Discovery of Novel Androgen Receptor Inhibitors as Prospective Therapeutics for Advanced Prostate Cancer

by

Huifang Li

BSc., China Pharmaceutical University, 2007

MSc., China Pharmaceutical University, 2010

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Abstract

Prostate cancer (PCa) is the most commonly diagnosed cancer in men, and the second leading cause of male cancer death in North America. The androgen signalling pathway plays a central role in the development and advancement of PCa as well as in its progression to a lethal castration-resistant stage (CRPC). The human androgen receptor (AR) is a master regulator of PCa progression and survival, and a well-validated drug target for PCa. All clinically used AR inhibitors (antiandrogens) are initially effective to PCa; however, they invariably cause resistance. Thus, there is a continuing need for developing novel anti-AR drugs for the treatment of PCa and CRPC.

Although the mechanism of resistance to antiandrogens is not completely clear, it involves mutation-driven antagonist-to-agonist transformation of the AR response, and the emergence of AR splice variants (ARVs) lacking the entire ligand-binding domain (LBD) of the protein. This dissertation describes the discovery and development of novel AR inhibitors directed towards the conventional androgen binding site (ABS) of the receptor, as well as the discovery of an entirely novel class of inhibitors targeting the DNA-binding domain (DBD) of the AR. Both types of AR inhibitors were identified through virtual screening and molecular modeling, followed by *in vitro* and/or *in vivo* validation of developed drug prototypes. The objective of developing novel chemotypes for ABS binders and AR DBD inhibitors is to help circumvent drug resistance problem in the field of PCa.

Preface

1. Work described in section 3.2.1 of Chapter 3 has been published [**Li H**., Hassona M.D., Lack N.A., Axerio-Cilies P., Leblanc E., Tavassoli P., Kanaan N., Frewin K., Singh K., Adomat H., Böhm K.J., Prinz H., Guns E.T., Rennie P.S., Cherkasov A. Characterization of a new class of androgen receptor antagonists with potential therapeutic application in advanced prostate cancer. Mol. Cancer Ther. 2013, 12: 2425-35.]. Drs. Cherkasov and Rennie are senior authors, and they supervised this project throughout the concept formation to manuscript revision. I worked on the *in silico* part, as well as drafted and revised the manuscript. Drs. Lack and Leblanc are major contributors to the *in vitro* evaluation of the inhibitors. Dr. Hassona and Mr. Adomat contributed the *in vivo* data. Dr. Tavassoli, Ms. Frewin and Ms. Singh helped with biological experiments, while Mr. Axerio-Cilies and Dr. Kanaan helped with the computation.

2. Work described in section 3.2.2 of Chapter 3 has been published [**Li H**., Ren X., Leblanc E., Frewin K, Rennie P.S., Cherkasov A. Identification of novel androgen receptor antagonists using structure- and ligand-based methods. J. Chem. Inf. Model. 2013, 53: 123-30.]. Drs. Cherkasov and Rennie supervised this project on molecular modeling and biological evaluation, respectively. Xin Ren and I contributed to the computational part equally, and I drafted and revised the manuscript. Dr. Leblanc and Ms. Frewin tested the compounds.

3. A version of Chapter 4 has been published [**Li H**., Ban F., Dalal K., Leblanc E., Frewin K., Ma D., Adomat H., Rennie P.S., Cherkasov A. Discovery of Small-molecule Inhibitors Selectively Targeting the DNA-Binding Domain of the Human Androgen

Receptor. J. Med. Chem., 2014, 57 (15): 6458-67.]. Drs. Cherkasov and Rennie are supervisory authors, and they led this project throughout the concept formation to manuscript revision. I was the leading computational chemist, responsible for design and conduction of computational experiments, part of the experimental evaluation, and wrote the manuscript. Dr. Ban mentored me on the modeling and design portions of the project. Dr. Leblanc managed the *in vitro* screening of inhibitors and mentored me on bioassays. Dr. Dalal contributed to the characterization of identified inhibitors. Ms. Frewin and Mr. Ma contributed to the biological evaluation and characterization. Mr. Adomat helped to examine chemical properties of the developed compounds.

4. Materials and methods described in Chapter 2 have been published in above mentioned research papers.

List of publications:

1. **Li H**., Ban F., Dalal K., Leblanc E., Frewin K., Ma D., Adomat H., Rennie P.S., Cherkasov A. Discovery of Small-molecule Inhibitors Selectively Targeting the DNA-Binding Domain of the Human Androgen Receptor. J. Med. Chem., 2014, 57 (15): 6458-67.

2. **Li H**.,* Hassona M.D.,* Lack N.A.,* Axerio-Cilies P., Leblanc E., Tavassoli P., Kanaan N., Frewin K., Singh K., Adomat H., Böhm K.J., Prinz H., Guns E.T., Rennie P.S., Cherkasov A. Characterization of a new class of androgen receptor antagonists with potential therapeutic application in advanced prostate cancer. Mol. Cancer Ther. 2013, 12: 2425-35.

* These authors contributed equally to this work.

3. **Li H**.,* Ren X.,* Leblanc E., Frewin K, Rennie P.S., Cherkasov A. Identification of novel androgen receptor antagonists using structure- and ligand-based methods. J. Chem. Inf. Model. 2013, 53: 123-30.

[*] These authors contributed equally to this work.

4. Dalal K., Roshan-Moniri M., Sharma A., **Li H**., Ban F., Hessein M., Hsing M., Singh K., LeBlanc E., Dehm S., Guns E.S., Cherkasov A., Rennie P.S. Selectively Targeting the DNA Binding Domain of the Androgen Receptor as a Prospective Therapy for Prostate Cancer. J. Biol. Chem. 2014, 289:26417-29.

5. Ban F., Leblanc E., **Li H**., Munuganti R.S., Frewin K., Rennie P.S., Cherkasov A Discovery of 1H-indole-2-carboxamides as Novel Inhibitors of the Androgen Receptor Binding Function 3 (BF3). J. Med. Chem., 2014, 57 (15): 6867-72.

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3βHSD: 3β-hydroxysteroid dehydrogenase.

ABS: the androgen binding site in the ligand binding domain.

ADME: absorption, distribution, metabolism, excretion.

ADT: androgen deprivation therapy.

AF1: activation function 1 in N-terminal domain.

AF2: the activation function 2 in the ligand binding domain.

AKT: protein kinase B.

AR: androgen receptor.

ARE: androgen response element.

ASO: antisense oligonucleotides.

BF3: the binding function 3 in the ligand binding domain.

BMI: body mass index.

CRPC: castration-resistant prostate cancer.

D4A: Δ4-abiraterone.

DBD: the DNA-binding domain of androgen receptor.

DHT: 5α-dihydrotestosterone, an endogenous ligand of AR.

DRE: digital rectal exam.

EBD: ETS DNA-binding domain.

EGF: epidermal growth factor.

eGFP: enhanced green fluorescent protein

ER: estrogen receptor.

ERG: ETS-related gene.

ETS: E-26 transformation-specific.

FKBP5: FK506 binding protein 5.

GPCR: G protein-coupled receptor.

GPU: graphics processing unit.

GR: glucocorticoid receptor.

HSP90: heat shock protein 90.

HTS: high throughput screening.

IGF: insulin-like growth factor

IL: interleukin.

LBD: the ligand-binding domain of AR.

LBDD: ligand-based drug discovery.

LHRH: luteinizing hormone releasing hormone.

MD: molecular dynamics.

MTD: maximum tolerated dose.

mTOR: mammalian target of rapamycin.

MR: mineralocorticoid receptor.

NLS: nuclear localization signal.

NMR: nuclear magnetic resonance.

NTD: the N-terminal domain of AR.

PCa: prostate cancer.

PDK1: 3-phosphoinositide dependent protein kinase-1.

PTEN: phosphatase and tensin homolog.

PI3K: phosphatidylinositol 3-kinase.

PIP2: phosphatidylinositol 4, 5-triphosphate.

PIP3: phosphatidylinositol 3, 4, 5-triphosphate.

PK: pharmacokinetics.

PPARγ: peroxisome proliferator-activated receptor-gamma.

PR: progesterone receptor.

PSA: prostate-specific antigen.

QSAR: quantitative structure-activity relationship.

QSPR: quantitative structure-property relationship.

QSTR: quantitative structure-toxicity relationship.

R1881: a synthetic androgen.

RMSD: root mean square deviation.

RNA: ribonucleic acid.

RTK: receptor tyrosine kinase.

SAR: structure-activity relationship.

SBDD: structure-based drug discovery.

SHBG: sex hormone binding globulin.

SHR: steroid hormone receptor.

shRNA: short-hairpin RNA.

siRNA: small-interfering RNA.

SRD5A: 5α-reductase.

STAT3: signal transducer and activator of transcription 3.

TMPRSS2: transmembrane protease, serine 2.

VS: virtual screening.

XPA: xeroderma pigmentosum group A.

Acknowledgements

This dissertation features the results of my research in the course of my PhD study, and it wouldn't be completed without help and support from many talented and inspiring people. First and foremost, I would like to thank my supervisor Dr. Artem Cherkasov for offering me the opportunity to work in his lab, which allows me to continue my pursuit in the field of drug discovery. His sharp vision, efficient workstyle, enthusiasm and commitment to work have greatly influenced me and motivated me to keep pushing myself to higher levels. I would like to thank my supervisory committee Drs. Paul S. Rennie, YZ Wang and Steven S. Plotkin for their mentorship in each stage of my PhD tenure. I am grateful to Dr. Rennie for his insightful guidance, useful suggestions and strong support in the past few years both as a supervisory committee and leader of our AR inhibitor group. I am thankful to Dr. Wang and Dr. Plotkin for their important advices and their support of my research.

I would like to thank all members in Drs. Cherkasov and Rennie labs, and friends from the Vancouver Prostate Centre for their help, support and collaboration. I appreciate the experience to work in a multidisciplinary environment, to communicate and learn from people with a different background and expertise.

I would like to acknowledge the UBC bioinformatics program, its program coordinator Ms. Sharon Ruschkowski, program head Dr. Steven Jones and friends from the bioinformatics program for their help and support throughout my PhD.

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Words cannot express gratefulness to my parents, brother and other family members for their unconditional love that helped me to go from China to Canada to pursue my PhD study. Without their love, support and encouragement, it wouldn't be possible for me to complete my PhD degree. In the past few years away from home, they are the drive of me to overcome the difficulties of living in in a foreign country and to take every opportunity to improve myself.

I appreciate all the help, encouragement, and support from my dear friends in and out of Canada. I feel truly lucky for having a handful of close friends, who have accompanied me through different stages of my 'PhD life'. They shared my joy, complaints, and pressures, and helped me to stay on track. I believe the friendship with those awesome friends will become unforgettable memory in my life.

Dedication

I dedicate this dissertation to my beloved parents and brother. My father made me believe that I am destined for “great things”; my mother kept helping me to grow up as a person with self-esteem, independence and confidence; my brother showed me that life is full of possibilities and taught me how to never, ever give up.

To my beloved parents and brother

Chapter 1: Introduction

1.1 Prostate

The prostate is a part of male reproductive and urinary systems that is a walnut-sized gland, located between the bladder and testis and in front of the rectum. The prostate produces the fluid portion of semen as well as other substances to maintain and nourish sperm. The prostate tends to grow larger with age and raise prostate health problems, including prostatitis, benign prostatic hyperplasia and prostate cancer (PCa).

1.2 Prostate Cancer

PCa is the most commonly diagnosed malignancy in men, and the second leading cause of male cancer death in North America.[1] It is mostly prevalent in developed countries, such as United States, Canada, and Northern and Western European countries, and the lowest incidence rates are found in Asia and North Africa.[2] There is a remarkable incidence difference between developed and developing countries.[2, 3] In 2014, PCa accounts for 27% (233,000) of all newly diagnosed male cancers in United States;[1] it accounts for 24% (23,600) of all new male cancer cases in Canada (Canadian Cancer Statistics 2014). The incidence of PCa in Asian countries has also increased substantially in recent years.[2-4]

1.2.1 Risk Factors of PCa.

Although the etiology of PCa is still obscure, some risk factors have been found to be linked to the incidence and progression of PCa. Well-established demographic risk factors include increasing age, race/ethnicity and family history,[5] which are non-modifiable. Other modifiable risk factors such as lifestyle and behavioral factors have been found to be associated with PCa.[5, 6]

Increasing age is a significant risk factor of PCa.[7] PCa is rare in men under 50, while men who are older than 50 have an increasing risk of PCa. In the U.S., over 80% of PCa cases were diagnosed in men over age 65. Epigenetic alteration with aging, such as DNA methylation and histone modification, may represent some of the molecular mechanisms responsible for increased incidence in older men.[8]

Race/ethnicity is a clear but not well-understood risk factor for PCa. African-Americans have the highest incidence rate, and the disease tends to be diagnosed at younger ages and develops faster.[9] After African-American men, PCa is mostly commonly found in Caucasian men, followed by Hispanics and Native Americans.[10] Asian men have the lowest rates of PCa,[4] and it is worth noting that incidence rates among Asian men living in North America were much higher than men living in Asia, suggesting the effect of race is influenced by country of residence.

Various studies suggest the family history is also a major risk factor of PCa.[11] Men whose fathers or brothers have PCa, have 2 to 3 times higher risk of the disease than men who don't have a family history of PCa.[12] The younger a man's relatives are when they have PCa, the greater his risk is for developing the disease.[11] PCa risk also appears to be higher for men with a family history of breast cancer.[13]

Epidemiologic, preclinical and clinical studies indicate that diet is strongly associated with the incidence and progression of PCa.[14, 15] Published data suggested high consumption of carbohydrates, saturated fats, red meat and processed food promotes PCa risk, while the intake of plant phytochemicals, omega-3 fatty acids, soy protein and green tea has a protective effect and reduces the risk.[16] The western diet with high fat is suggested to increase the production of hormones including androgens and estrogens, and the Asian diet

with high fiber and low fat is associated with lower circulating levels of hormones and thus lower risk of PCa.[4, 17]

Obesity is usually measured by the body mass index (BMI, weight in kilograms divided by the square of height in meters, kg/m^2), and it has been found to be linked with a range of cancer types.[18] Accumulating studies have shown that elevated BMI is significantly associated with higher risk of PCa-specific mortality and increased risk of biochemical recurrence after primary treatment.[19, 20, 21] Physical activity has been shown to be linked to reduced risk of breast and colon cancers, while there is weak or no relation with the risk of PCa.[22, 23] Other risk factors such as alcohol, smoking, and diabetes are unclear for their roles in PCa.

1.2.2 PCa Diagnosis and Test.

There are three types of tests commonly used to check the prostate gland, including the digital rectal exam (DRE), prostate specific antigen (PSA) testing, and prostate biopsy.

DRE is an early, simple way to check the prostate and screen for PCa, which allows the doctor to feel the enlargement or hard lumps of the prostate with a gloved and lubricated finger.

The PSA test was introduced into clinical practice in U.S. in mid-late 1980s, and in Canada in 1989 along with DRE to help detect PCa. PSA is a substance secreted almost exclusively by the prostate gland to help make semen and nourish sperm. The PSA test is a blood test taken from the arm, and elevated levels of the PSA indicate something wrong with the prostate, including PCa. Notably, numerous health conditions including prostatitis and benign prostate hyperplasia can also cause increased blood PSA levels. Thus, men with a low PSA level can have cancer, while men with a high PSA level can be cancer free.

Consequently, the PSA test leads to overdiagnosis and overtreatment to low-risk disease as well as inadequate treatments to high-risk disease.[24] It is still unclear whether the PSA test does more harm than good, and thus, the use of PSA test to screen PCa remains debatable.

The prostate biopsy is conducted if the symptoms or test results suggest the occurrence of PCa. Small tissue samples are taken from the prostate, which are used to determine if any suspicious tissue is cancerous. A positive test result means PCa is present, and a Gleason Score will be given by examining the tissue sample in comparison with normal prostate cells.

1.2.3 Clinical States and Therapies for PCa.

The progression of PCa from the point of diagnosis to death is described by a series of clinical states based on the degree of disease, hormonal level, and metastasis, including clinically localized disease, rising PSA after primary treatment, clinical metastases (non-castrate) and castration-resistant state **(Figure 1.1)**.[25] These clinical states are well-recognized by physicians and patients, and one patient may progress from one state to the next, and a patient maintained in a state is tantamount to cure.[26]

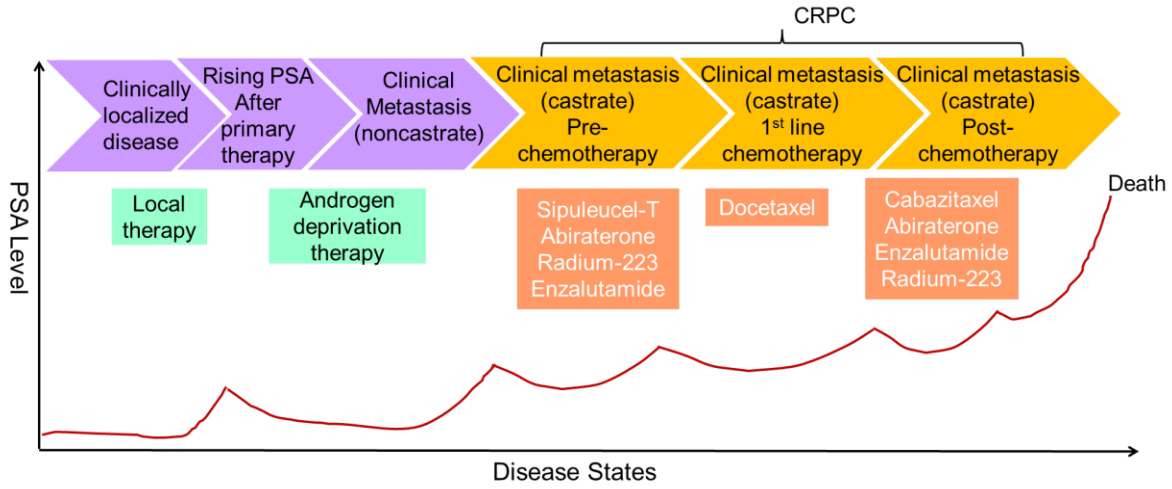


Figure 1.1. Clinical states and treatments of PCa.

The clinical states of PCa patients include clinically localized disease, rising PSA after primary therapy, clinical metastasis and castration resistant prostate cancer (CRPC) (including three stages: prechemotherapy, 1st chemotherapy and post-chemotherapy). The localized disease is treated by local therapy, and androgen deprivation therapy is used for rising PSA after primary therapy and clinical metastasis. Therapies for CRPC include chemotherapy and antiandrogens.

Approximately 85% of newly diagnosed PCa are localized to the prostate gland, and remaining cases can be attributed to invasive or metastatic disease types.[27] If PCa is diagnosed early (when it is in local or regional stage), the disease could be cured by local therapies (prostatectomy, radiation and cryotherapy) with a corresponding five-year survival rate approaching 100%.[28]

A portion of patients have recurrence with a rising PSA after initial local therapies, and treatments in this state aim to prevent the disease from becoming detectable or symptomatic. Since the seminal work by Huggins and Hodges in 1941 that discovered PCa is an androgen-dependent disease,[29] the androgen deprivation therapy (ADT) has become the mainstay for high-risk local or metastatic PCa. The ADT aims to reduce levels of circulating androgens or prevent the male hormones from reaching the androgen receptor (AR) by orchiectomy, luteinizing hormone releasing hormone (LHRH) agonists/antagonists (also called chemical castration), and antiandrogens.

Approximately 10-20% of PCa patients have *de novo* metastatic disease[30] - a more advanced, incurable form of PCa, once it metastasizes to the bone and lymph nodes. It has been demonstrated that in both relapsed and metastatic PCa, ADT alone or in combination with other modalities, provides an initial response manifested by decreasing PSA.

After long-term application of ADT, the PCa patients become insensitive to the treatment, and disease relapse invariably occurs after a median of 14-20 months,[30] with the transition to a more aggressive form of PCa termed as hormone-refractory, or androgen-independent, and most recently, castration-resistant (CRPC) state.[31] CRPC is characterized by rising PSA and further progression, which has a poor prognosis and remains challenging. In metastatic CRPC states, there are three stages: pre-chemotherapy, first-line chemotherapy and post-chemotherapy.

With improved understanding of the biology of CRPC, more therapeutic agents have been developed in recent years. Before 2010, only Docetaxel-based chemotherapy demonstrated improved survival, and after 2010, new ADT drugs were approved for the treatment of CRPC in pre- and post-chemotherapy. In addition, other agents include the recently introduced immunotherapy Sipuleucel-T and radioisotope Radium-223 targeting the bone metastasis. Docetaxel is still the first-line chemotherapy agent, and a newer drug Cabazitaxel is effective in many Docetaxel-resistant cancers and can extend survival by an average of 2 to 4 months despite lack of curative efficacy.

1.3 The Androgen Signaling Pathway

The androgen signaling pathway plays a critical role in the development and progression of PCa, and a comprehensive consideration of this pathway is important for understanding the role of AR as a therapeutic drug target for PCa, including the physiological function of androgens, and the structure, function and mechanism of AR.

1.3.1 Androgens

Endogenous androgens (testosterone and 5α-dihydrotestosterone (DHT)) are male sex hormones responsible for the development of male phenotype during embryogenesis and for

male sexual maturation during puberty. In adult men, androgens remain critical for the development and maintenance of the reproductive system, and affect many other tissues such as the muscle and bone.[32] The action of androgens in reproductive tissues (prostate and testis) is known as the androgenic effect, while its effect in muscle and bone is known as the anabolic effect.

Approximately 90-95% of testosterone is produced in testis, and 5-10% of testosterone is synthesized in the adrenal gland. The synthesis of testosterone is regulated by the hypothalamus-pituitary-testis axis. The LHRH (also known as gonadotropin-releasing hormone) secreted by the hypothalamus stimulates the release of luteinizing hormone (LH) by pituitary, which binds to luteinizing hormone receptor in the testis and induces the production of testosterone. A majority of testosterone binds to carrier proteins such as the steroid hormone binding globulin (SHBG) and albumin, and only 1-2% of testosterone exists in an unbound form. The free form of testosterone enters prostate cells, and then it is converted into a more active metabolite DHT by the 5α-reductase enzyme. Importantly, DHT binds to AR with higher affinity, and its biological activity exceeds that of testosterone up to 10 times, which makes DHT the primary ligand for the AR. Aberrant regulation of androgens leads to diseases such as androgen insensitivity syndromes and PCa.

