR antagonists to become agonists in response to certain LBD mutations of the PR, but this was again dependent on the presence of cAMP and was not related to mutations discovered in the clinic that were known to lead to tumour resistance [61,62].

Thus, it is possible to speculate that despite a number of somewhat related examples of mutation-caused drug resistance, there has been comparatively little evidence showing cases of definitive antagonist to agonist drug conversion, as it has been abundantly observed for AR LBD mutants.

SARMs and plasticity of nuclear receptors

A further indication of the plastic nature of the AR, is the promiscuous activity of a class of drugs known as selective androgen receptor modulators (SARMs). These compounds are characterized by their ability to function as agonists in some tissues and antagonists in others. A comprehensive review by McEwan, 2013 details the growing interest in SARMs and describes the underlying chemistry and biology of these novel drug molecules [63]. There are also equivalent compounds for both the ER and PR known as SERMs and SPRMs respectively. The existence of these types of compounds are not new; however, it has not been until recently that their exact mechanisms of action have begun to be described. In fact, both tamoxifen and raloxifene, major cancer drugs targeting the ER and also PR, were initially described as antagonists but are now known to act differently depending on the location of action [64]. Mifeprestone, a drug targeting the PR and commonly used to terminate pregnancy, has also been shown to have a mixed agonist–antagonist effect [65].

SARMs, in contrast, have been investigated less than SERMs or SPRMs and most of the research has centered on the search for drugs that emphasize the agonist effects but limit those effects to certain tissues. These would be alternatives to androgen therapies currently used to treat hypogonadism in men or decreased libido in women, which can often have terrible side effects [66]. SARMs could alleviate these side effects by being able to be delivered orally or via injection without the risk of dramatically raising systemic androgen levels and avoiding toxicity of the liver and other organs. Despite these developments, there has been little investigation into SARMs that focus more on the antagonistic effects desirable as a treatment for prostate cancer. Current SARMs are partial agonists in androgenic tissues such as the prostate and full agonists in anabolic tissues, making them unsuitable as a cancer treatment, but may have potential for other androgen based diseases such as benign prostatic hyperplasia [67].

As explained previously, emerging evidence on the effects that certain AR point mutations have on current anti-androgens suggests that those ligands may not be pure antagonists as previously thought [47]. Further research has demonstrated that these drugs, such as bicalutamide and hydroxyflutamide can act as antagonists in some contexts and as agonists in others [68]. In addition to this, other work has demonstrated that coregulators may play an important role in the mixed effect of SARMs, where varying concentrations of co-activators in different tissues can mediate the activity of the receptors in response to the ligand [66]. In general, the exact pharmacology behind the mixed effect of SARMs is still poorly understood. However, the current understanding supports the idea that the AR is rather plastic, in the sense that it can accept a variety of ligands (Fig. 2a) and switch between conformations easily, making it an especially difficult target.

Similarities and differences of nuclear receptors

The similarities between the structures and functions of the main nuclear receptors has led to many inferences in their targeting by small molecules and mechanisms of their drug resistance. Much of the current evidence suggests a common pattern of resistant plasticity of nuclear receptors, where different subtypes, disordered structures, and availability of many co-regulators can alter the effectiveness of targeted treatments. The similar effects of SARMs, SERMs and SPRMs also suggest a common pattern of promiscuity of nuclear receptor ligands.

Despite these commonalities however, the AR appears to stand out in regards to the number of documented LBD resistant mutations. The Androgen Receptor Gene Mutations Database from McGill university catalogs all the known AR mutants and puts the total number at over 1,000 with ~150 of those mutants being found in PCa tissues [69]. This indicates the remarkable prevalence of AR single point variations, many of which cause the agonist switching and therefore represents an immense clinical risk for PCa patients. This has motivated the need to screen for these mutations, to characterize them and to develop the corresponding prognostic means for clinical outcomes [70]. It is unclear, however, why mutation-driven drug conversion is far less prevalent and profound in ER and PR, compared to AR, while similarity between these receptors could imply much closer resemblance in their biology and mechanisms of resistance.

As mentioned previously, much of the structural and mechanistic insight about the AR has simply been inferred from the ER and other receptors based on analogy,focusing here on the classical, genomic signalling pathway while recognizing the existence of other non-genomic pathways. However, the high number of crystal structures of the AR has enabled more rigorous functional studies on the AR in an attempt to give a mechanistic interpretation of the receptor's activity in the context of both native ligand and drug interactions. While the exact AR activation process is not fully understood, there is strong evidence originated from molecular dynamics simulations that the receptor's active conformation is associated with an inward movement of helix 12 closing around the ABS (which, in turn, can be induced or inhibited by a ligand) [71,72]. As discussed earlier, these movements in helix 12 are comparable between the AR and ER, so it is unclear why there is not the same extent of activating mutations as in the AR. The subtleness of the activation process best described in [53] may provide some insight into the mixed effects of AR ligands as only a small perturbation in the structure due to mutation or the effect of a co-regulator may be enough to change the receptor to its active state from inactive or vice versa.