1.3.2 Androgen Receptor

The AR gene was first identified to be located on chromosome X at Xq11.2 in 1981,[33] and the human AR cDNA was first cloned and sequenced in 1988.[34] The AR gene is more than 90 kb long and codes for a 110 kDa protein constituted of 919 amino acids.[35] The human AR (NR3C4, nuclear receptor subfamily 3, group C, gene 4) belongs to the steroid hormone receptor (SHR) family, which also includes estrogen receptor (ER), glucocorticoid

receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR). The AR is mainly expressed in androgen target tissues, such as the prostate, muscle, liver, and central nervous system (CNS). It is a key ligand-activating transcription factor in healthy prostate and during neoplastic transformation. In normal prostate, AR regulates prostate secretory function, stimulates epithelial cell renewal, and maintains cells in a differentiated state.

Same as other SHRs, the AR is organized into four functional parts including the N-terminal domain (NTD), the DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD) (**Figure 1.2**). These domains play different roles in the AR activity, and an understanding of these domains is essential for the development of AR-targeted therapeutics.

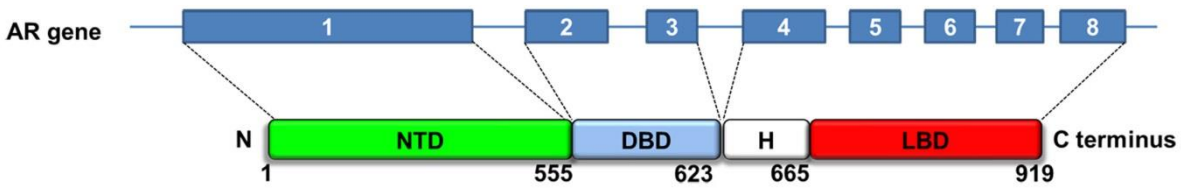


Figure 1.2. Structural organization of the AR gene and protein.
The AR has four functional domains including the N-terminal domain (NTD) encoded by exon 1, the DNA-binding domain (DBD) encoded by exons 2-3, the hinge region (H) encoded by exon 4 and the ligand-binding domain (LBD) encoded by exons 4-8.

The NTD is encoded by exon 1, and it accounts for more than half of the size of the AR (residues 1-555) and is highly variable in the sequence compared to other SHRs.[36] This part of the protein is very flexible and intrinsically disordered in solution,[37] which has hampered the elucidation of its three-dimensional (3D) structure. The NTD has the main

transactivation function region, termed the activation function 1 (AF1), which contains two transactivation units, TAU1 (residues 100-370) and TAU5 (residues 360-485).[38] The AF1 is indispensable for the full activity of the AR, and is essential for constitutively active truncated AR splice variants (ARVs) that do not contain the LBD portion. Interestingly, two motifs in NTD, the FQNLF (residues 23-27) and the WHTLF (residues 433-437), have been shown to interact with the activation function 2 (AF2) functionality in AR LBD region, leading to N/C intra- and/or intermolecular interactions, which are important for the transcription of some target genes.[39]

The DBD (residues 556-623) is encoded by exons 2 and 3, and is highly conserved among the SHRs. The DBD consists of two zinc fingers and a loosely structured carboxyl-terminal extension (CTE) region. The first zinc finger is proximal to the NTD, responsible for the DNA recognition. The α-helix of the zinc finger, termed recognition helix, directly interacts with nucleotides in the DNA major groove. There is a P(roximal)-box region (residues 577-GSCKV-581) in the recognition helix, which is identical in other SHRs and responsible for the specific recognition of the DNA response element. The second zinc finger contains the D(istal)-box region (residues 596-ASRND-600) which is the dimerization interface (**Figure 1.3**). The CTE is critical for the 3D organization of the DBD and it mediates the selectivity of the receptor to androgen response elements (AREs) on the DNA.[40] The AR DBD and CTE can recapitulate the DNA binding and dimerization as the full-length AR. The structure of the DBD has been resolved crystallographically[41] and will be discussed in greater details in Chapter 4.

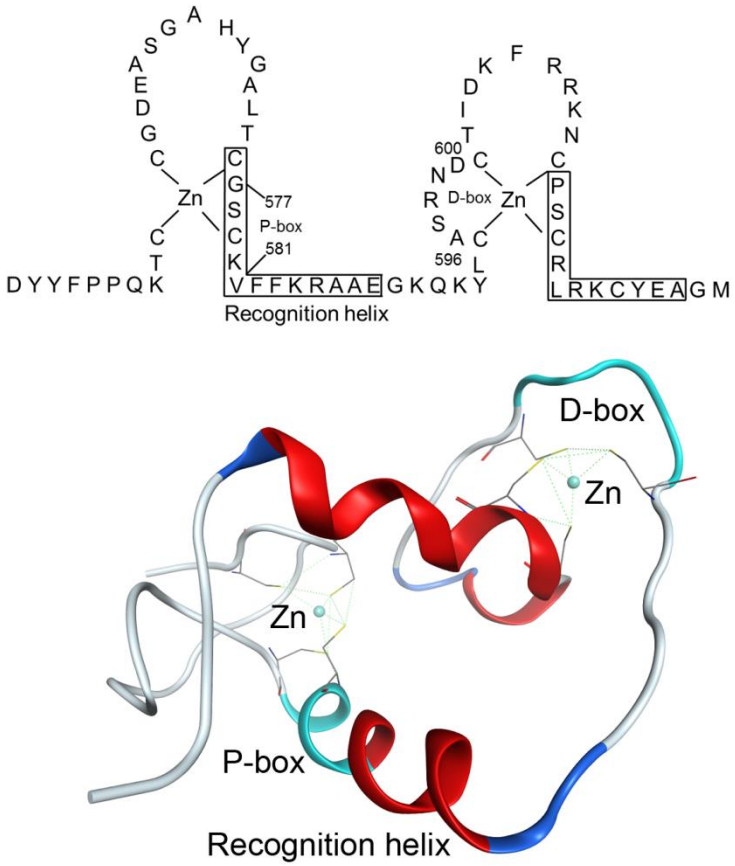


Figure 1.3. The AR DBD construct and 3D structure.

(Upper): A schematic of the AR DBD is given to show two zinc fingers in the core of AR DBD, the P-box and D-box residues, and residues (in boxes) that constitute two alpha helices. (Lower): The 3D structure of the AR DBD monomer with P-box and D-box residues colored in cyan (PDB code: 1R4I).

The hinge region (residues 624-665) is a flexible linker that connects the LBD and DBD segments. The hinge is known for its role in nuclear translocation through carrying a ligand-dependent nuclear localization signal (NLS, residues 617-633) spanning the hinge and DBD.[42] Upon androgen binding, the NLS is exposed to bind importin-α and translocate the AR from cytoplasm to nucleus.[43, 44] The NLS consists of two clusters of basic amino acid

residues (617-RKCYEAGMTLGARKLKK-633), and this motif is highly conserved among other SHRs.

The LBD (residues 666-919) is encoded by five AR exons (exons 4-8) varying from 131 to 288 bp in size. The LBD consists of 11 α-helices (H) and 2 β-sheets that are arranged in three layers to form an antiparallel "α-helical sandwich" (**Figure 1.4**). It is worth noting that the H2 is missing in the AR, but the same numbering for other helices was adopted for easy comparison with other SHRs. The androgen binds to a buried pocket in the LBD, termed the androgen binding site (ABS), which is formed by the N-terminal regions of H3, H5, and the C-terminal regions of H10 and H11. Upon binding of an androgen, the LBD undergoes conformational changes and the H12 is repositioned to stabilize the complex. This motion allows the formation of a shallow surface groove by residues of H3, loop 3-4, H4, and H12, referred to as the AF2, which is the major interface for the intra- and/or intermolecular N-C interactions and coactivator recruitment.[45] It has been demonstrated that the AF2 preferentially binds to the FXXLF motifs, including the 23-FQNLF-27 present in the NTD, and interacts poorly with LXXLL motifs, which are commonly found in AR coactivators.[46] In recent years, another surface groove consisting of H1, loop3-4, and H9 residues was identified on the AR LBD, called the binding function 3 (BF3). It was reported to allosterically regulate the AF2 activity;[47] however, the complete functional role of BF3 is not clear yet.

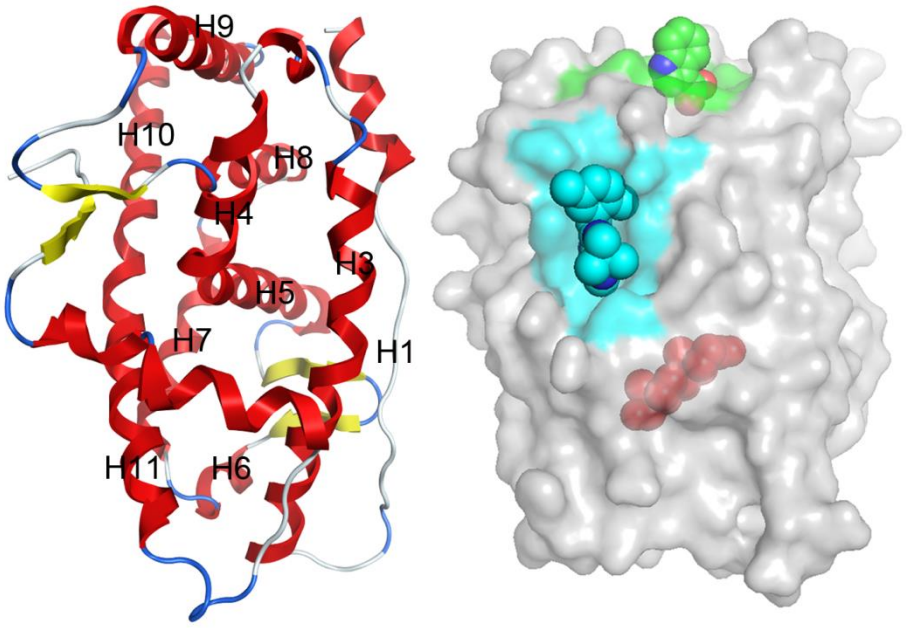


Figure 1.4. The 3D structure of AR LBD.

(Left): The AR LBD structure is composed of 11 α-helices. (Right): A surface representation of the AR LBD showing the ABS (ligand in red), AF2 (cyan) and BF3 (green) sites with corresponding ligands in each different binding site (PDB code: 2PIP).

1.3.3 Activation Mechanism of AR

The human AR is known as an intracellular ligand-activated transcription factor. In its unliganded state, the AR is inactive and primarily resides in the cytoplasm, where it is bound to cochaperones such as the heat shock protein 90 (HSP90). Upon androgen binding, the AR becomes activated and dissociated from cochaperones. The activated AR will then recruit coactivators, and undergoes a series of conformational changes, as well as translocates into the nucleus.[48] In the nucleus, AR dimerizes via the DBD portions, and further binds to AREs in the promoter and enhancer region of target genes, interacts with different coregulators, and initiates the transcription of regulated genes (**Figure 1.5**). Notably, AR regulates over 1000

genes,[49] including AR itself and its target genes such as PSA, transmembrane protease serine 2 (TMPRSS2) and FK506 binding protein 5 (FKBP5).

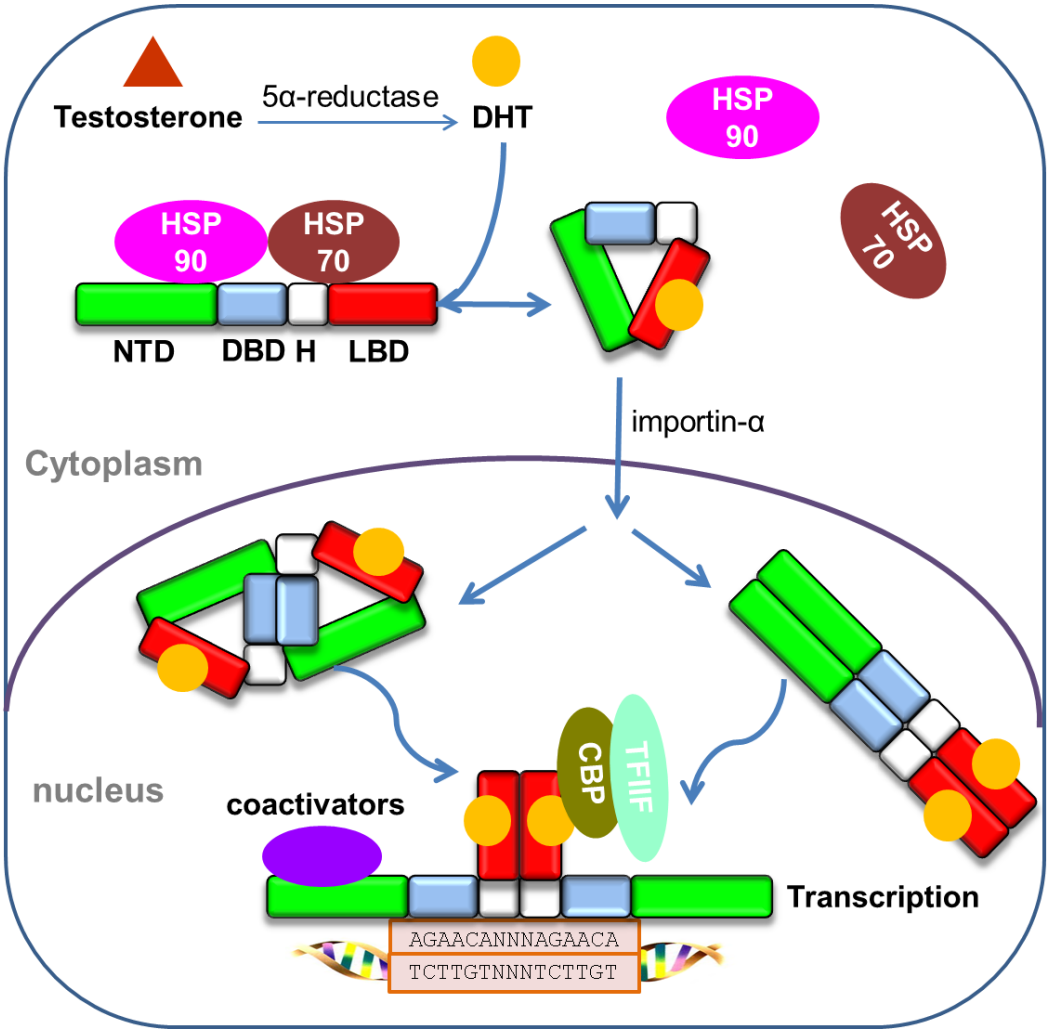


Figure 1.5. The AR signaling pathway.

The classic AR transactivation signaling pathway, involving the androgen conversion, AR activation, nuclear translocation, dimerization and coregulator recruitment.

Although the mechanism of AR action is still not entirely clear, increasing evidence indicate that the function of AR is also regulated by a complex network of coregulators and cell signaling pathways. The AR serves as a scaffold in the promoter and enhancer region,

for the assembly of coregulators or other transcriptional factors to activate or repress the transcription. The AR coregulators are proteins recruited by the receptor, including co-activators such as the p160 family,[50] and co-repressors such as NCOR.[51, 52] In addition, the AR could also be activated by kinase signaling pathways in an androgen-independent manner, such as the phosphinositide 3-kinase (PI3K)/AKT/mamalian target of rapamycin (mTOR) signaling pathway.[53-55]

1.4 Current Advances in the Development of AR Inhibitors

Given the importance of the AR signaling pathway, the human AR has been recognized as a pivotal drug target in both PCa and CRPC. The ever-increasing number of AR inhibitors has been reported in the literature with different mechanisms of action, including antiandrogens,[56-62] selective androgen receptor modulators (SARMs),[63, 64] AR LBD surface site (AF2, BF3) inhibitors,[65-68] AR NTD binders,[69-72] and other AR-targeted avenues.[73-75]

1.4.1 Antiandrogens

Antiandrogens are AR inhibitors that prevent the AR function by competing with androgens for binding to the receptor. So far, all existing anti-AR drugs for the treatment of PCa are known as antiandrogens. The antiandrogen can be classified as steroidal or non-steroidal based on the structure. Steroidal antiandrogens, such as Cyproterone acetate, demonstrate partial agonistic activity, poor oral bioavailability, potential hepatotoxicity, lack of tissue selectivity, and cross-reaction with other SHRs. In addition, the structural modification of steroidal ligands is limited by the steroidal scaffold. Thus, steroidal antiandrogens are rarely used in the clinic.

In comparison, non-steroidal antiandrogens demonstrate better AR specificity and oral bioavailability and are more suitable for structural modifications and advancements. A variety of non-steroidal antiandrogens has been reported to date, including toluidides, hydantoins, phthalimides and quinolinones (**Figure 1.6**). The toluidides are the first reported non-steroidal antiandrogens, represented by the first-generation drugs Flutamide (Eulexin®, Schering-Plough), Nilutamide (Nilandron®, Sanofi-Avenis), and Bicalutamide (Casodex®, AstraZeneca). These substances have already been clinically used for decades, and they all act on the ABS part of the AR, where they compete with DHT for binding and therefore block the coactivator recruitment by the AR. Flutamide and Nilutamide were found to render hepatotoxicity, while Bicalutamide exhibited fewer side effects and had longer half-life in patients, so it eventually replaced Flutamide and Nilutamide as a PCa treatment.[48]

Patients initially respond well to these drugs; however, around 30% of patients undergo a paradoxical decline in PSA levels when the drug is discontinued. This phenomenon is called "antiandrogen withdrawal syndrome", particularly associated with Flutamide.[76] Moreover, after long-term administration of these drugs, the resistance inevitably develops as the disease progresses into a hormone-refractory state - the CRPC. One of well-documented mechanisms of antiandrogen resistance is the occurrence of mutation-driven receptor promiscuity and mutation-caused bioconversion of AR antagonists into agonists. Thus, T877A and W741L/C mutations in the AR LBD are well-characterized substitutions that drive Flutamide and Bicalutamide to agonise the AR, respectively.[77-79]

The second-generation antiandrogens, represented by clinically used Enzalutamide (Xtandi®, Astellas) and the investigational drug ARN509,[57, 59, 60] were developed on the basis of a hydantoin compound RU59063, which has a very high binding affinity to AR. Same as

the first-generation antiandrogens, Enzalutamide and ARN509 also bind to the AR ABS with superior affinity, and they demonstrate improved anti-AR potency, better pharmacokinetics (PK) profile, and improved *in vivo* efficacy. Compared to other conventional antiandrogens, they exhibit additional mechanisms of anti-AR action by preventing AR nuclear translocation, impairing DNA binding, and affecting coactivator recruitment. As Enzalutamide demonstrated significant overall patient survival benefit (~5 months) with metastatic CRPC,[80] it was approved by the FDA for CRPC in 2012. However, it should be noted that despite of the survival benefit of Enzalutamide in pre- and post-chemotherapy, resistance invariably develops to this drug within 1-2 years in patients with metastatic CRPC. Similarly, the resistance has already been reported for its analogue, the investigational drug ARN509. In particular, a mutation F876L in the helix 11 of the AR LBD was identified to render the agonistic effect of Enzalutamide and ARN509.[81-83]

Of note, the marketed drug Abiraterone acetate (Zytiga®, Johnsoon & Johnson, New Jersey, USA) is mainly known as an androgen synthesis inhibitor targeting CYP17 and other enzymes; however, it is also considered as an antiandrogen as it inhibits AR. The anti-AR profile of Abiraterone contributes to its antitumoral effects, and is clinically used for metastatic CRPC. However, resistance to Abiraterone has been found and mechanistic basis of resistance has not been completely elucidated. Recently, it was demonstrated in mice and patients with PCa that Abiraterone was converted into a more active Δ4-abiraterone (D4A), which blocks enzymes required for DHT synthesis including CYP17A1, 3β-hydroxysteroid dehydrogenase (3βHSD) and 5α-reductase (SRD5A). Same as Abiraterone, the D4A also inhibits AR with potency comparable to Enzalutamide.[84] The metabolite D4A has more

potent anti-tumor activity against xenograft tumors than abiraterone itself, which may imply more clinical and survival benefits by direct D4A treatment.

Although the resistance emerged to the second-generation antiandrogens Enzalutamide and ARN509, the field evolved rapidly with ongoing studies on next-generation antiandrogens. ODM-201 is a next-generation non-steroidal antiandrogen under clinical investigation. It has higher binding affinity to AR with the inhibition constant Ki value eight-fold lower than Enzalutamide, and impairs nuclear translocation. Remarkably, it doesn't activate mutated AR including F876L, T877A and W741L, which have been suggested to drive resistance to clinically used drugs.[85] ODM-201 showed more potent antitumor activity both *in vitro* and *in vivo* non-clinical models of CRPC. Unlike Enzalutamide and ARN509, ODM-201 doesn't penetrate the brain-blood-barrier, and no seizure was observed. The phase I/II trial showed a favorable safety profile and PSA response, and the phase III trial is ongoing.[61]

Galeterone (also called TOK-001 or VN/124-1) is an investigational drug in clinical Phase III study by Tokai Pharmaceuticals. It has been shown as a dual inhibitor, concomitantly inhibiting CYP17 and AR.[58, 62] In addition, galeterone causes AR degradation. Studies showed that galeterone blocks AR transactivation with 10-times higher activity compared to Bicalutamide while it doesn't agonise the mutated AR.

In summary, the development of antiandrogens as therapeutics for PCa is the most studied area with marketed and investigational drugs; however, a common resistance problem has been reported for all current drugs and the investigational drugs may have the same concern. Although multiple plausible mechanisms for resistance have been reported (see section **1.5** for details), it is worth noting that the mutation-driven transformation of

antagonists to agonists is a common limitation for the current antiandrogen therapies since the AR ABS is a hot spot for mutations. The structural basis for this problem is still unknown which limits further investigation.

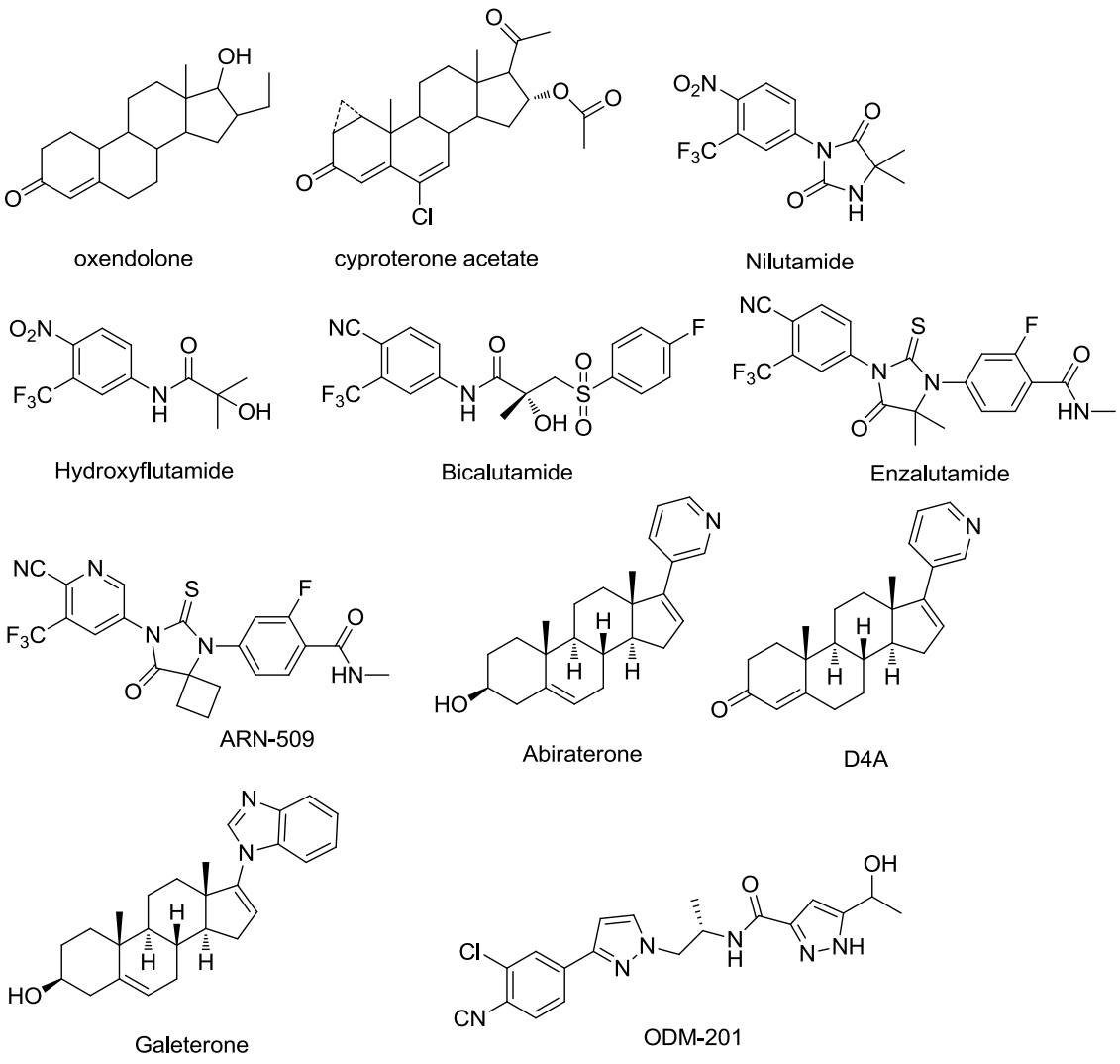


Figure 1.6. Steroidal and non-steroidal antiandrogens.

Chemical structures of steroidal antiandrogens Oxendolone and cyproterone acetate, and non-steroidal antiandrogens including first-generation antiandrogens Nilutamide, Hydroxyflutamide and Bicalutamide, second-generation antiandrogens Enzalutamide,

Abiraterone and its metabolite D4A, and investigational antiandrogens ARN-509, Galeterone and ODM-201.

1.4.2 SARMs

SARMs are tissue-selective AR modulators that act as antagonists in the prostate but agonise the receptor in the pituitary and muscle cells.[48] An ideal SARM has characteristics of high AR specificity, improved oral bioavailability and PK profile, low (or no) hepatoxicity, desirable tissue-selectivity, and differentiated androgenic and anabolic effects, and thus provides therapeutic opportunities for a variety of diseases. The antagonist activity in the prostate reduces the potential of PCa while the agonist activity in the muscle and bone can treat diseases such as muscle-wasting conditions, hypogonadism, and frailty.[86] SARMs could be classified into steroidal and non-steroidal. The limitations of steroidal SARMs hamper the clinical use, including poor PK, cross-steroidal activity, hepatotoxicity and insufficient discrimination of anabolic and androgenic effects.

The non-steroidal SARMs have been intensively studied in the past decade, with a variety of chemical classes published. The aryl propionamides were the first reported SARMs demonstrating sufficient *in vivo* tissue selectivity. This chemical series is structurally similar to Bicalutamide, and the replacement of the aryl sulfonyl in Bicalutamide with a phenoxyl group leads to the transformation of the antagonist to agonist, such as investigational drugs Andarine (S-4) and Enobosam (Ostarine/GTx-024/S-22).[87] These compounds are in clinical trials for the treatment of muscle-wasting and osteoporosis. Compounds from other chemical classes have also been reported as SARMs, such as hydantoins, quinolinones, and tetrahydroquinolones. Some of these chemicals have been

advanced into clinical trials for frailty and osteoporosis, such as BMS-564929 and LGD-2226 (**Figure 1.7**). So far, most SARMs are investigated for their therapeutic use for muscle-wasting conditions rather than PCa.

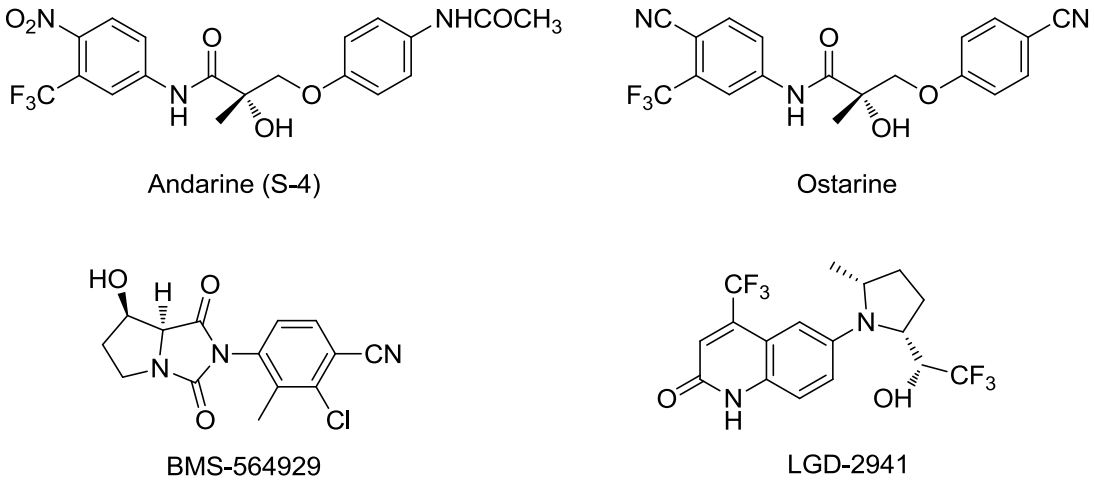


Figure 1.7. Chemical structures of representative SARMs.

1.4.3 AR LBD Surface Site Inhibitors

Due to limitations of conventional antiandrogens, targeting alternative sites on the AR may help avoid the rising problem of drug resistance driven by ABS mutations. For example, the AR LBD surface sites, AF2 and BF3, are important for the coregulator recruitment and AR transcription. Crystal structures demonstrated that small molecules are able to bind these large, hydrophobic exposed sites, indicating the druggability of these surface pockets.[88]

The AF2 site in the AR LBD is well-known for interacting with coregulators which is vital for AR transcription, and thus AF2 inhibitors are developed to disrupt the coregulator modulation of AR transcription. The reported small-molecule peptidomimetic (D2 in **Figure 1.8**) binds to AF2 by mimicking the LXXLL motif-containing coregulators,[68] which effectively disrupts interactions of AR and LXXLL-containing cofactors. It demonstrated

potent inhibition of AR and PCa cell proliferation, and suppression of tumor growth *in vivo*. This type of inhibitors has a distinct mechanism of action from conventional antiandrogens which may provide opportunities to circumvent the mutation-driven antiandrogen resistance.

In 2007, Fletterick's group reported that some AF2 binders could preferentially bind to a previously unknown allosteric area - the BF3 site, which opened up an opportunity to develop BF3 inhibitors by blocking the protein-protein interaction.[89] A few chemical classes of BF3 inhibitors have been reported to date which demonstrated potent *in vitro* and/or *in vivo* activities, such as dihydropyrimidines,[90] 1H-indole-2-carboxamides,[91] benzimidazoles,[66] and indoles[67]. Notably, the recently reported indole-based AR BF3 inhibitors demonstrate excellent inhibition of AR transcription, PSA expression, and the androgen-induced proliferation of androgen-sensitive LNCaP and enzalutamide-resistant MR49F PCa cell lines. Moreover, the reported lead compound (compound 23)[67] effectively reduces AR-dependent gene expression in PCa cells, and importantly, inhibits tumor growth in both LNCaP and MR49F xenograft models. Thus, the BF3-directed AR inhibitors may potentially become alternative therapeutic agents to treat antiandrogen resistant forms of PCa.

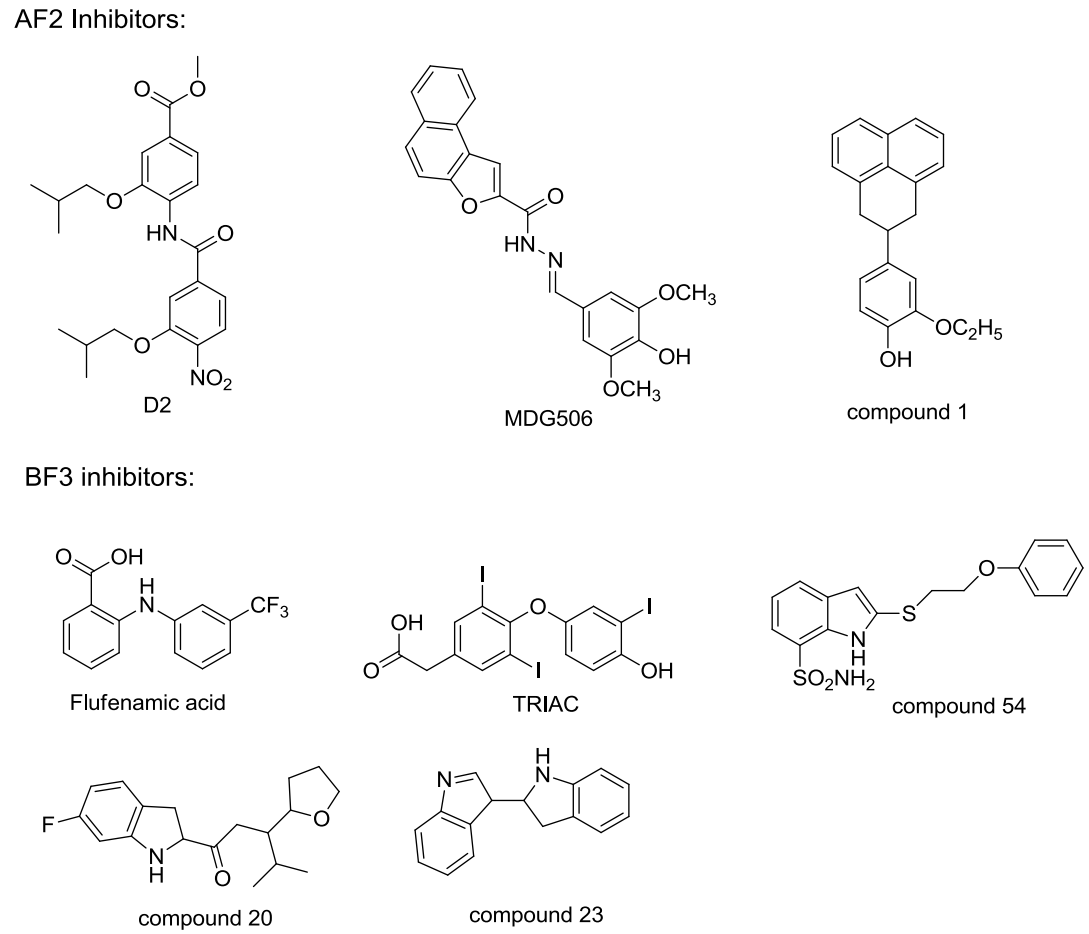


Figure 1.8. Chemical structures of AR LBD surface site inhibitors.

1.4.4 AR NTD Inhibitors

The AR NTD is essential for AR activity, and it has less than 15% homology with other SHRs. In contrast to conventional antiandrogens, possible AR NTD inhibitors may not only possess significant AR specificity, but more importantly, can inhibit ARVs. Thus, AR NTD represents an intriguing target for PCa and CRPC.[92, 93]

Although the AR NTD is intrinsically disordered which impeded the structure-based development of therapeutics, small-molecule inhibitors targeting the AR NTD were discovered,[69, 70, 72] represented by EPI-001 (**Figure 1.9**). The EPI-001 was identified from

high-throughput screening of a library of marine sponge extracts, and it covalently binds to the AF1 in the intrinsically unstructured AR NTD.[69] EPI-001 and its isomers block the transcriptional activity of AR as well as ARVs, and reduce the tumor growth of CRPC xenografts. However, recent study demonstrated that EPI-001 is not a specific AR NTD inhibitor, and it was found to be a peroxisome proliferator-activated receptor-gamma (PPARγ) modulator with inhibitory effects on AR expression and activity in PCa.[71] ESSA Pharma Inc. has filed an investigational new drug (IND) application of EPI-506 with FDA, and its clinical effects are expected.

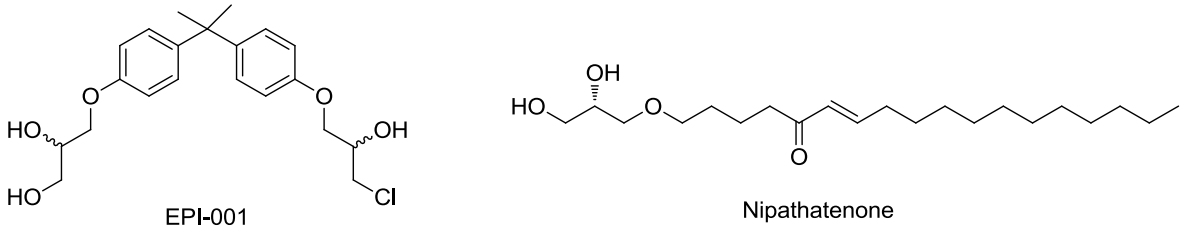


Figure 1.9. AR NTD inhibitors.

1.4.5 Others

In addition to conventional and novel targeting of AR described above, there exists a variety of strategies of developing new interventions of AR, such as AR degradation, drug repurposing for non-competitive AR inhibitors, and AR-targeted RNA interference (**Figure 1.10**).

The ASC-J9 was identified from natural products as an AR degradation enhancer,[75, 94] which selectively degrades AR protein through interruption of the AR-AR selective coregulator interaction. Additionally, the ASC-J9-promoted AR degradation renders

suppression of AR transcription, AR-mediated PCa cell growth, and tumor growth in androgen-dependent and castration-resistant xenograft models.

The pyrvinium pamoate (PP) is an FDA-approved drug which was identified as a non-competitive AR inhibitor through drug repurposing.[56] PP was reported to inhibit the transcriptional activity of both full-length AR and ARVs, without preventing DHT binding. However, PP displayed generic toxicity and cross-reactivity with other SHRs,[95] and the fact that PP targets multiple proteins may explain the undesired off-target effects. Recently, it has been reported that PP may inhibit the transcription initiation via the DBD,[96] but the exact mechanism of PP is unclear.

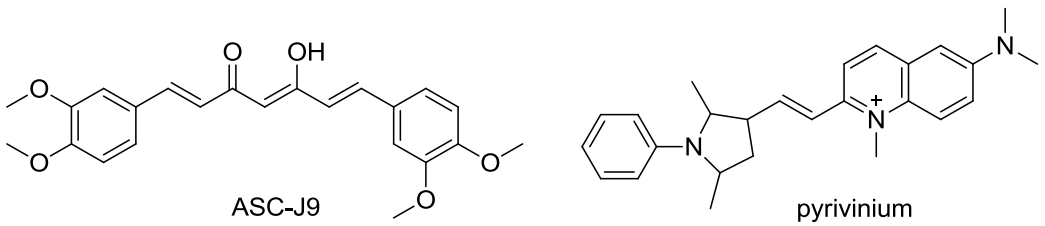


Figure 1.10. Chemical structures of miscellaneous compounds targeting AR.

The RNA interference is a potentially new therapeutic strategy by silencing critical genes in PCa, such as AR. The short-hairpin RNA (shRNA) against AR could inhibit tumor growth and suppress PSA,[97] and the lipid nanoparticle small-interfering RNA (siRNA) system and antisense oligonucleotides (ASO) have been shown to be effective in silencing the wild-type AR and ARVs.[73, 74]

1.5 The Resistance Problem of Current Antiandrogens

As mentioned above, the resistance is the main problem for current anti-AR drugs, and thus, it is important to understand the mechanism of resistance for further development of

new drugs. Significant effort has been made to reveal the mechanism of resistance with ever growing evidences reported, but the full picture is still unclear. Some mechanisms are partly involved in the resistance, including AR overexpression, gain-of-function mutations, the emergence of ARVs, and alternative pathways.[98-102]

1.5.1 AR Overexpression

High AR expression in CRPC at both mRNA and protein levels represents a mechanism of acquired resistance to ADT. In 2004, Sawyers el al. demonstrated that the AR mRNA is universally upregulated in hormone-refractory xenograft model, and increased AR expression is sufficient to convert antiandrogen Bicalutamide from antagonist to agonist.[99] AR overexpression can occur due to gene amplification, and it has been reported that AR gene amplification is present in about 30% of patients with recurrent PCa who had initially responded to ADT for over 12 months. AR amplification was not found in untreated primary tumors, suggesting AR amplification is not involved in the genesis or progression of PCa in patients untreated with ADT.[103, 104] The AR amplification increases AR and mRNA expression,[103, 105] which is proposed to enhance the AR sensitivity, promote the survival and proliferation of tumor cells even if the residual androgen level is low after the castration. Since the rate of AR amplification is low, it cannot account for most cases of resistance.[106] In addition, high AR expression at mRNA level without increase in gene copy number has also been observed in CRPC.[107]

1.5.2 Gain-of-Function Mutations

AR mutations are rare in early-stage untreated PCa, but are found in approximately 10-30% of patients previously exposed to Bicalutamide and Flutamide.[108-110] More than 1000 mutations in the AR have been described so far, with the majority of them occur either in the

NTD or the LBD, and rarely in DBD or hinge regions of the receptor.[111-113] The resistant mutations occur in particular regions involved into ligand-receptor and protein-receptor interactions, which may affect the ligand or coregulator binding to the AR.[114] Mutations in the LBD have been intensively studied, and a number of substitutions confers a gain of function by allowing other ligands to bind and activate AR.[115, 116] A few well-documented mutations, such as T877A/S and H874Y, lead to promiscuous activation of the AR by various steroid hormones or antiandrogens through converting AR antagonists into potent agonists.[117, 118] For current antiandrogens Flutamide, Bicalutamide, and Enzalutamide, specific point mutations (T877A, W741L/C, F876L) have been identified to induce antagonist-to-agonist transformation (**Figure 1.11**) driving *in vitro* and *in vivo* resistance.[77, 81-83, 117] Thus, mutations in AR may account for one of the major mechanisms underlying CPRC progression.

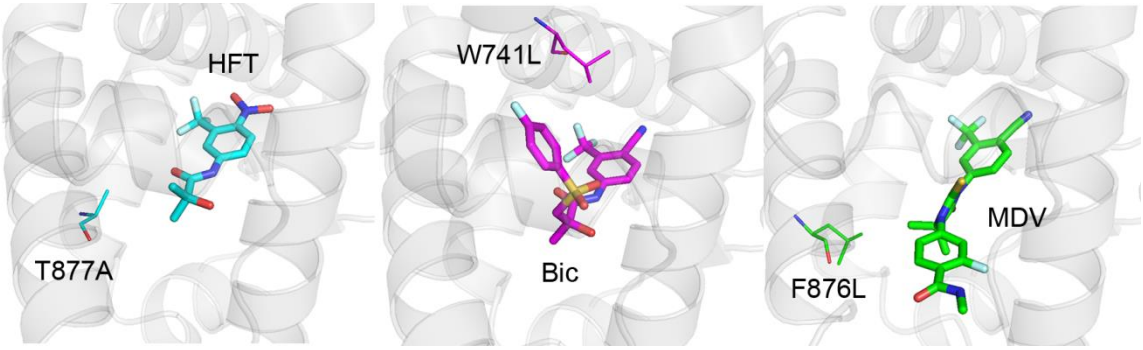


Figure 1.11. AR ABS mutants that induce agonistic effect of antiandrogens. Hydroxyflutamide (HFT), Bicalutamide (Bic) and Enzalutamide (MDV) is positioned in the AR ABS with mutated residues T877A, W741L and F876L highlighted, respectively.

1.5.3 The Emergence of ARVs

ARVs were first reported in patients with advanced PCa in 2009.[119, 120] Accumulating evidence demonstrated that CRPC cells and clinical samples are capable of expressing constitutively active, ligand-independent ARVs lacking the C-terminal LBD portion of the protein.[119-122] So far, there have been 17 ARVs discovered, and all of them do contain both NTD and DBD regions, with varying or completely absent LBD part (**Figure 1.12**).[123] It has also been shown that ARVs can regulate both canonical genes (full-length AR-dependent genes) as well as distinct ones. Importantly, ARVs could recapitulate the transcriptional program in the absence of androgens or full-length AR.[124, 125] It is bieleved that some ARVs such as AR-V7 are sufficient to drive resistance to antiandrogens, and none of current drugs are effective against ARVs.[125, 126] A recent study of AR-V7 in circulating tumor cells from metastatic CRPC patients (treated with Abiraterone and/or Enzalutamide), demonstrated that ARVs represent an important predictive biomarker of CRPC and one mechanism of resistance.[127, 128]

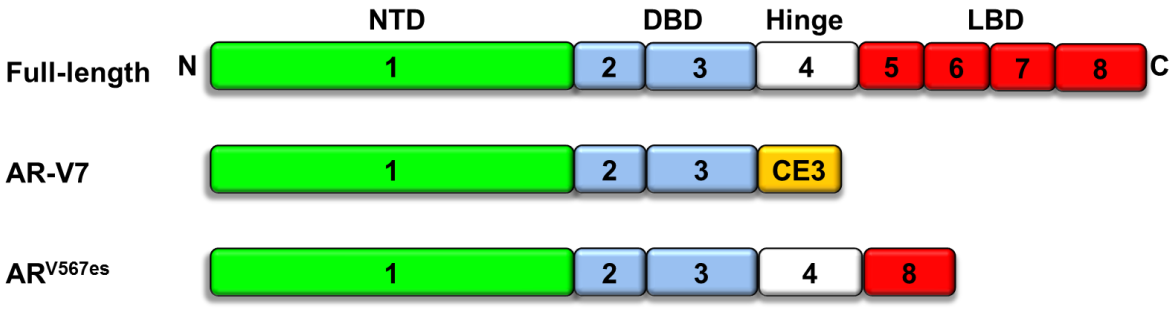


Figure 1.12. Full-length AR and two mostly studied ARVs.