Further studies have implicated the AR in telomere instability, complicating the picture even more. Zhou et al. showed that in LNCaP PCa cells, telomere stability was dependent on the AR as inhibition or knockdown of the receptor caused telomere dysfunction [73]. Additionally, the AR both binds to and is a component of telomeres, suggesting that targeted AR drugs may contribute to genomic instability and prostate cancer cell proliferation.

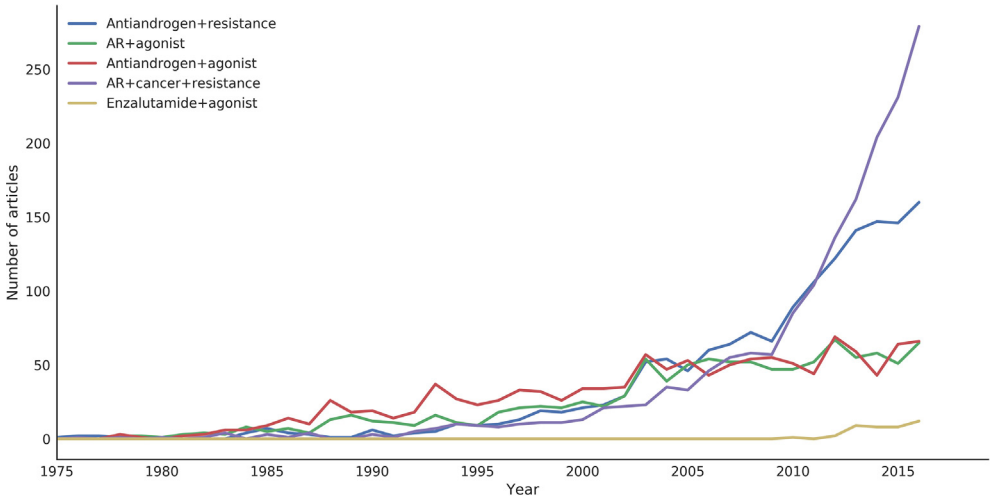


Fig. 5. Number of citations in Pubmed per year for different combinations of keywords for androgen receptor cancer resistance.

The AR is also found in many tissues in the body besides the adrenal glands and the prostate and has been implicated in other cancers beyond prostate [74]. Therefore, these mutations should also be considered in other cancer types to avoid resistance and to improve treatments for those cancers as well. Future AR targeted therapies will require clever strategies and should aim to avoid this mechanism of resistance, possibly by targeting other parts of the protein besides the androgen binding site. The success of this approach was demonstrated recently, where a new anti-androgen compound was identified that bound the activation function 2 (AF2) [75] and also compounds targeting the binding function 3 (BF3) site of the AR [76–80]. Both classes of compounds successfully bound to different sites of the protein and were able to effectively inhibit growth in prostate cancer cells resistant to enzalutamide. Additionally, a drug targeting the DNA binding domain of the AR was developed in 2014 that had anti-AR potency similar to enzalutamide with the potential to overcome these resistant mutations and could also be effective against AR-V7 splice variants [81–83].

Conclusions and future directions

Prostate cancer has been one of the most well studied cancers and has had targeted treatment options for decades mainly aiming to inhibit the AR, one of the most investigated drug targets in cancer. The majority of those treatments, despite great initial success, invariably acquired resistance. This can be seen with the sharp rise in the number of articles discussing the AR, resistance, antiandrogens, and agonist effects, and since its discovery in 2009, enzalutamide has seen increasing citations along with 'agonist' term (Fig. 5)[84]. Interestingly, these mechanisms of resistance, either overexpression, truncations, or point mutations, appear to only be present in therapeutic scenarios. This suggests that the extensive targeting of the AR and the pressure that puts on the receptor has led to the variety of ways for acquired resistance to develop. The remarkable adaptation of the AR has made PCa difficult to treat but has also led to transformative research on cancer treatment and resistance.

Some of the mechanisms of AR resistance are common among many cancers such as overexpression of the receptor or mutations that cause the receptor to be constitutively active or less sensitive to small molecule inhibitors. However, two mechanisms stand out for the AR: production of splice variants that are constitutively active and occurrence of LBD point mutations that turn a well-established antagonistic drug into a potent agonist. These are especially clinically relevant mechanisms and they need to be further characterized in full details in order to equip clinicians with the knowledge and means to address the phenomena of drug resistance in prostate cancer. This would be a significant step towards personalized medicine where patients could be monitored and then provided or taken off treatments based on their genomic profile. As the field of cancer therapeutics progresses with more targets being introduced and more focused drugs being developed, we should heed the lessons from the AR and pay particular attention to point mutations and splice variants and their potential to cause resistance to cancer treatment.

Declaration of Competing Interest

None.

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