1.5.4 Alternative Pathways

In addition to androgens, AR could also be activated by growth factor signaling pathways, such as epidermal growth factor (EGF), insulin-like growth factor (IGF), interleukin-4 and -6 (IL-4 and IL-6),[129-132] For example, the IL-6 level was found to be increased in CRPC patients, and the coregulator p300 mediates androgen-independent AR transactivation by IL-6.[133] Besides, the PI3K/AKT/mTOR signaling circle is a critical oncogenic pathway that plays a role in the tumorigenesis and resistance in a variety of cancers.[134] This pathway was found to be deregulated in advanced PCa and associated with ADT resistance.[135] Preclinical study elucidated that there is a dynamic interplay between PI3K/AKT/mTOR pathway and AR signaling during the development of the resistance.[136] Therefore, a PI3K/AKT pathway inhibitor has been synergistically used with antiandrogens to delay the CRPC progression.[137] Thus, these alternative pathways may play a role in CRPC.

1.5.5 Others

Other mechanisms have also been suggested to contribute to ADT resistance in CRPC, such as the synthesis of intratumoral androgens and bypass of AR,[138, 139] while the complete picture of resistance is yet to be elaborated.

1.6 Drug Discovery Research

Drug discovery and development is generally considered as a time-consuming and costly process. From the concept to market, a typical drug discovery campaign may take approximately 12-15 years, and could cost up to 2.6 billion USD.[140-142] The standard drug discovery pipeline includes the following phases: target identification, target validation, hit identification, lead optimization, preclinical trials, and clinical evaluation (**Figure 1.13**).

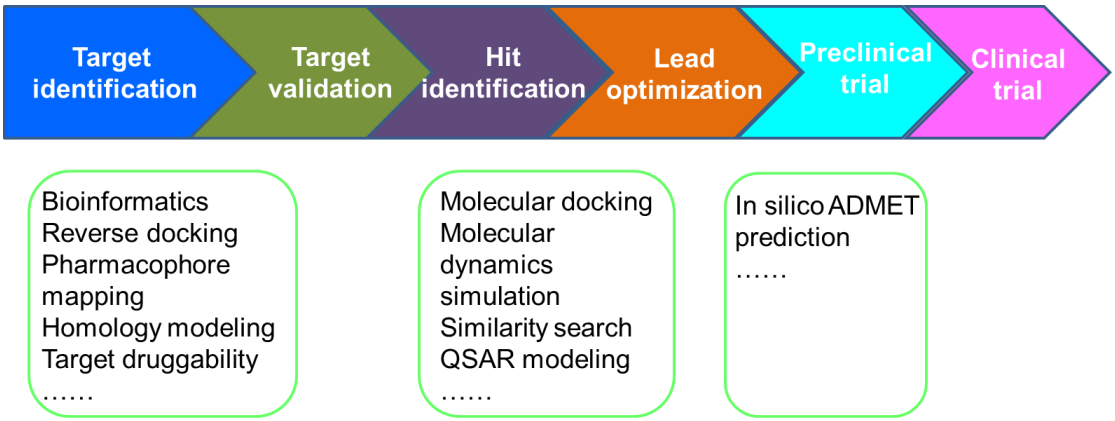


Figure 1.13. The drug discovery process and CADD components.

1.6.1 Target Identification and Validation

The initial phase of drug discovery is to identify and validate a target, which typically is a protein (enzyme, receptor, transporter, etc) that has a proven function in the pathophysiology of a disease and could be modulated by a small molecule (drug).[143] Among all identified drug targets, receptors represent the largest group (44%), including the G protein-coupled receptors (GPCRs), ion channels, and receptor tyrosine kinases. Enzymes make up the second largest group (29%), and transporters are the third group (15%).[144] The sequencing of human genome over the past decade provided a great impact on the target identification and validation, with new potential drug targets identified as well as druggable space broadened and better understood.[145, 146]

With the time, the knowledge has emerged that an ideal drug target should generally possess the following characteristics: (1) it should be involved into biological pathway which is closely related to a disease; (2) modulation of the target is less important in normal conditions; (3) the target protein should be characterized functionally and structurally; (4) it should not be expressed throughout the body; (5) the protein should be druggable; (6) high

throughput screening (HTS) assays should be available to identify potential drugs for the protein; (7) it is desirable to have target-specific biomarker to monitor the therapeutic benefit; (8) targeting the protein has low likelihood of potential side effects; (9) an appealing drug target is generally distinct from known targets with a good intellectual property condition.[143, 147]

It should be noted that not all potential drug targets are druggable, not suitably interacting with a drug or no binding site. On the other hand, druggable targets (if judged by their three-dimensional structures) do not necessarily have therapeutic potential if the binding doesn't link to a disease or result in therapeutic benefits. So drug targets worth exploiting are those that are both druggable and disease-modifying, such as the human AR.[148, 149]

Target identification and validation is of utmost importance and should be executed before launching a costly drug discovery campaign.[150] Many sources and techniques could be used to identify potential targets, including literature, bioinformatics analysis, gene expression profiling, phenotype analysis, genetic association and functional screening among others. Once a drug target is identified, it needs to be validated using techniques such as the *in vitro* cell-based study, antisense technology, and transgenic animal models.[143, 151-153]

1.6.2 Hit Identification and Lead Optimization

With a viable target in hands, the hit identification and lead optimization phases could be carried out. A 'hit' refers to an active molecule initially identified by screening a compound database or library and represents a starting point for the hit-to-lead optimization campaign. A 'lead' is an analogue or derivative of a 'hit' with improved potency, selectivity and physiochemical properties, which meets criteria for *in vivo* testing.

The identification of hit compounds in the drug discovery process generally comes from different screening paradigms, including HTS and virtual screening (VS). Millions of molecular structures are initially processed to identify hits, and a successful screening will yield a number of hits belonging to several chemical classes (structural scaffolds). The next step is to compare various hits and define the most promising chemical series for further development – the hit to lead optimization. For this process, hundreds of compounds are screened to narrow down to one or two candidates. This phase involves intensive investigation on the structure-activity relationship (SAR) among hit analogues and/or derivatives. From the derived SAR, there emerges an iteration process of design, synthesis and experimental evaluation of chemicals. A lead optimization program continues to produce better preclinical candidates through fine tuning unfavorable properties while maintaining the potency.

Once a candidate molecule is selected, its preclinical testing could be launched to assess the bioactivity and safety through animal studies, which is high-risk with a low success rate.[141, 151] The clinical trials (phase I to III) take approximately 6 years and cost around 200 million USD, and over 90% of drug candidates fail in clinical trials.[152, 154, 155] Due to this high attrition rate, new technologies should be incorporated into the drug discovery process to help lower the risk and expenses.

1.7 Computer-Aided Drug Discovery

Although the drug discovery process has been expedited with new science and technologies, the output is still typically not proportional to the investment. Computer-aided drug discovery (CADD) is recognized as an effective method to shorten the development cycle, reduce the risk, and save expenses. Thus, it has been widely used in academia and

industry throughout the drug discovery process to assist the target identification and validation, hit identification, lead optimization and preclinical study.[156] Herein, a brief review is presented on common CADD tools used in different stages of drug discovery.

1.7.1 CADD in Target Identification and Validation

Target identification and validation mainly rely on laborious and time-consuming wet-lab experiments. In recent years, to identify targets in a cost and time-effective manner, computational methods and platforms have been developed as an alternative/complementary way for target identification and validation. In addition to genomics-based approaches, other computational tools were developed, such as homology modeling and target druggability prediction.

Despite progresses in the nuclear magnetic resonance (NMR) spectroscopy and x-ray crystallography, the readily accessible structures in the Protein Data Bank (PDB) represent only a small portion of the druggable genome.[148, 149] In the absence of a protein structure, homology modeling is a well-established computational tool to construct the target protein structure based on its amino acid sequence (query) and 3D structures of homologous PDB proteins (template).[157] Over years, the reliability of homology modeling has been greatly improved, and the application of a homology model plays an important role in structure-based drug discovery.

The binding site in a target is generally identified through NMR, x-ray crystallization and mutagenesis studies. For some targets, only the 3D structure of an apo form is available or could be homology modelled while binding sites are unknown. Thus, for a potential target of interest, plausible binding sites for the drug discovery need to be detected. With the availability of the 3D structure of the target, a variety of computational methods could be

used for the binding site prediction, including template-based, geometry-based, energy-based, and molecular dynamics-based.[158, 159]

1.7.2 CADD in Hit Identification and Lead Optimization

In contrast with the traditional drug discovery approach which relies on labour-intensive and costly experiments such as HTS, CADD is finding increasingly broad use in hit identification and lead optimization process for its obvious advantages of low cost and high efficiency. With the emergence of high performance computing facilities such as clusters and graphics processing unit (GPU), CADD becomes an indispensable part of modern drug discovery. In the hit identification and lead optimization stage, CADD is generally used for following purposes: 1) to virtually screen databases of millions of compounds within weeks, and quickly identify hits with higher hit rate than HTS;[160] 2) to study protein-ligand interactions and to guide hit-to-lead optimization by improving the activity or physiochemical properties;[161] 3) to design novel chemicals based on emerging SAR.

In general, CADD methodologies can be classified into two categories, the structure-based drug discovery (SBDD) and ligand-based drug discovery (LBDD).[158] The SBDD is typically used with the availability of 3D structure of the target (from x-ray crystallography, NMR, or homology modeling), which leverages the structure and binding interactions to determine key protein-ligand interaction features. The SBDD methods include molecular docking, molecular dynamics (MD) simulation, pharmacophore search and so on. The LBDD mainly exploits the structure and bioactivity information of both known actives and inactives, for example, similarity search and quantitative structure-activity relationship (QSAR) modeling. It is generally used when there is at least one known bioactive ligand, while no or little information of 3D structure of the target is available. With both target and ligand

information available, the SBDD and LBDD can be integrated to complement each other. In the absence of known ligands or target structures, the SBDD is particularly useful by developing homology models, which remarkably contributed to the drug discovery for this type of targets. Below is a brief summary on the most representative SBDD and LBDD methods.

Molecular docking: On the premise of a 3D structure of the target and a known or predicted binding site, molecular docking is a classic SBDD approach to study the protein-ligand interaction and to predict the binding affinity. In particular, it is a powerful technique for docking-based VS. Briefly, molecular docking applies the "key-in-lock" principle by fitting the ligand into the binding site and finding the best match between them, and to predict the corresponding binding affinity expressed through a scoring function. Currently, most docking programs employ rigid docking in which the protein is fixed and only conformational changes of ligands are considered, with no solvation effect taken into account. The docked poses are generally accurate while scoring functions are still questionable.[162]

Molecular dynamics (MD) simulation: Compared to docking, MD simulation provides an insight into the dynamic protein-ligand interplay in the presence of solvent and under almost physiological condition (certain temperature, pressure, pH) which is more accurate, but also significantly more expensive.[163-165] MD simulation could be used to sample potential binding sites in the target, and more importantly, it provides a means to study protein-ligand interaction in greater details, especially when the experimental x-ray crystallization of protein-ligand complex is expensive or impractical. The binding free energy calculation is an important step as post-processing on the MD simulation which generally provides better correlation with experimental data than docking scores by molecular docking. Despite

limitations on the force field side of computations, ambiguity of solvent models, high computational cost, etc., MD simulations have become an important tool in drug discovery, particularly in biologics study, for instance, antibody design.

Similarity search: Similarity search is a ligand-based approach utilizing the idea that similar structures are likely to have similar properties. Under the circumstance of an identified active compound, it is a very efficient way to use the hit as a surrogate for performing a similarity search against a large pool of chemicals, which may result in compounds with improved activity and/or scaffold hopping (chemical scaffolds departing from the original query).[166] Fingerprint-based similarity search is a commonly used ligand-based method for analogues of a known active. The fingerprint is a bit-string encoding a set of features in a molecule, and analogues were identified by comparing bit-strings of molecules with known actives.[166] The similarity between molecules could be quantified by Tanimoto Coefficient (Tc, $Tc = \frac{c}{a+b-c}$, where a and b represent bits in each fingerprints of two molecules, and c is the common bits they have).[167]

QSAR modeling: QSAR is a knowledge-based approach to map the chemical structure (descriptors) to activity using a variety of statistical or machine learning techniques.[168] Properly validated QSAR model could be used to predict the biological activity of unknown compounds, and screen large chemical databases. Molecular descriptors are at the core of QSAR modeling, which transforms the chemical information in a molecule into a useful number through a logic or mathematical procedure.[169] A large number of descriptors reflecting different dimensionalities has been developed from 1D to 6D, and with the dimension increasing, descriptors account for more subtle molecular effects for more complex QSAR modeling.

1.7.3 CADD in Preclinical Research

Drug metabolism and pharmacokinetics (DMPK) including the absorption, distribution, metabolism, excretion and toxicity (ADMET) are important aspects for lead compounds to be advanced into preclinical and clinical stages. To improve the DMPK/ADMET profile of lead compounds while maintaining their biological activity, computational approaches could be used to predict desired properties, to fine-tune metabolic profiles and to facilitate lead optimization.

Molecular filters are generally applied to chemical libraries preceding the screening to discard compounds with potentially poor DMPK/ADMET profile. Lipinski's rule of five is a commonly used filter to remove compounds with possibly unfavorable bioavailability, using filtering criteria of molecular weight (< 500), logP (< 5), number of hydrogen bond donors (< 5), and number of hydrogen bond acceptors (< 10).[170] However, it has been found that many existing drugs violate certain criteria. Some improvements have been made to complement the rule of five, including the addition of the rotatable bond (< 10), polar surface area (< 140 Å), number of aromatic rings, and number of structural alerts.[171]

Structure-based methods are also used to predict the metabolism of lead compounds. The metabolic stability of a compound is affected by cytrochrome P450 enzymes, and prediction of a compound binding to dominating P450 enzyme isoforms by molecular docking could provide an indication of the likelihood of metabolic reaction. In addition, the binding pose of the compound with P450 enzyme may predict the specific site for metabolism.[158] In addition, ligand-based methods, such as quantitative structure-property relationship (QSPR) and quantitative structure-toxicity relationship (QSTR) were also used to predict the ADME properties.

1.8 Objective

Once PCa progresses to CRPC stage, existing ADT therapies are ineffective due to the development of resistance, which highlights the urgent need for novel therapeutics to address the resistance problem. The persistence of AR is a central mechanism in CRPC resistance, and the AR represents one of the most effective drug targets for CRPC. Thus, the objective of this work is to develop novel AR inhibitors for the treatment of CRPC to overcome/circumvent the resistance.

We started with targeting the conventional ABS to identify structurally distinct AR inhibitors using the power of *in silico* screening, and we obtained potent AR ABS inhibitors that were validated *in vitro* and *in vivo*. Though the developed compounds demonstrated significant promise, we were aware of limitations of targeting ABS, such as the site promiscuity upon mutations. In particular, the emergence of ARVs lacking the LBD and evidences of its correlation with antiandrogen resistance suggested that targeting the LBD including ABS won't win the battle of resistance. Thus, we started pursuing our objective from a new angle.

The new strategy was to explore the AR DBD as a potential drug target, which is a functional domain in both full-length AR and ARVs. Thus, targeting AR DBD provided potential to not only inhibit full-length AR, but also suppress constitutively active ARVs. Prior to our study, there were no reports on utilization of AR DBD for the drug development. Thus, the discovery of AR DBD inhibitors involved the target identification and validation, hit identification, lead optimization and upcoming preclinical evaluation. Although the developed lead compound needs further preclinical assessment, it already demonstrated all desired profiles and may serve as a prospective therapeutics for CRPC.

1.9 Thesis Layout

Chapter 1 provides the background information on PCa, AR, current anti-AR drugs, the resistance problem, drug discovery process, CADD and the objective of the work. Chapter 2 presents material and methods applied in this work. Chapter 3 and 4 summarize results of two research projects (development of AR ABS inhibitors and AR DBD inhibitors), which have been published in three papers as indicated in the preface. Chapter 5 is the conclusion.

Chapter 2: Materials and Methods

All methods applied in Chapter 3 and 4 were summarized in this chapter, and only specific workflows were presented in following chapters.

2.1 *In Silico* Methods

2.1.1 Protein and Ligand Preparation

The crystal structure of the protein was prepared using the Protein Preparation Wizard within Maestro 9.3 (Schrödinger, LLC). The hydrogens were added; bond orders were assigned; and missing side chains for some residues were added. The structure was subjected to energy minimization until the root mean square deviation (RMSD) relative to the starting geometry reached 0.3 Å.

A library of commercially available molecules (~3 million) from the ZINC database[172] were imported into the Molecular Operating Environment (MOE 2011, Chemical Computing Group Inc). All the molecules were protonated/deprotonated by a washing process, added partial charges and minimized with the MMFF94x force field to a gradient of 0.1 kcal/mol Å. After the minimization, the database was exported as an sdf file.

2.1.2 Virtual Screening

The docking program Glide (grid-based ligand docking with Energetics) in Maestro 9.3 was used for the VS.[173, 174] The bound conformation of the ligand or residues in the predicted binding site was used to define the active site for the VS. For Glide docking, the grid was defined using a 20 Å box centered on the selected ligand or residues. No constraints were applied and all the settings were kept as default. The ZINC database was docked using Glide standard precision (SP) mode, and the predicted binding pose of the entire database was

ranked by glidescore. Compounds with potentially low binding affinities (high glidescore values) were discarded.

To avoid any bias in docking programs, compounds with favorable glidescores were subsequently docked by eHiTs (Electronic high-throughput screening),[175] and compounds with eHiTs score higher than a cutoff value were removed. The RMSD of docked poses generated by Glide and eHiTs were then calculated to keep compounds with consistent docked poses, and compounds with high RMSD values (>2 Å) were removed.

Remaining compounds were further narrowed down by applying physicochemical parameters, including the number of acceptor (≤ 5), donor (≤ 5), logP (≤ 5), molecular weight (≤ 300), rotatable bonds (≤ 3), charges (=0) and rings (≤ 3).[170, 176] The ligand efficiency was used to rank the compounds, and top ranked compounds were classified into clusters by Fingerprint cluster in MOE 2011, using a similarity and overlap threshold of 60%. Finally, the docked poses were visualized and compounds with favorable interactions were selected. All tested compounds were purchased from commercial vendors such as Enamine, Vitas-M, and Life Chemicals with purity ≥95%.

2.1.3 Similarity Search

The Instant JChem 5.9.0 (ChemAxon) is a similarity search tool which supports query and sorting functionality, and is capable of handling large volumes of data. The Zinc database was imported into the Instant JChem. The identified hit was used as a query to identify analogues by structure search criteria such as similarity.

2.1.4 PLIF Analysis

The protein-ligand interaction fingerprint (PLIF) module in the MOE was used to transform the protein-ligand interaction into uniform fingerprint. Six types of interactions in

PLIF were translated into fingerprints, including sidechain H-bond donor (D) and acceptor (A), backbone H-bond donor (d) or acceptor (a), ionic interactions (I) and surface contact interaction (C). H-bonds between polar atoms were calculated using a method based on protein contact statistics, whereby the atom pairs were scored by the distance and orientation. The score was expressed as a percentage probability of being a good H-bond. Surface contact interaction was determined by calculating the difference of the solvent exposed surface area of residues in the absence and presence of the ligand. The solvent exposed surface area was determined by adding 1.4 Å to the vdw radii of each heavy atom, and computing the fraction of total surface which did not lie within the radius of any other. In order to cover all possible interactions, interactions expressed by the percentage of at least 1% for H-bond and 5 $Å^2$ for surface contact were recorded as an effective fingerprint.

2.1.5 MD Simulation

The coordinates of the x-ray crystal structure were used for MD simulation. The geometry optimization and electrostatic potential calculations of the ligand were performed using HF/6-31G in Gaussian 09. The force field parameters of the ligand were generated by Antechamber program in AMBER 12[177] using generalized amber force field (gaff)[178] and restrained electrostatic potential (RESP). All structures were modeled by LEaP within AMBER 12 using standard ff12SB force field, including all missing hydrogen atoms of the protein. To keep the whole system neutral, chloride (Cl-) or sodium (Na+) ions were added using the t-leap procedure, and solvated with the TIP3P water model in a truncated octahedral box with a 10 Å distance around the solute.

MD simulations were carried out within AMBER 12 on westgrid (compute calculation Canada). Firstly, it is a two-step minimization. The first-step energy minimization was

performed on solvent with the protein-ligand complex restrained for a 2000-step steepest decent minimization and then 3000 steps of conjugate gradient minimization. The second-step minimization was performed on the whole system without restraints for the same steps. After the energy minimization, the system was heated up from 0 to 298 K over 40 ps with a harmonic restraint weight of 500 kcal/(mol·Å^2) on the whole system, followed by a 60 ps density equilibrium simulation by restraining the solute with a harmonic restraint weight of 10 kcal/(mol·Å^2). Then production MD simulation was conducted for 100 ns without any restraints under the NPT ensemble condition at a temperature of 298 K and pressure of 1atm. During the simulation, the periodic boundary conditions (PBC) and particle-mesh Ewald (PME) for long range electrostatics were employed. Short range interactions were cut off at 10 Å, and bonds involving hydrogen were held fixed using SHAKE. The time step was set to 2 fs, and the snapshot was taken every 500 steps to record the trajectory.

2.1.6 Binding Free Energy Calculation

The MM-PB/GBSA components within the AMBER package were employed to compute the binding free energy.[179] These two methods were performed in parallel by running a python script "MMPBSA.py.MPI". Snapshots at 10 ps intervals in production phase were extracted for energy calculation within the whole simulation time. Average binding free energy on all snapshots was used for analysis. Based on the hypothesis, binding free energy was divided into the binding energy in vacuum and solvation free energy. The former was calculated by molecular mechanical algorithm with sander program and included internal energy, vdw energy and electrostatic energy; the latter consisted of polar and nonpolar solvation free energy. Poisson-Boltzmann (PB) and generalized Born (GB) model were applied to calculate the contribution of polar solvation free energy, whereas solvent

accessible surface area (SA) method was used to evaluate the nonpolar part of solvation free energy. The entropic penalty was omitted in terms of the same simulation condition and considerable computational cost.

2.2 *In Vitro* Assays

2.2.1 EGFP Cellular AR Transcription Assay

A rapid, non-destructive AR transcriptional assay was used to screen for chemicals with AR transcriptional inhibition and measure the IC_{50} values.[180] Briefly, the human LNCaP PCa cells, were stably transfected with an androgen responsive probasin-derived promoter (ARR2PB) fused to an enhanced green fluorescent protein (eGFP) reporter. The LNCaP-eGFP cells were grown in phenol red free RPMI 1640 media supplemented with 5% charcoal stripped serum (CSS). After 5 days, the cells were plated into a 96-well plate (35,000 cells/well) with 0.1 nM R1881 and certain concentrations of test compounds. Fluorescence (excitation 485 nm, emission 535 nm) was measured after 3 days of incubation with the compounds, and was used to quantify the AR transcriptional inhibition by the compounds.[65, 66, 95, 181-183]

2.2.2 PSA Assay

The PSA level was measured in parallel with the AR transcriptional inhibition using the eGFP AR transcription assay using the same plates. After incubation of LNCaP cells with 0.1 nM R1881 and the test compound for 3 days, 150 µl of the media was taken from each well and added to 150 µl of PBS. The PSA level was then evaluated using the Cobas e 411 analyzer instrument (Roche Diagnostics) according to the manufacturer's instructions.[181, 182]

2.2.3 Cell Viability Assay

The PC3, LNCaP, and enzalutamide-resistant cells (MR49F) were seeded into a 96-well plate (3,000 cells/well) in RPMI 1640 containing 5% CSS, and treated with 0.1 nM R1881 and compounds in various concentrations for 4 days. Then the cell density was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay according to the manufacturer's protocol (CellTiter 961 Aqueous One Solution Reagent, Promega). The percentage of cell growth was normalized to control wells with and without 0.1 nM R1881, and calculated as % growth = (sample – vehicle)/(0.1 nM R1881-vehicle) * 100.[65, 66, 95, 181-183]

2.2.4 Androgen Displacement Assay

The androgen displacement activity was assessed using the Polar Screen Androgen Receptor Competitor Green Assay Kit as per the instructions of the manufacturer.

2.2.5 Biolayer Interferometry (BLI) Assay

The biotinylated AR LBD was produced in situ using AviTag technology. The AviTag sequence (GLNDIFEAQKIEWHE), which was followed by a six residue glycine serine linker (GSGSGS), was incorporated into the N-terminus of the AR LBD (669-919). *Escherichia coli* BL21 containing both biotin ligase and AR LBD vectors were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of DHT and biotin at 16 °C overnight. The bacteria were then lysed by sonication, and the resulting lysate was purified by immobilized metal ion affinity chromatography (IMAC) with nickel-nitrilotriacetic acid (Ni-NTA) resin and cation-exchange chromatography (HiTrap SP). Purified biotinylated AR LBD (50 μg/mL) was bound to the superstretavidin sensors

overnight at room temperature. The sensor was kept in assay buffer [150 mM lithium sulfate, 50 mM HEPES, 1 mM DTT, and 10 µM DHT].[65, 66]

2.2.6 Site-Directed Mutagenesis Study

The residues in the predicted binding site were mutated using the Quickchange™ Site-Directed Mutagenesis Kit as per the instructions. The introduced mutations were verified by sequencing.[95, 183]

2.2.7 Transient Transfection

The PC3 cells were starved in RPMI 1640 media (Gibco, USA) supplemented with 5% CSS for 5 days, and then seeded into a 96-well plate (5,000 cells/well). After 24 hours, plasmids such as wild-type human AR (50 ng/well), AR mutants, AR-V7 or other nuclear receptors, and 50 ng ARR_3tk-luciferase plasmids were co-transfected into PC3 cells using 0.3 µL/well transfection reagent (TT20, Mirus). After the transfection for 48 hours, cells were treated with compounds at various concentrations for 24 hours. The AR, GR and PR activation was stimulated with 0.1 nM R1881, 1 nM dexamethasone and progesterone, respectively. ER-α transcriptional activity was measured using a MCF-7 cell line bearing stable transfection of an ERE-Luciferase reporter, with transcriptional activity stimulated by 1 nM Estradiol. Cell lysis was carried out with 60 µL 1X passive lysis buffer/well (Promega). 20 µL of cell lysate from each well were mixed with 50 µL of luciferase assay reagent (Promega) and luminescence was recorded on a TECAN M200pro plate reader.[95, 183]

Chapter 3: Development of AR ABS Inhibitors

3.1 Background and Rationale

CRPC has been historically thought to be androgen independent; however, accumulating evidences demonstrate that CRPC continues to depend on the transactivation of the AR, further evidenced by survival benefit from Abiraterone and Enzalutamide in pre- and post-chemotherapy patients.[80, 102, 184] This provides rationale that the human AR is still a drug target for CRPC. All current anti-AR drugs such as Bicalutamide and Enzalutamide target the ABS, and more investigational drugs such as ODM-201 and Galeterone are developed as next-generation antiandrogens targeting the ABS.[58, 61, 62, 185] Thus, the ABS represents the most validated drug target for the development of effective therapeutics for PCa and CRPC.

3.1.1 Characteristics of the ABS

In all reported structures of the AR LBD, the protein maintains a three-layered sandwich fold consisting of 11 α-helices and 2 β-sheets; the only observed structural differences lie in subtle conformational changes of ABS residues.[48] The AR ABS is a buried site that is mainly composed of hydrophobic side chains, including L701, L704, L707, Q711, M742, M745, V746, M749, F764, M787 and L873, which interact with AR binders through Van der Waals interactions. There are two pairs of conserved polar patches (R752 and Q711, N705 and T877) located in opposing ends of the site, which may play a pivotal role in anchoring steroidal AR binders (**Figure 3.1**). For non-steroidal compounds, generally, the R752 and Q711 make hydrogen-bond interactions with AR binders through a conserved

water molecule. The ABS is known for its remarkable plasticity that it adjusts the volume to accommodate ligands of various sizes.[79, 186]

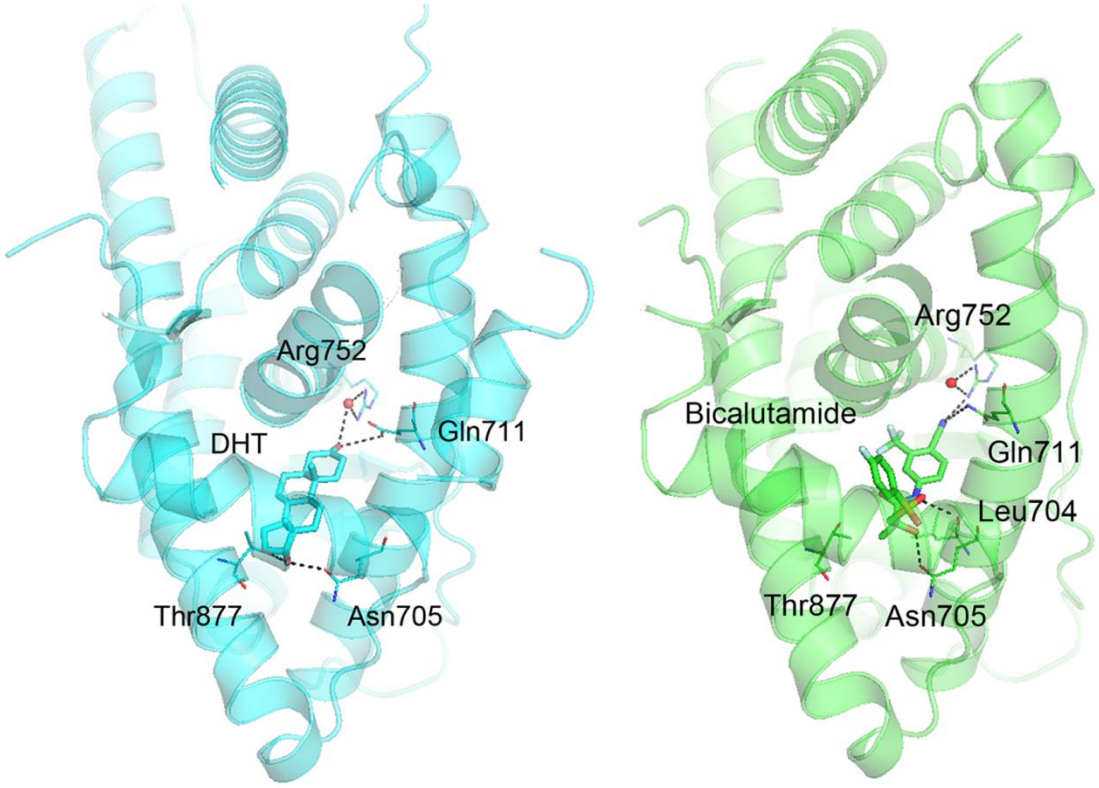


Figure 3.1. Crystal structures of AR LBD with AR binders.

(Left): AR LBD co-crystalized with DHT (PDB code: 3L3X); (Right): AR LBD with Trp741L mutation co-crystalized with Bicalutamide as an agonist (PDB code: 1Z95).

3.1.2 AR LBD Antagonism vs. Agonism

So far, the agonism and antagonism of AR has been mainly elaborated from a mechanistic perspective. In brief, the H12 serves as a "switch" to open or close the LBD according to the binding of antagonists or agonists. When agonists bind, the H12 is positioned to cover the LBD in a "close" state, and the AF2 is exposed that enables the recruitment of coactivators. Upon antagonist binding, the LBD adopts an "open" state by

repositioning the H12 to occupy the AF2 site, which prevents the coactivator or recruits repressor binding and thus blocks the receptor transactivation.[48]

The agonistic form of AR has been disclosed by a plethora of co-crystal structures of wild-type AR LBD with agonists or mutated AR LBD with antagonists acting as agonists (**Figure 3.2**),[79, 186-193] demonstrating AR LBD is in a "close" state with coactivators such as SRC binding to the AF2. The antagonism of AR is not clear yet due to the infeasibility of resolving wild-type AR LBD with antagonists. However, the antagonism of ER (**Figure 3.3**), PR and GR has been unveiled by co-crystal structures of wild-type LBD and antagonists,[194-197] demonstrating the H12 reorganized and occupied the AF2. Based on the antagonism of other SHRs, it was hypothesized that AR may share a similar antagonism.

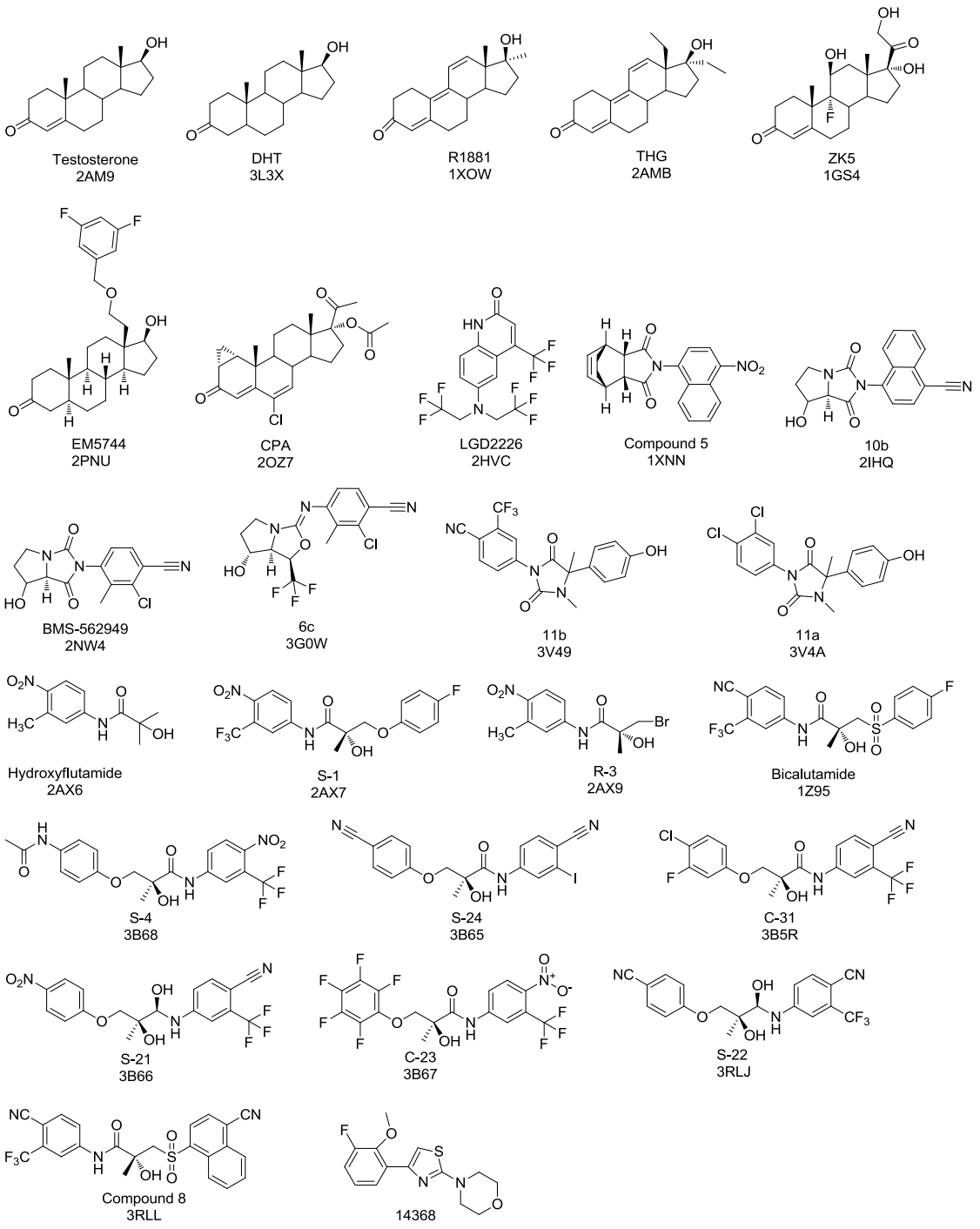


Figure 3.2. AR binders co-crystallized with AR LBD.

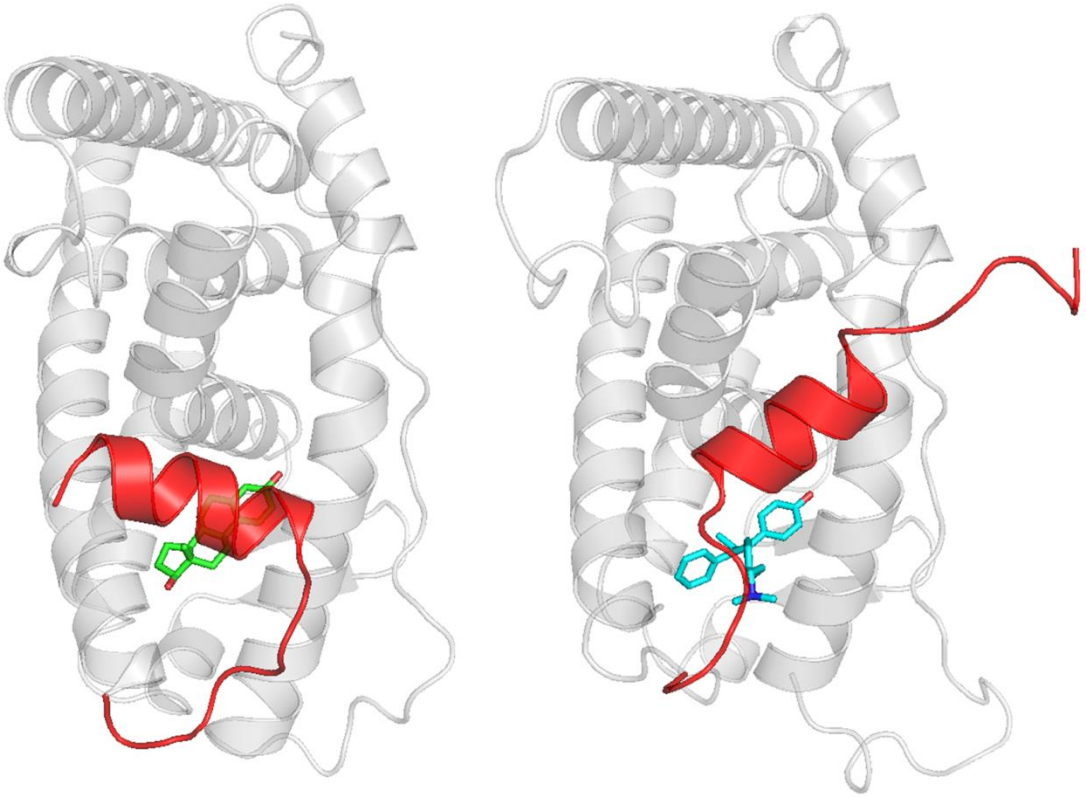


Figure 3.3. Crystal structure of ER in agonistic and antagonist forms

(Left): ER co-crystalized with estradiol in the agonistic form (PDB code: 1ERE); (Right): ER co-crystalized with tamoxifen in the antagonistic form (PDB code: 3ERT). The H12 was colored in red.

3.1.3 Challenges and Opportunities of Targeting the AR ABS

Although the AR ABS is a well-established drug target and multiple antiandrogen drugs are clinically used, developing effective small-molecule AR ABS inhibitors is still challenging in the absence of an antagonistic conformation of AR. Crystallographic study of SHRs in the antagonistic forms revealed that the H12 positioning is drastically diverse,[194-197] which makes the homology modeling of H12 in AR infeasible. In addition, existing problems

of antiandrogens add difficulty to the development of AR ABS inhibitors, such as the mutation-induced AR promiscuity, antiandrogen withdrawal symptom and inefficacy of inhibiting ARVs.

On the other hand, the availability of co-crystal structures of AR LBD with various ligands as well as a large number of known AR ABS binders provides a wealth of information for developing new AR ABS inhibitors.[79, 186-193, 198-200] As all current antiandrogens share high chemical similarity (**Figure 1.6**), the common structural motif might account for the invariable development of resistance. Thus, it could be hypothesized that the discovery of structurally distinct AR ABS inhibitors may help circumvent the resistance of current drugs.

3.2 Results

3.2.1 Structure-Based Development of AR ABS Inhibitors

PLIF Analysis on AR ABS

A good understanding of the AR ABS-ligand interaction is the prerequisite for developing ABS inhibitors. Given that the antagonistic form of AR LBD is not available and there is a wealth of information on crystal structures of AR LBD in the agonistic form, representative crystal structures of human AR LBD in complex with steroidal and non-steroidal ligands were firstly studied using the PLIF to analyze the protein-ligand interaction. A total of 23 crystal structures of AR LBD with unique ligands were superimposed, and then the PLIF was calculated. A summary of residues making contacts with the ligand was presented in **Figure 3.4**, which mainly demonstrated a high tendency of H-bond interaction between ligands and polar residues and water molecules. Of note, R752 and N705 have the highest frequency to interact with ligands, and then residues L704 and Q711. The rest

residues have a low rate of contacts with ligands, indicating these interactions may not be essential.

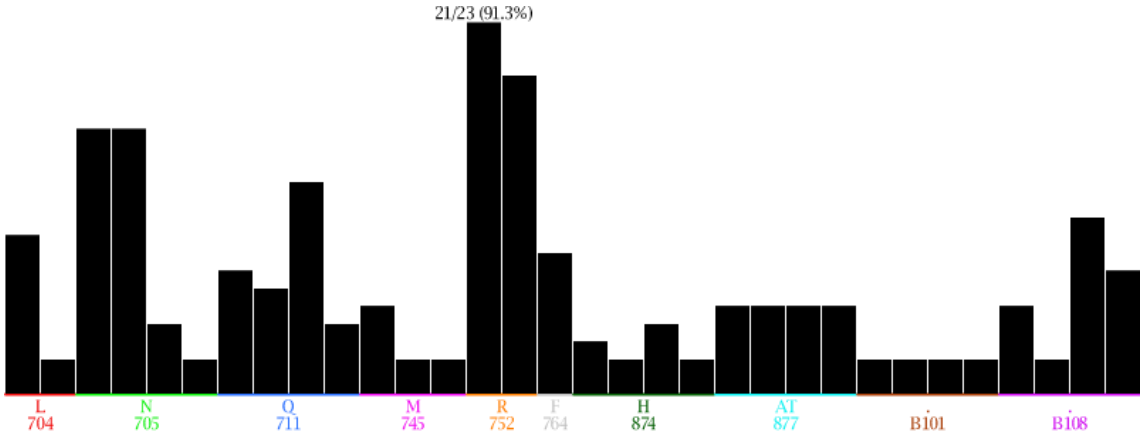


Figure 3.4. A histogram showing the number of ligands interacting with each residue.

The protein-ligand interaction fingerprint (PLIF) was used to analyze 23 co-crystal structure of AR LBD with 23 different ligands, which aims to reveal key residues and interaction types between the AR LBD with diverse ligands.

MD Simulation on AR LBD-Ligand

To better understand the AR LBD-ligand system, MD simulations were performed on the AR LBD-Testosterone (PDB code: 2AM9) and AR LBD (W741L)-Bicalutamide (PDB code: 1Z95). Both systems reached equilibrium in the first few nanoseconds of the simulation (**Figure 3.5**), and kept stable in following simulations. In comparison to crystal structures, the ligands were maintained in very similar conformations (RMSD < 1 Å), and no big conformational changes of AR LBD were observed (RMSD < 2 Å). The binding free energy was calculated using MMPB/GBSA, and binding free energy from PBSA showed correlation with experimental data that AR LBD-Testosterone has higher binding affinities

(WT AR LBD-Testosterone: -38.59 kcal/mol, W741L AR LBD-Bicalutamide: -36.21 kcal/mol).

A hydrogen bond analysis on the trajectory was performed by calculating the distance between binding site residues and the ligand, and the occupancy of the hydrogen bond during the MD simulation. Consistent with the PLIP analysis, all hydrogen bonds between key residues and ligands were captured during MD simulation. Furthermore, it revealed that the hydrogen bond with Asn705 is the strongest and the most stable for both AR LBD with Testosterone and Bicalutamide, while hydrogen bonding with remaining residues such as Arg752 is relatively weak (**Table 3.1**). This analysis provides insights into the AR LBD-ligand interaction and selection of virtual hits from prospective screening.

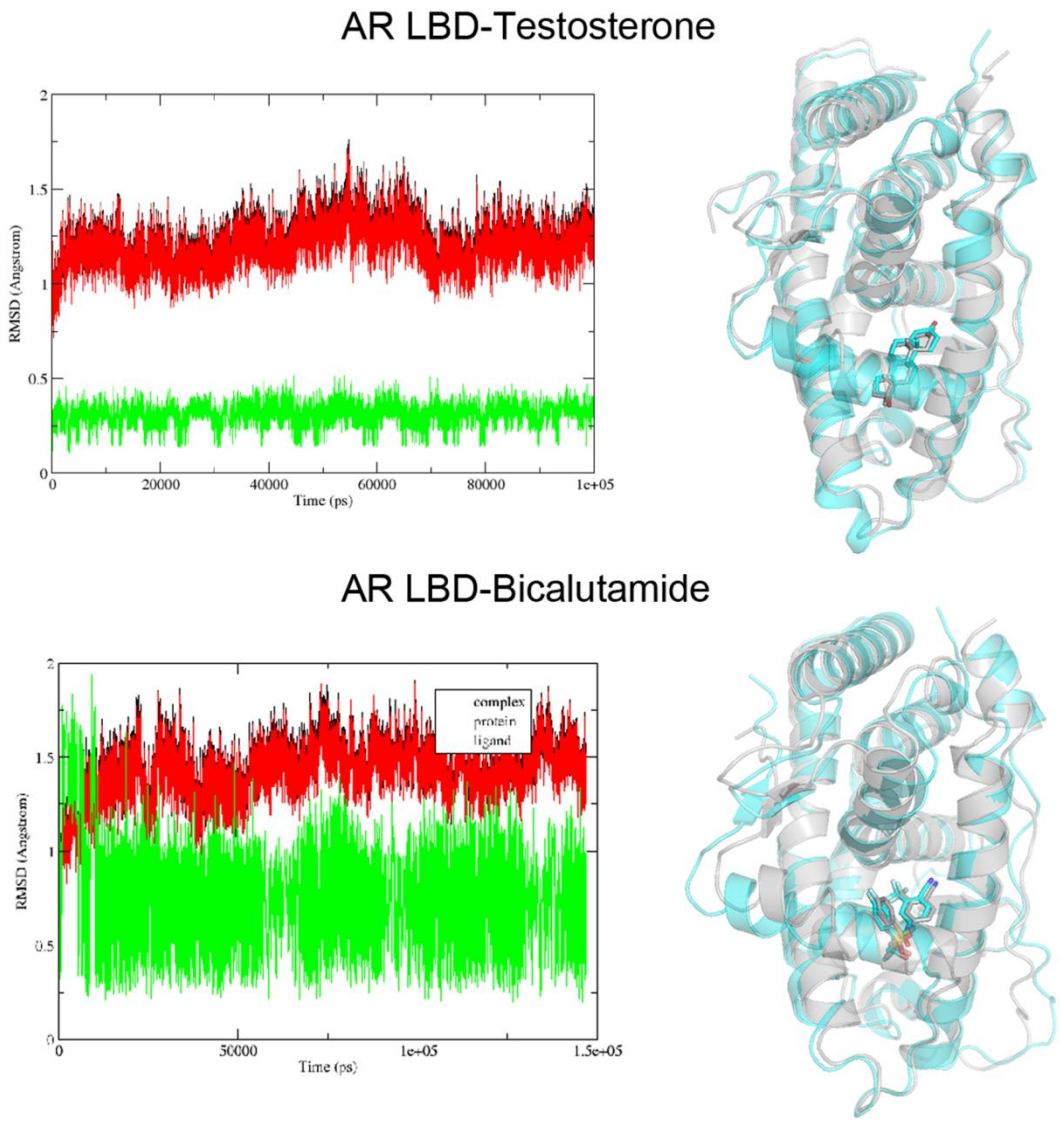


Figure 3.5. MD simulation on AR LBD-Testosterone and Bicalutamide.

(Left): RMSD values of protein backbone (red) and ligand heavy atoms (green) using the corresponding crystal structure as a reference. (Right): The superimposition of last snapshots (cyan) from the simulation with crystal structures (grey).

Table 3.1. Hbond interactions between binding site residues and ligands.

	Testosterone		Bicalutamide	
Residue	**Distance**	**Occupancy**	**Distance**	**Occupancy**
Asn705	2.76	93.22	2.67	98.1
Gln711	3.17	8.3	3.15	3.98
Thr877	2.84	6.44		
Arg752	3.17	0.58	3.13	41.11
Leu704			3.30	36.74

In Silico Screening for Chemically Diverse and Effective AR Binders

Three million purchasable compounds from the ZINC database[172] were docked into two selected crystal structures of the AR (PDB codes: 2PNU and 3L3X) using Glide SP program. About 700,000 compounds that successfully docked with Glide SP score < -7 in both runs were then re-docked into the AR ABS using eHiTs program.[201] A total of 130,000 structures were selected that were consistently docked by both algorithms (i.e. having RMSD < 2 Å for the docking poses produced by Glide SP and eHiTs). These compounds were further evaluated by several on-site rescoring approaches including Glide XP,[202] eHiTs score,[203] London dG scoring and pKi criteria computed by MOE program, and X-score.[204]

With this information, a cumulative scoring of 7 different predicted parameters was generated where each molecule received a binary 1.0 score for every 'top 10% appearance'. The final cumulative vote (with the maximum possible value of 7) was then used to rank the remaining entries. Based on the cumulative score, 50 compounds were selected for experimental evaluation. The overall workflow is summarized as shown in **Figure 3.6**.

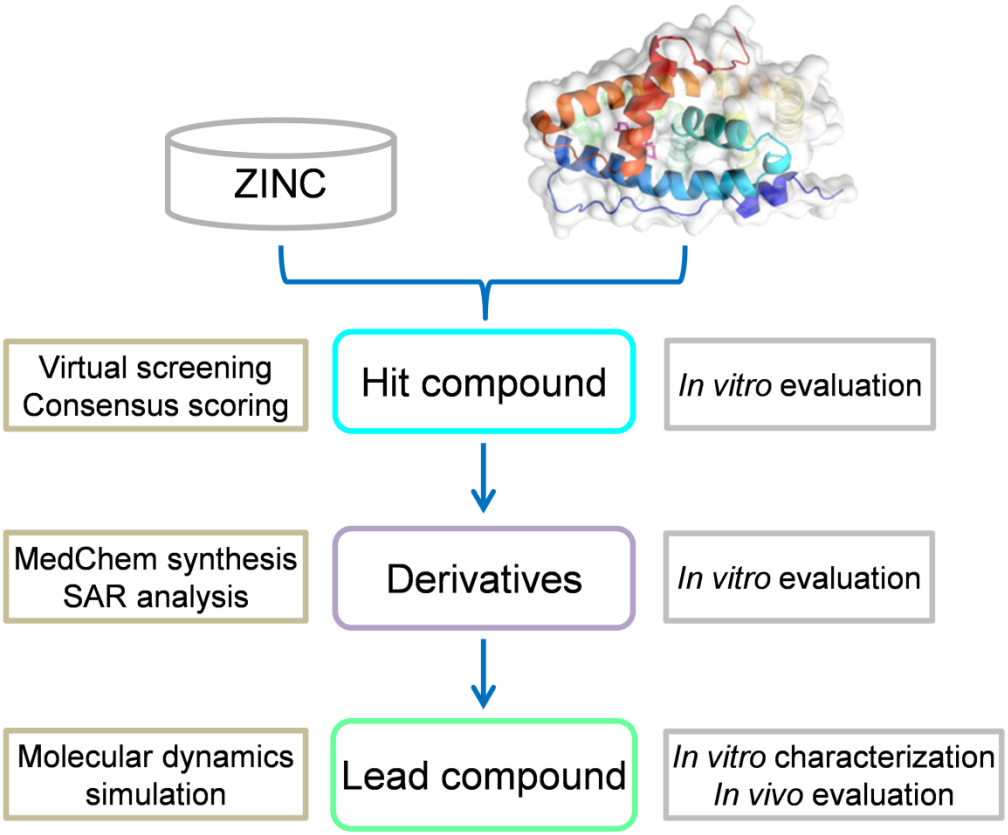


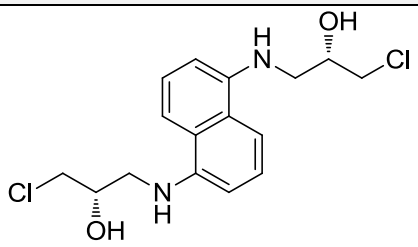
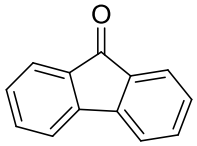
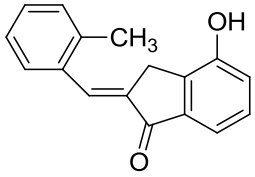
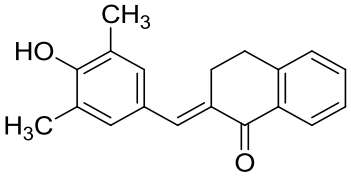
Figure 3.6. The structure-based workflow for the discovery of AR ABS inhibitors. Molecular docking-based virtual screening and a consensus scoring were used for identifying potential AR ABS inhibitors (hit compounds), which was evaluated using *in vitro* assays. Based on the hit, derivatives were synthesized and evaluated experimentally. The lead compound selected was characterized using *in vitro* assays and also evaluated for its *in vivo* activity.

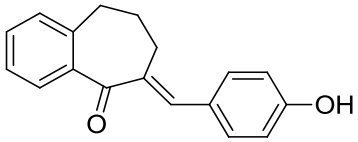
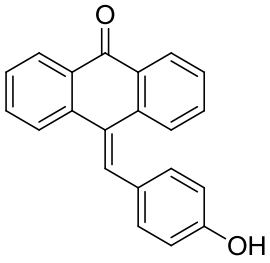
Identification of 10-Benzylidene-10H-Anthracen-9-Ones as Effective AR Antagonists.

The selected 50 chemicals were initially assessed with a non-destructive eGFP reporter assay to quantify levels of AR transcriptional activity in LNCaP eGFP cells, which stably express an androgen-responsive probasin promoter in front of an eGFP reporter.[180] Among

the tested chemicals, 6 compounds demonstrated effective transcriptional inhibition (>85%) when administered at a single 50 µM dose (**Table 3.2**). These compounds were then tested for their ability to displace DHT from the AR using a polar screen competitor green assay kit, and were found to have IC_{50} values ranging from 0.63 µM to 50 µM. These compounds did not affect SRC2-3 peptide displacement, suggesting they do not interact with the AF2 co-activation site of the AR (data not shown).[205]

Table 3.2. AR ABS binders identified from structure-based VS.

VPC-ID	Structure	DHT Displacement activity IC_{50} (µM)
2055		10
3002		5.9
3008		>10
3015		>10

VPC-ID	Structure	DHT Displacement activity IC_{50} (μM)
3018		>50
3022		0.625-2.5

The most active compound VPC-3022 was further measured for the transcriptional inhibition in the eGFP assay at various concentrations, and it demonstrated characteristic dose-dependent behavior, with an IC_{50} value of 4 μM (**Figure 3.7**). Since the endogenous AR in LNCaP cells harbors a T877A mutation, we used HeLa-AR cells transfected with an ARR_3tk-luciferase reporter in order to validate VPC-3022 for its inhibitory efficacy on wild-type AR.[206] VPC-3022 demonstrated inhibition of wild-type AR in a concentration-dependent manner with an IC_{50} of 1μM, consistent with the activity from our eGFP transcriptional inhibition assay in LNCaP cells. To rule out possible false positives from the AR transcriptional eGFP assay, we validated the results by quantifying the expression of PSA.[207] As expected, VPC-3022 induced a dose-dependent decrease of secreted PSA with a corresponding IC_{50} value of 3.6 μM.

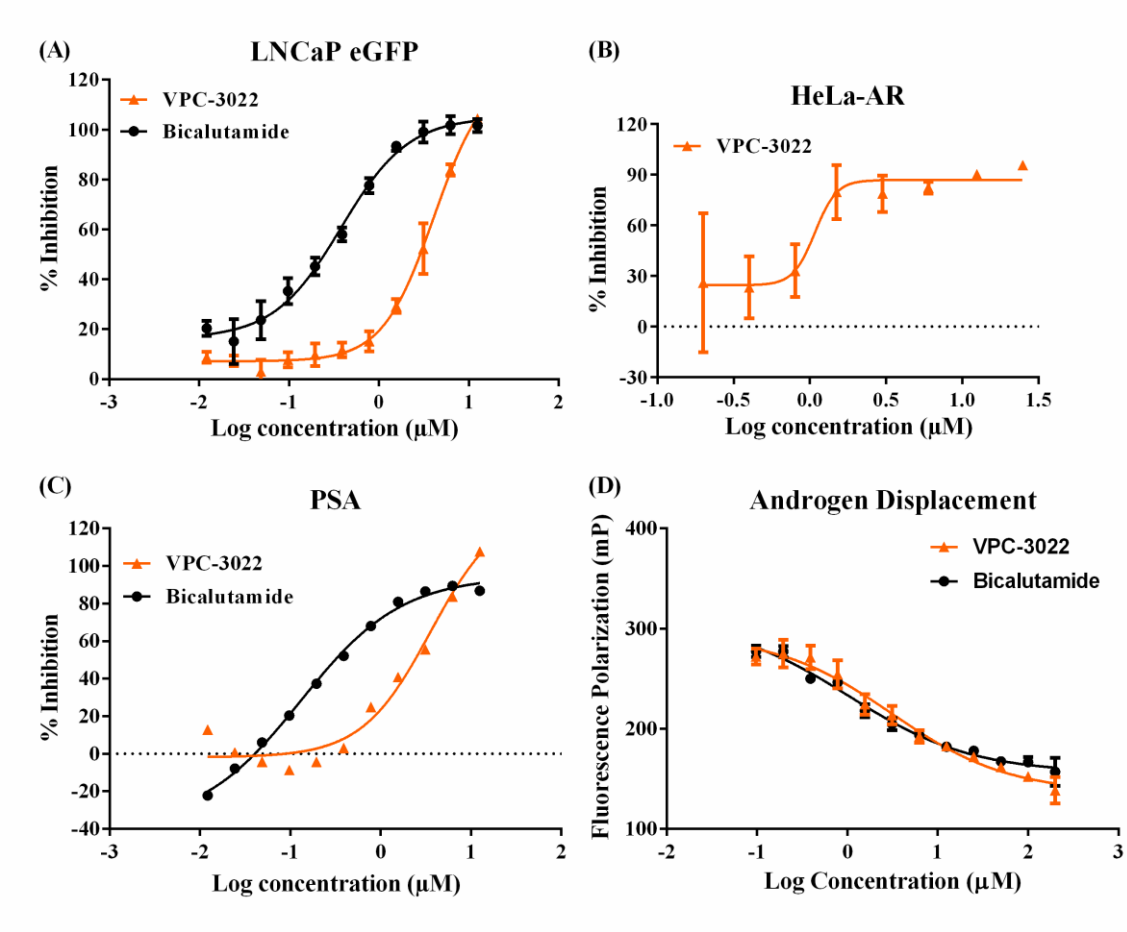


Figure 3.7. Dose-response curves of VPC-3022.

(A) The AR inhibitory activity measured by the eGFP cellular AR transcription assay; (B) AR transcriptional inhibition in HeLa-AR cells by luciferase assay; (C) PSA inhibition by the studied compound VPC-3022; (D) Androgen competition binding curves of VPC-3022.

We have further investigated interaction of the most active compound VPC-3022 with the AR C-terminal ligand binding domain (LBD) using the biolayer interferometry (BLI). The BLI results demonstrated direct and reversible interaction between the AR and VPC-3022. To evaluate the overall effect of VPC-3022 on prostate cancer cell viability, we have conducted the cell viability (MTS) assays using three cell lines: LNCaP, PC3 as well as

enzalutamide-resistant LNCaP cells (MR49F). As shown in **Figure 3.8**, VPC-3022 exhibit profound concentration-dependent suppression of cell survival, especially with those that have resistance to the current antiandrogen Enzalutamide. Although the particular mechanism(s) of enzalutamide-resistance in these cell lines has not yet been clearly elucidated, it involves retention of the AR.[208] More specifically, at a concentration of 1.5 μM, VPC-3022 inhibited the cell proliferation of enzalutamide-resistant cells by almost 100% and LNCaP cells by 50%, but only a small effect was observed on AR-negative PC3 cells.

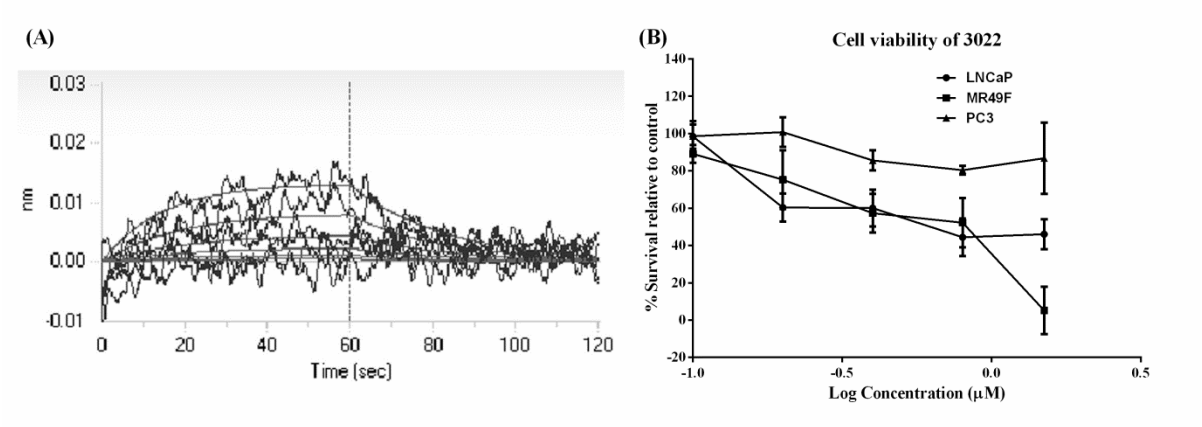


Figure 3.8. BLI and cell viability of VPC-3022.

(A) Dose-response curves (0.09-3 μM) showing direct AR binding of compound VPC-3022 in serially dilution; (B) Effects of VPC-3022 on the cell growth of three PCa cell lines. AR-positive LNCaP and MR49F, and AR-negative PC3 cells were treated with VPC-3022 in a concentration-dependent manner in the presence of 0.1 nM R1881. Error bars indicate standard deviation.

Interestingly, the VPC-3022 compound was also found to induce an almost complete degradation of the AR in various cell lines (**Figure 3.9**), including HeLa-AR and LAPC4 with wild-type AR, LNCaP with mutated AR (T877A) and enzalutamide-resistant MR49F.[208]

At the same time, VPC-3022 did not alter AR mRNA transcript level, and the addition of the transcriptional inhibitor cyclohexamide didn't affect the AR degradation, strongly suggesting the loss of AR is due to degradation of the protein. Given these results, VPC-3022 was selected as a parental compound for further structural modifications to improve the anti-AR effect and increase the therapeutic potential.

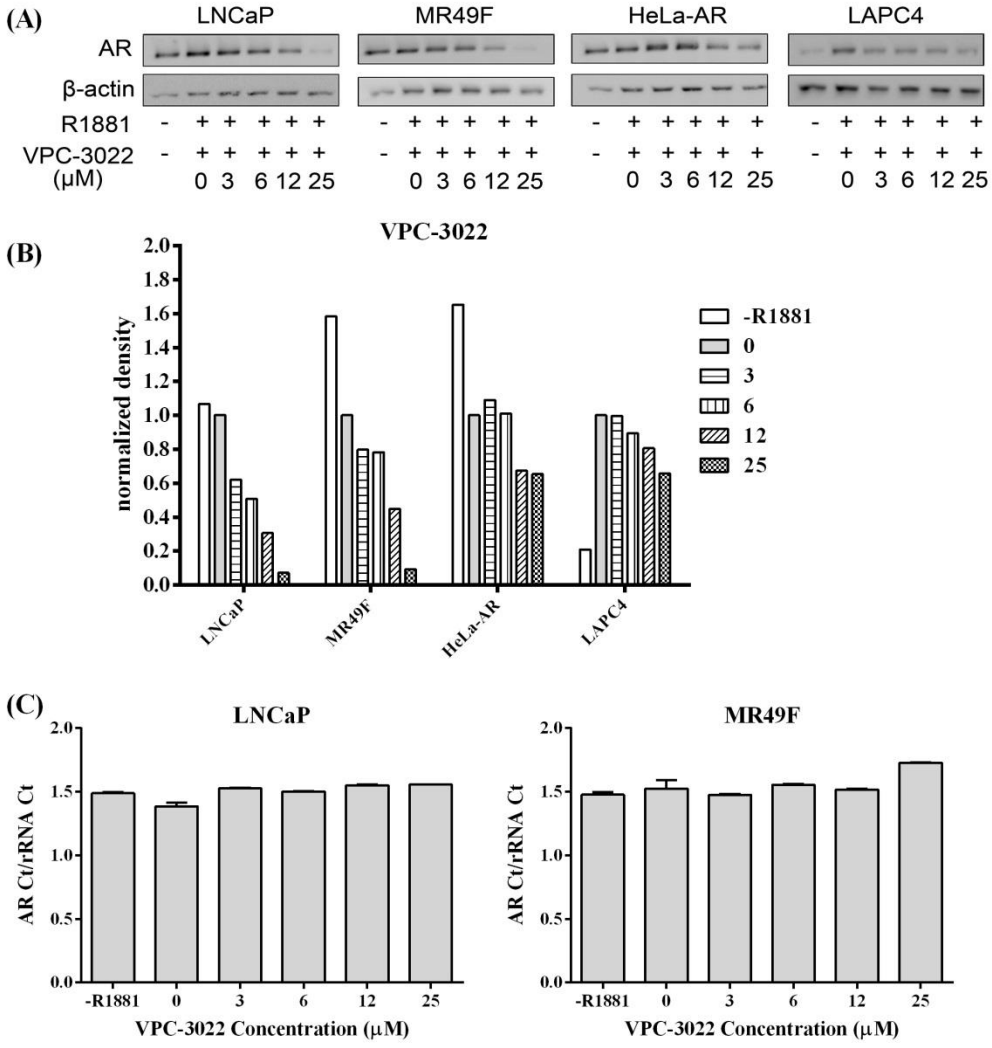


Figure 3.9. VPC-3022 degrades the AR in PCa cells.

(A) LNCaP, MR49F, HeLa-AR and LAPC4 cells were treated with VPC-3022 for 24 hours at the indicated concentrations in the presence of 0.1 nM R1881. Whole-cell extracts

were prepared from each treatment and subjected to western blot analysis with anti-AR or β-actin antibodies. (B) Band density of AR relative to β-actin measured by ImageJ Program. (C) AR mRNA levels are unaffected upon treatment with VPC-3022. Total RNA was extracted after treatment of LNCaP and MR49F with VPC-3022 for 24 hours at the indicated concentrations in the presence of 0.1 nM R1881. Quantitative RT-PCR analysis of AR expression was performed and normalized to 18S rRNA levels. Error bars indicate standard deviation.

Binding Mode Prediction of the Hit Compound.

In order to develop an effective strategy for structural optimization of VPC-3022, its predicted interaction with the AR ABS was investigated in greater details. The docking pose of VPC-3022 (**Figure 3.10**) reflects mainly a hydrophobic character of its anchoring to the ABS and the overall orientation of the compound in the site resembles positioning of the native ligand - DHT. Due to the infeasibility of experimental evaluation of antagonistic configurations of the AR, we have conducted a MD simulation of VPC-3022/ABS complex. The established 10 ns MD trajectories reflected overall stability of the complex with a resulting RMSD value around 2.5Å (data not shown). The MD-optimized positioning of VPC-3022 inside the ABS revealed possible formation of a hydrogen bond with residue R752, which was not captured by the original docking experiment. Based on this predicted binding mode inside the target site, a series of analogues were developed by retaining the anthracenone moiety and modifying substituents on the benzylidene group.

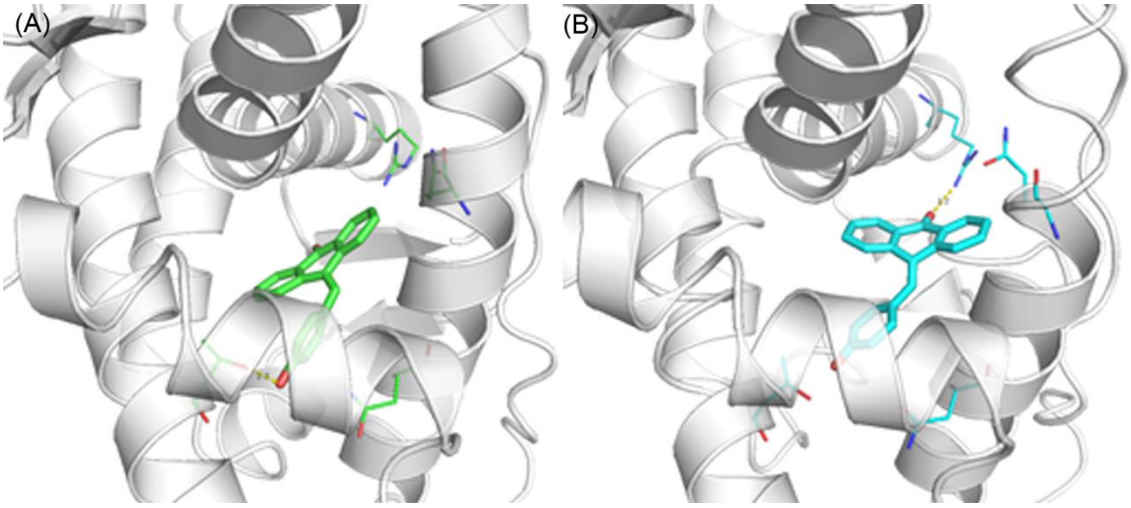


Figure 3.10. Binding mode of VPC-3022 in the ABS.

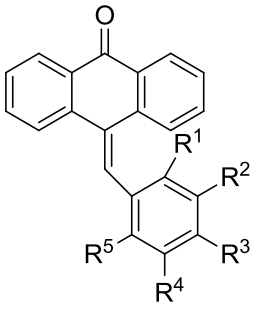
(A) Docked pose of VPC-3022 in the AR ABS; (B) conformation of VPC-3022 after MD simulation and a hydrogen bond contact found.

Development of the 10-Benzylidene-10H-Anthracen-9-Ones as Antiandrogen Prototype.

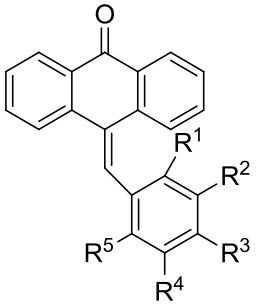
A series of 10-benzylidene-10*H*-anthracen-9-ones were synthesized by an aldol-type condensation reaction of 10*H*-anthracen-9-one with appropriately substituted benzaldehydes under basic conditions in the presence of pyridine/piperidine (**Table 3.3**) [209]. The created compounds were subsequently evaluated for their ability *a)* to displace DHT from the receptor, *b)* to inhibit the AR in the eGFP and PSA assays, *c)* for their direct interaction with the AR, as detected by the BLI, *d)* to inhibit the cell proliferation, and *e)* to degrade AR in PCa cells. In these experiments, except a weak agonist (VPC-3037), the rest of tested 10-benzylidene-10*H*-anthracen-9-ones demonstrated potent androgen displacement from the AR and effective, dose-dependent inhibition of its transcriptional activity, with corresponding IC_{50} values estimated in 0.2-50 μM range. Importantly, two particular analogues - VPC-3033

and VPC-3045 demonstrated ten-fold enhanced anti-AR potency compared to the parental compound VPC-3022. The direct binding to AR LBD and cell viability were also examined for the analogues, as exemplified by VPC-3033 in **Figure 3.11**, which show similar binding pattern to VPC-3022 and strong inhibition of cancer cell proliferation.

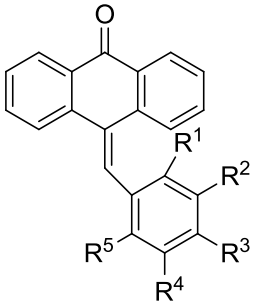
Table 3.3. Structures and activities of developed derivatives



VPC-ID	R^1	R^2	R^3	R^4	R^5	eGFP IC_{50} (μM)	DHT displacement activity IC_{50} (μM)
3022			-OH			3.3	0.625-2.5
3023		$-OCH_3$	$-OCH_3$	$-OCH_3$		1.6	10
3024		$-COOCH_3$	-OH			7.9	40
3025						2.2	0.625-2.5
3026	2-Methylthiophene					6.9	0.625-2.5
3027		$-C(CH_3)_3$	-OH	$-C(CH_3)_3$		16.2	40
3028	$-OCH_3$	$-OCH_3$				8.3	>40
3029			$-COOCH_3$			9	>40



VPC-ID	R^1	R^2	R^3	R^4	R^5	eGFP IC_{50} (μM)	DHT displacement activity IC_{50} (μM)
3030	5-methyl-1,3-benzodioxole					4.3	2.5-10
3031			$-NO_2$			3.1	>40
3032		-OH	-OH	-OH		48.8	10
3033				-OH		0.2	0.625-2.5
3034	$-OCH_3$				$-OCH_3$	6.9	10
3035	-OH	$-OCH_3$				4.6	0.625-2.5
3036		$-CH_3$	-OH	$-CH_3$		1.1	2.5-10
3037		$-OCH_3$		$-OCH_3$		weak agonist	2.5-10
3038	-OCH3	-OH	$-OCH_3$			1.4	10-40
3039		-OH	$-OCH_3$			1.2	10-40
3040			$-OCH_3$			2.6	40
3041			$-OCH_3$	$-OCH_3$		2.8	10-40
3042			-OH	-OH		2.3	>40
3043			-OH	$-OCH_3$		1.7	40
3044		$-OCH_3$	-OH	-OCH3		3.8	10-40



VPC-ID	R^1	R^2	R^3	R^4	R^5	eGFP IC_{50} (μM)	DHT displacement activity IC_{50} (μM)
3045	-OH		$-OCH_3$			0.2	10
3046			$-OCH_2$-phenyl			10.9	>40
3047			$-CF_3$			1.1	10
3048		-Cl		-Cl		6.6	40
3049	$-OCH_3$					2.9	2.5

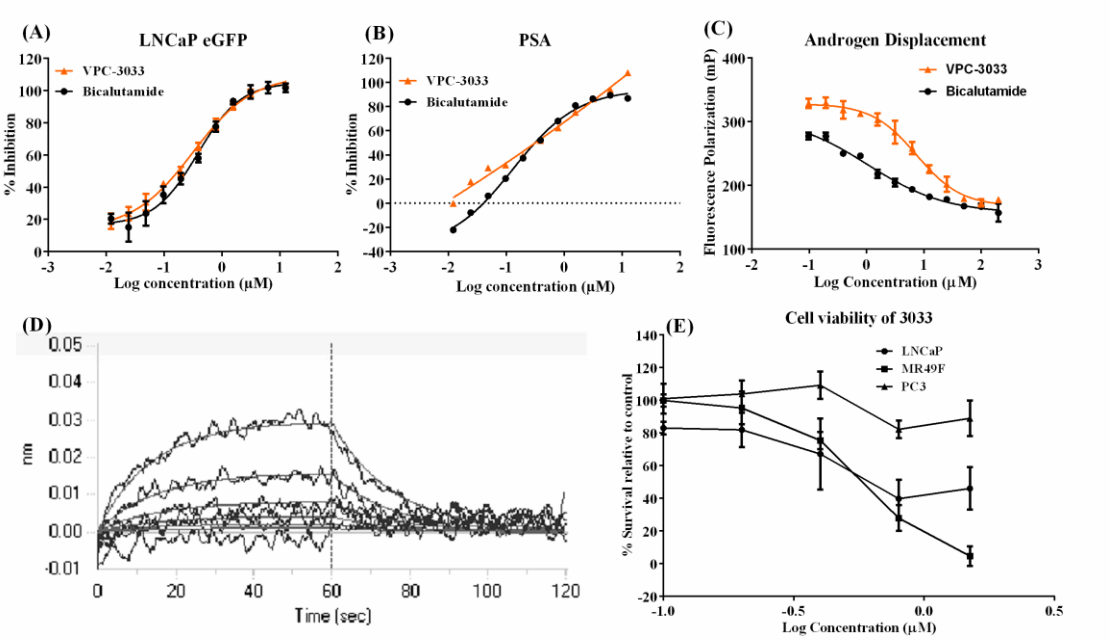


Figure 3.11. Dose-response curves of VPC-3033.

(A) The AR inhibitory activity measured by the eGFP cellular AR transcription assay; (B) PSA inhibition by the studied compound VPC-3033; (C) androgen competition binding curves of VPC-3033; (D) direct AR binding of compound VPC-3033 in serially dilution; (B) effects of VPC-3033 on the cell growth of three PCa cell lines.

The AR-degrading ability of these chemicals appeared to be also enhanced - the western blot presented in **Figure 3.12** illustrates almost complete elimination of the receptor from LNCaP, MR49F, HeLa-AR, and LAPC4 cells at concentrations of 6-25 μM of VPC-3033. Of note, two other synthesized analogues, VPC-3031 and VPC-3041, also demonstrated significant AR degradation at concentrations of 6-25 μM. To confirm the AR degradation by these compounds, the mRNA expression was measured in LNCaP and MR49F cells treated at the same concentrations of the compounds as in the western blot assays. The mRNA levels were not affected by the treatment of these compounds at different

concentrations. The combined evidence suggests that this class of compounds induces degradation of AR in PCa cells.

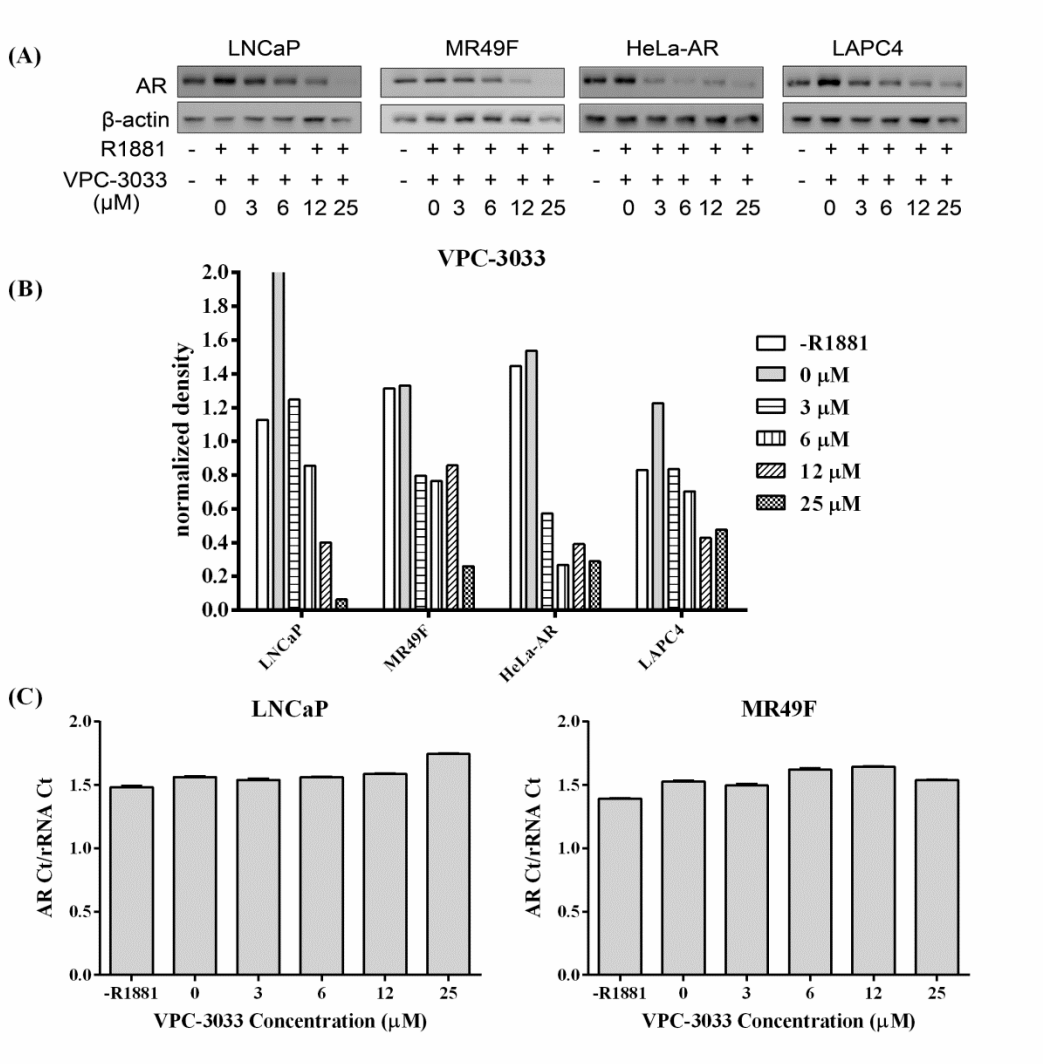


Figure 3.12. VPC-3033 degrades the AR in PCa cells.

(A) LNCaP, MR49F, HeLa-AR and LAPC4 cells were treated with VPC-3033 for 24 hours at the indicated concentrations in the presence of 0.1 nM R1881. Whole-cell extracts were prepared from each treatment and subjected to western blot analysis with anti-AR or β-actin antibodies. (B) Band density of AR relative to β-actin measured by ImageJ Program. (C) AR mRNA levels are unaffected upon treatment with VPC-3033. Total RNA was extracted

after treatment of LNCaP and MR49F with VPC-3033 for 24 hours at the indicated concentrations in the presence of 0.1 nM R1881. Quantitative RT-PCR analysis of AR expression was performed and normalized to 18S rRNA levels. Error bars indicate standard deviation.

As this chemical class has been reported to act as anti-microtubule agents with tubulin polymerization inhibitory activity, the tubulin polymerization activity was examined. Importantly, as reported previously, the tubulin polymerization inhibitory activity for the highly active AR inhibitors was not very pronounced, even for the most potent compound VPC-3033, further supporting the conclusion that the observed anti-AR activity is not simply an artefact as a consequence of the inhibition of tubulin polymerization.

Binding Mode of the Lead Compound in AR ABS.

Previous reports indicated that mutation of W741 to leucine or cysteine will generate additional space in the AR ABS that allows accommodation of the bulky phenyl ring of bicalutamide and converts it from antagonist into agonist that stimulates transcriptional activity and cancer growth.[210] It has been simulated by docking that binding of VPC-3033 to the AR ABS occurs at a notable distance from the W741 residue and therefore a mutation here is not likely to have an effect on the compound's AR binding and activity (**Figure 3.13**). Similarly, the well-documented agonist-converting T877A mutation, as found in the AR present in LNCaP cells, should not influence binding of VPC-3033 to this site, as the compound does not form any critical contacts with T877 or its mutant.[210] In support of this, our 10-benzylidene-10*H*-anthracen-9-one derivatives, including VPC-3033, demonstrated effective inhibition of the LNCaP cell line as well as cell lines with wild type AR. When

tested in the LNCaP xenograft model, the lead compound VPC-3033 demonstrated effective *in vivo* potency against the PCa tumor growth.

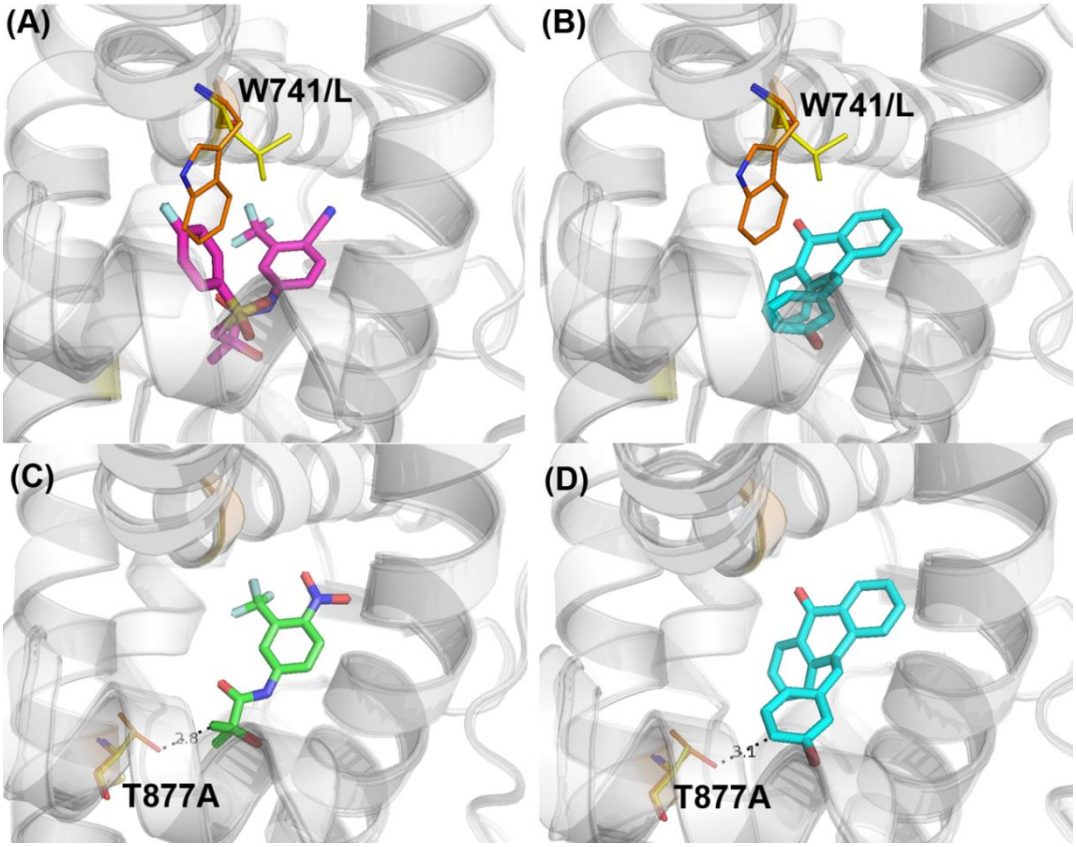


Figure 3.13. VPC-3033 in AR ABS compared with Bicalutamide and Hydroxyflutamide. VPC-3033 may not be affected by W741L and T877A which converts Bicalutamide and Hydroxyflutamide, respectively. Docking modes of VPC-3033 in AR ABS with W741L and T877A (B, D) were compared with Bicalutamide (A) and Hydroxyflutamide (C), which showed that VPC-3033 makes no direct contacts with these two residues.

In Vivo Evaluation of VPC-3033 Revealed Its Ability to Suppress AR Function.

The lead compound VPC-3033 was investigated for its effect on the tumor growth using an LNCaP xenograft model.[211, 212] Results from preliminary acute toxicity studies indicated that doses up to and including 50mg/kg could be tolerated by the studied mice with

no decrease in body weight. The measured serum levels suggested that the compound could be administered effectively via intravenous tail vein injection and that the compound could be detected for up to 24 hours (**Figure 3.14**). At higher doses, the serum Cmax was estimated to be between 10-100 µM. While the clearance was fairly quick, based on the *in vitro* data, the plasma concentration was well within the predicted therapeutic window. A dose of 10 mg/kg was chosen based on these preliminary studies and previous literature reports of antiandrogens.[213] At this dose, the growth of tumor was effectively suppressed compared to the vehicle control over a three-week intravenous dosing regimen ($p < 0.01$ from day 14 onwards). The results clearly indicate that VPC-3033 could effectively inhibit androgen sensitive LNCaP xenograft growth *in vivo*, suggesting this class of compounds is effective as AR antagonists.

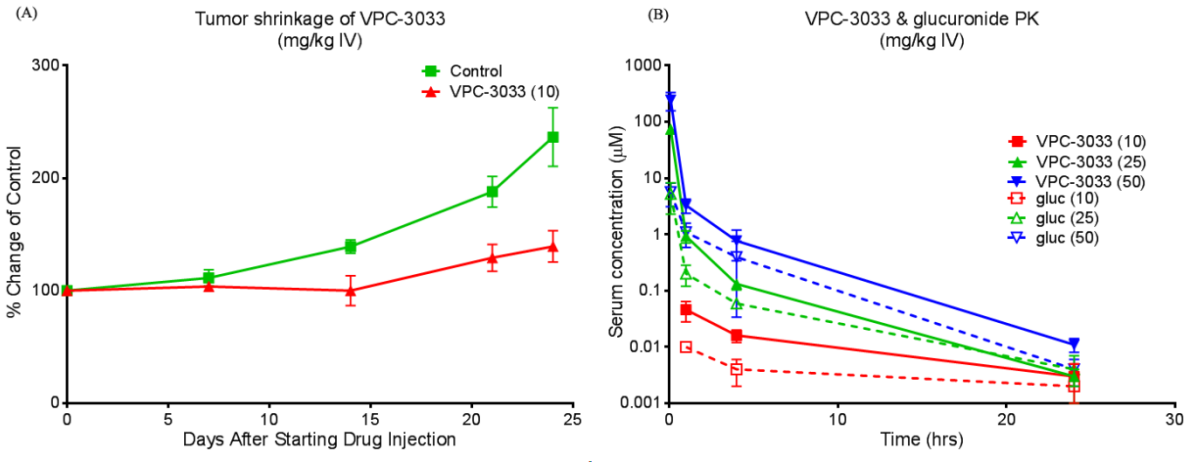


Figure 3.14. *In vivo* effects of VPC-3033.

(A) A limited PK analysis of VPC-3033 following single 10, 20 and 50 mg/kg iv injections displays circulating levels achieved and relatively rapid clearance for both the compound and a major glucuronide metabolite that was observed. Cmax would be expected to be 1-2 orders of magnitude higher than the initial 1hr point collected. (B) The *in vivo* effect of VPC-3033

(10 mg/kg) on tumor volume. The effects of VPC-3033 were determined using LNCaP mice xenografts (n=6). Data is presented as Mean ± SEM. A p value < 0.05 was considered significant (*) change, and a p value < 0.001 was considered very significant (**) change compared to vehicle control.

3.2.2 Structure- and Ligand-Based Discovery of AR ABS Inhibitors.

Structure-based virtual screening has shown to be a powerful tool to identify AR ABS inhibitors as shown in section 3.2.1 as well as by other groups.[181, 214] On the other hand, there is a large number of AR ABS binders reported, which could be used for ligand-based drug discovery.[215] It has been shown that virtual screening data fusion using structure- and ligand-based methods is a more effective tool, with the potential to outperform individual method.[216] Herein, a combination of structure- and ligand-based virtual screening methodologies (**Figure 3.15**) was used in this study to achieve a higher hit rate and to provide wider structural coverage of the chemical space.

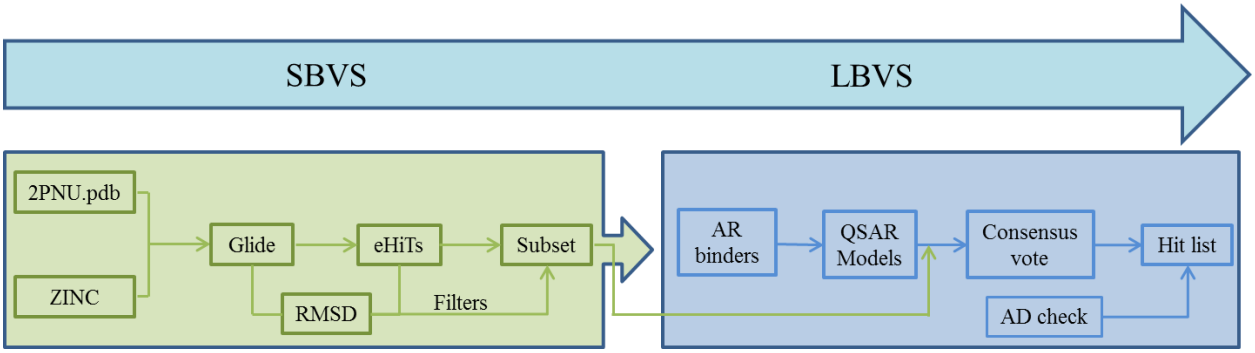


Figure 3.15. The structure- and ligand-based pipeline for AR ABS inhibitors.

Docking-based virtual screening was applied to the database (in green box), and the subset was subjected to a second round screening by QSAR models (in blue box) which were developed based on AR binders.

Structure-Based Virtual Screening.

From our initial structure-based screening against AR ABS (**section 3.2.1**), we obtained moderately active hit compounds. Then we sought to do a more systemic structure-based study on the ABS system and get better understanding of the AR ABS site, and we performed an evaluation on the crystal structures and the docking program.

Self- and cross-docking: The AR ABS is a flexible site and it can accommodate compounds of very different sizes and chemotypes. As there are a plethora of crystal structures of AR C-terminal LBD available, self- and cross-docking routines were used to evaluate AR structures that are most representative for VS. Based on the volume of the AR ABS cavity and types of bound ligands, four high-resolution crystals (PDB code: 2AM9, 3L3X, 2PNU and 1Z95) were selected.[79, 198-200] Those PDB entries corresponded to the AR in complex with testosterone, DHT, bulky steroidal derivative EM5744 and non-steroidal AR antagonist bicalutamide, respectively. The RMSD values between docked poses and bound conformations of the crystallographic ligands were calculated and reported as performed in other papers.[217] The bound conformations of smaller native ligands in 2AM9 and 3L3X structures could be well reproduced by docking, while bulkier EM5744 and bicalutamide could not be accurately fitted into smaller 2AM9 and 3L3X. However, the self- and cross-docking of these ligands into 2PNU and 1Z95 were accurate and reproducible in both docking programs (**Table 3.4**).

Table 3.4. The RMSD values between docked and bound conformations.

Glide	2AM9	3L3X	2PNU	1Z95	eHiTs	2AM9	3L3X	2PNU	1Z95
testosterone	0.79	0.22	0.48	3.14	testosterone	0.70	1.04	3.35	1.11
DHT	0.66	0.27	0.55	1.08	DHT	0.92	0.75	0.56	1.11
EM5744	NA	NA	0.28	1.5	EM5744	7.56	4.63	1.00	1.85
Bic*	4.81	NA	1.29	0.34	Bic*	3.08	4.15	0.65	2.21

NA: Ligands were skipped by glide sp docking program. * represents Bicalutamide.

Pre-screening with the directory of useful decoys (DUD): The four crystal complexes were also evaluated by pre-screening with the directory of useful decoys (DUD) dataset.[218] The docking enrichment factor (EF) established for known AR binders from the DUD were similar and suitable in cases of 2AM9 and 3L3X structures (**Table 3.5**), while docking with 1Z95 target resulted in low enrichment. The AR ABS structure corresponding to 2PNU protein database entry yielded the highest EF indicating its better ability to differentiate AR binders. Therefore, consistent with the self- and cross-docking evaluations, the crystal structure 2PNU was selected for VS.

Table 3.5. The EF values of the screening of DUD using two crystal structures.

PDB code	2AM9		3L3X		1Z95		2PNU	
Docking program	Glide	eHiTs	Glide	eHiTs	Glide	eHiTs	Glide	eHiTs
$EF_{1\%}$	26	34.7	26	32.2	11	35.9	26	35.9
$EF_{20\%}$	3.6	4.7	3.6	4.7	2.8	4.6	4.1	5.0

Virtual screening: Molecular docking was initially performed with Glide SP program using a subset of 3 million compounds from ZINC database. The Glide SP docking score cutoff (> -8) was used to identify low- or no affinity ligands to be discarded. The compounds left (702,151) were further re-docked into 2PNU using eHiTs, and only compounds with eHiTs score less than -3 (571,666) were kept. The docked poses were compared with those from the Glide experiment, and compounds with low RMSD values (< 2 Å) were kept. These compounds were further filtered following the Lipinski's rule of five[16] to keep compounds

with potential good bioavailability. Finally, 136,595 compounds were advanced into ligand-based VS.

Ligand-Based Virtual Screening.

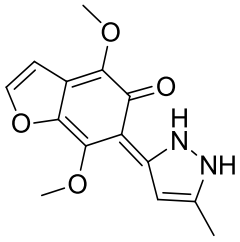
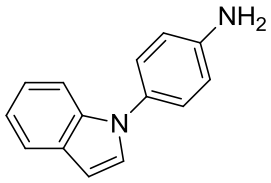
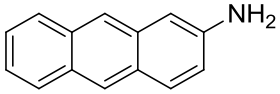
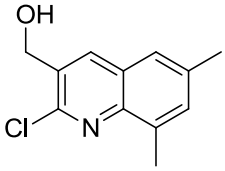
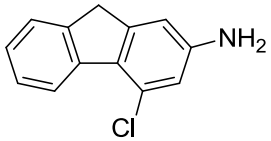
The ligand-based VS mainly relies on QSAR models, and this part of work was performed collaboratively in our lab.[182] Briefly, QSAR models using available AR binders and a set of selected descriptors were generated using a collection of machine learning algorithms in WEKA. These models were validated by an external test set and 9 validated QSAR models were applied to screen the pre-processed ZINC database described above. A consensus voting protocol was implemented to rank the QSAR results and select hit compounds. Based on the cumulative voting counts, the dataset was shrunk to 2,198 compounds with a consensus score of 8 and 9. Among these chemicals, half of these compounds (1,202 entries) belong to steroidal category and were excluded. By discarding broken structures, a list of 800 compounds was retained for virtual hit selection. Following the workflow of structure- and ligand-based VS, the list of virtual hits was further checked through receptor-ligand interaction. The docked poses were visualized to select compounds forming favorable interactions with the receptor. And finally, 37 diversified compounds were selected for further bioactivity testing.

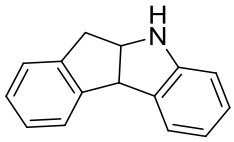
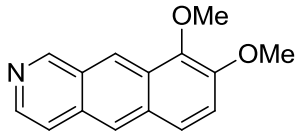
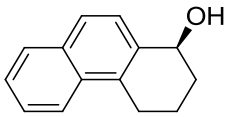
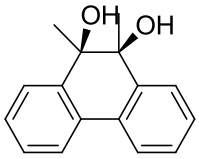
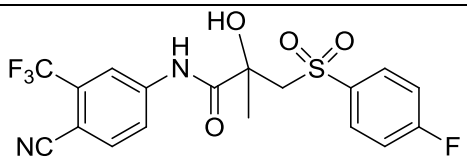
Cell-Based and *in Vitro* Evaluation of Virtual Hit Compounds.

Compounds that bind to the AR ABS can be identified by their capability to compete with a fluorescently-tagged androgen using the androgen displacement assay kit (Polar Screen^{TM}). An initial 50 μM screen with our selected 37 compounds identified 9 structurally diverse chemicals which demonstrated significant androgen displacement. The AR binding affinity was further evaluated by measuring the IC_{50} of these 9 chemicals. All of them are

active compounds with IC_{50} less than 20 µM, and particularly, 6 compounds demonstrated an IC_{50} under 5 µM (**Table 3.6**).

Table 3.6. AR ABS inhibitors identified from structure- and ligand-based VS.

VPC-ID	Structure	Androgen Displacement IC_{50} (µM)	eGFP IC_{50} (µM)	PSA IC_{50} (µM)	TC[a]
12002		12.29	2.04	N/A	0.27
12007		1.84	1.06	0.703	0.18
12051		1.23	1.95	2.299	0.18
12052		3.25	1.76	1.063	0.25
12058		2.02	2.54	2.1	0.23

VPC-ID	Structure	Androgen Displacement IC_{50} (µM)	eGFP IC_{50} (µM)	PSA IC_{50} (µM)	TC[a]
12060		3.43	0.38	0.173	0.18
12061		3.49	1.92	2.569	0.19
12063		18.63	9.35	N/A	0.20
12068		5.16	16.18	N/A	0.28
Bic		1.06	0.38	0.131	1

[a]Tanimoto coefficient

Subsequently, we evaluated the effect of these compounds on the transcriptional activity of AR using LNCaP cells, which stably transfected with an androgen-responsive probasin-derived promoter fused to an eGFP reporter (LN-ARR2PB-eGFP). Out of these 9 chemicals, 7 demonstrated significant transcriptional inhibition (IC_{50} < 5 µM), and one

compound (VPC-12060) showed low submicromolar activity (IC_{50} = 0.378 μM, **Figure 3.16**) which is comparable to clinical antiandrogen Bicalutamide (IC_{50} = 0.382 μM).

To confirm the inhibition of AR transcription by these compounds, we also evaluated the amount of PSA protein secreted in the media by eGFP LNCaP cells. PSA is a protein that is naturally transcribed under the control of the AR using the probasin promoter. A concentration-dependent reduction in PSA secretion was induced by these compounds, with IC_{50} correlated with the eGFP inhibition. For VPC-12060, the PSA level (PSA IC_{50} = 0.138 μM) is comparable to that of Bicalutamide (PSA IC_{50} = 0.131 μM). Other active compounds also showed low PSA level, together with high AR transcriptional inhibition and strong androgen displacement potency.

To determine whether the identified AR inhibitors affect cell proliferation, we have carried out the cell proliferation assay with two PCa cell lines: AR-positive LNCaP cells and AR-negative PC3 prostate cancer cells. The chemical VPC-12060 can antagonize the proliferative effect of the androgen R1881 without any significant effect on the growth of AR-negative PC3 cells at the same concentrations, which suggests that the inhibition of proliferation observed in AR-positive LNCaP cells is mediated through the antagonism of AR.

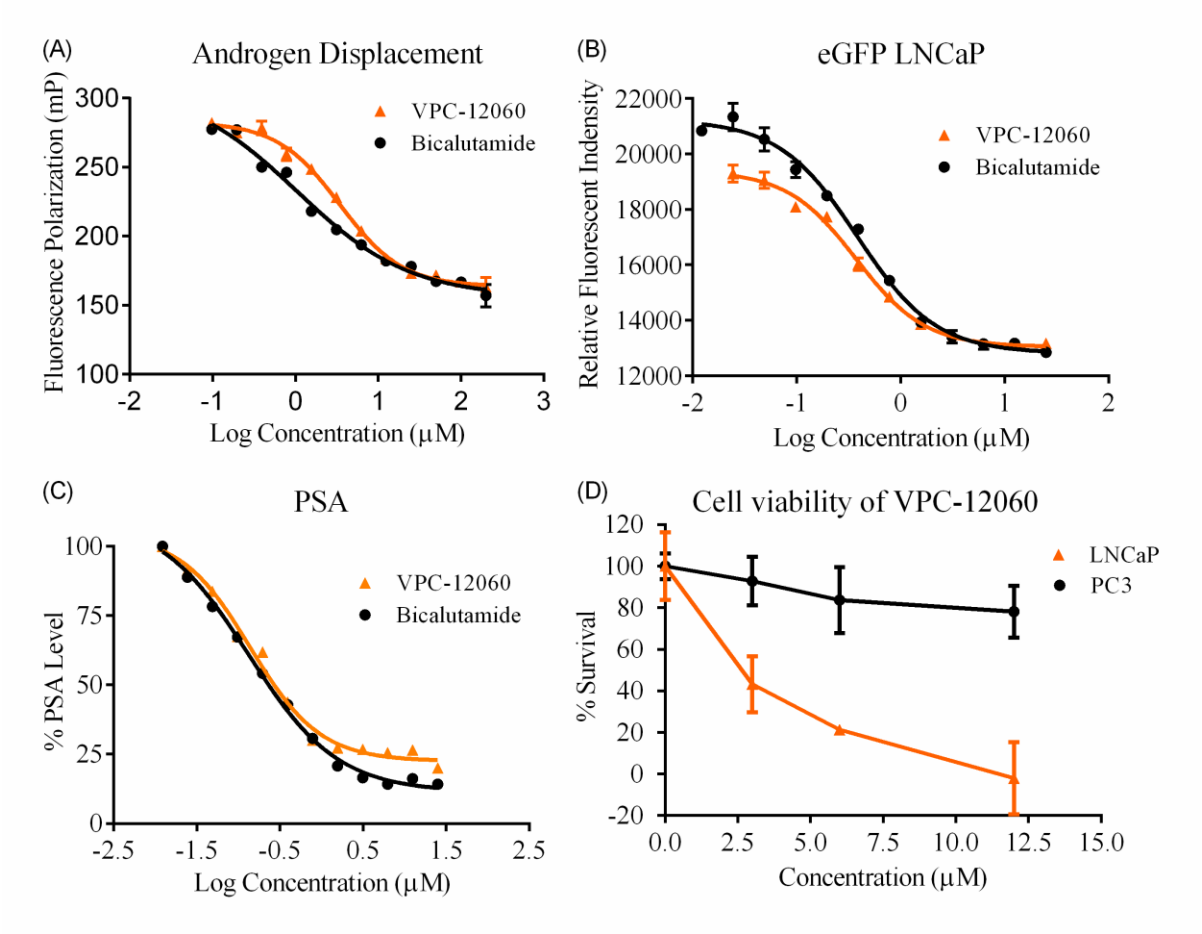


Figure 3.16. Dose-response curves of VPC-12060.

(A) Androgen displacement activity using a fluorescence polarization assay; (B) AR transcriptional inhibition by an eGFP AR transcription essay in LNCaP cells; (C) PSA suppression; (D) cell viability using MTS assay.

Molecular Docking Analysis of Hit Compounds.

The identified non-steroidal AR binders are structurally distinct from current AR antagonists as shown by the tanimoto coefficient in **Table 3.6**, and demonstrated sufficient binding to the AR ABS. To understand the essential features responsible for the potency of these compounds, the receptor-ligand interaction at the binding site was examined based on

the docked poses. The AR ABS is well-characterized as a hydrophobic cavity that forms strong hydrophobic interactions with a steroidal core of androgens. In that prospective, the identified compounds all possess hydrophobic scaffolds, and the docked poses of these compounds adopt similar orientations in the AR ABS compared to crystallographic ligands (**Figure 3.17**). As observed from the figure, polar groups of VPC-12060 form hydrogen bonds with Leu704. For the majority of identified compounds there is only one polar group capable of forming hydrogen bond interactions with the AR ABS residues. The binding modes of these compounds suggest that van der Waals interactions may be essential for the ligand coordination, and consistent with the observation from PLIF and MD simulation, the hydrogen bonding may not play a determining role.

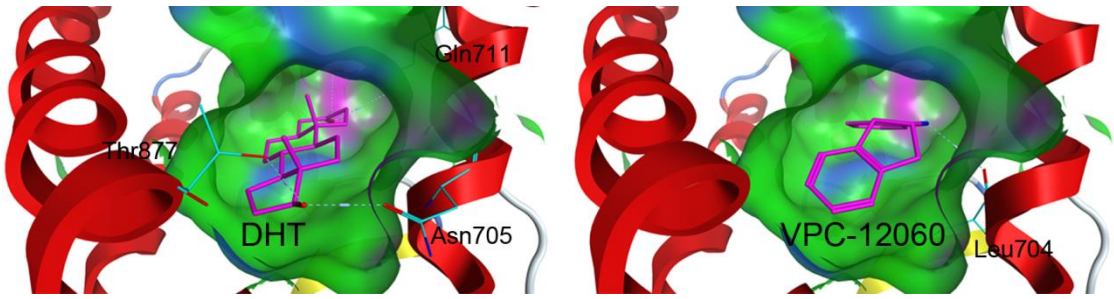


Figure 3.17. The molecular binding modes of DHT and VPC-12060 in the AR ABS. The surface of AR ABS is mainly hydrophobic (green) with some polar residues (magenta). DHT forms two pairs of hydrogen bonds with polar residues, while these contacts are missing in the binding of VPC-12060 with AR ABS.

3.3 Discussion

As the resistance invariably develops to current antiandrogens in PCa, even to the newly approved second-generation Enzalutamide, novel AR antagonists with new scaffolds and mechanism of action are greatly needed. In this work, two approaches using structure- as

well as structure- and ligand-based VS were performed to identify small-molecule inhibitors for the human AR, which can effectively interact with AR ABS and possess chemical scaffolds that are significantly different from currently used antiandrogens. Following experimental evaluation of virtual hits, a number of active AR antagonists were identified.

Surprisingly, the compound 3-hydroxy-10-benzylidene-10*H*-anthracen-9-one (VPC-3033), was found to not only effectively interact with the AR ABS and exhibit a profound ability to inhibit the growth of PCa cells, but also cause degradation of AR in PCa cells. The remarkable inhibitory effect on cells that have developed resistance to the second-generation antiandrogen Enzalutamide,[57] indicated its potential as a possible second line therapy following the development of resistance to current antiandrogens. Importantly, the chemical scaffold of 10-benzylidene-10*H*-anthracen-9-one is very different from structures of currently used antiandrogens. When this compound was evaluated in a tumor xenograft model, a significant effect on the suppression of tumor growth was seen compared to the vehicle control.

In conclusion, the established *in vitro* and *in vivo* inhibitory activity of the lead VPC-3033 from this study makes this compound an excellent prospective antiandrogen. The preliminary structure-activity relationship information obtained around its analogues may serve as a useful basis for the development of an entirely new class of drugs for CRPC.

Chapter 4: Development of AR DBD Inhibitors

4.1 Background and Rationale

4.1.1 The Need for AR Inhibitors with Novel Mechanism of Action

The AR LBD is a very compelling target site with confirmed binding groves for small-molecules, including the conventional ABS targeted by clinically used antiandrogens, as well as AF2 and BF3 sites targeted for disrupting protein-protein interactions of the AR. Though being an appealing target, the AR LBD is associated with the following therapeutic limitations: 1) The AR antagonism is quite complicated and there is no clear picture yet;[191, 219] 2) there exists a high probability of resistant mutations in AR LBD;[82, 83] 3) the emergence of ARVs lacking the entire LBD drives resistance to current antiandrogen drugs, suggesting AR inhibitors targeting the ABS, AF2 or BF3 of the LBD may be all ineffective to ARVs.[125, 127] Due to these limitations, novel strategies are needed to target AR beyond the LBD.

The AR NTD represents a major transactivation domain, so targeting the NTD will effectively block the transactivation of both full-length AR and ARVs.[92, 93] However, since the NTD is intrinsically disordered and there are no successful AR NTD drugs available yet, it is not viable to perform either a structure- or ligand-based rational drug discovery. In addition, mutations in AR NTD frequently occur, which may also induce a mutation-based resistance to NTD inhibitors. Taking all these limitations into account, the AR LBD or NTD are not ideal drug targets, and new strategies with a novel mechanism of action are needed to develop effective inhibitors to avoid the mutation and ARVs-related resistance.

4.1.2 AR DBD as a Drug Target

The DBD is the most conserved functional domain among SHRs, and it is composed of two alpha helices packed in a perpendicular fashion via their hydrophobic faces. A recognition helix consisting of a P-box region inserts directly into the major groove of the conserved ARE half-site, forming crucial substructure for DNA binding. Residues Lys580, Arg585, and Val581 in the recognition helix form key hydrogen-bonding and van der Waals interactions with the DNA. The D-box region in AR DBD is responsible for the DBD dimerization through a network of hydrogen bonding interaction through Ala596, Ser597 and Thr592, which is also important for AR transcriptional activity and gene regulation.[41]

We found the AR DBD represents a suitable target capable of addressing unmet medical need, and postulated that targeting AR DBD may provide an effective way for treating CRPC for the following reasons. First of all, the AR DBD is involved in a key biological pathway, as the DBD dimerization and binding to DNA via DBD are critical for the normal functioning of both full-length AR and ARVs.[220] Secondly, targeting AR DBD will help circumvent resistance from gain-of-function mutations as well as ARVs.[96] Mutations are rarely found in AR DBD, and mutations in DBD generally render loss of transcription possibly due to disruption of DBD-DNA binding. The binding to DNA via DBD is a critical step for both full-length AR and ARVs, thus targeting the AR DBD through disrupting the DBD-DNA binding may promise the inhibition of all forms of the AR. Thirdly, the AR DBD is functionally characterized, and structurally solved with the availability of the x-ray crystal structure (PDB code: 1R4I, **Figure 4.1**),[41] which provides a foundation for the discovery of novel small-molecule inhibitors through a rational, structure-based drug design approach. The AR DBD was not previously studied for drug discovery, and is distinct from

any previously studied targets, including the AR ABS, AF2, BF3, and NTD. Thus, targeting AR DBD supports a favorable intellectual property situation, and more importantly, it may open a whole new area for developing CRPC therapeutics.

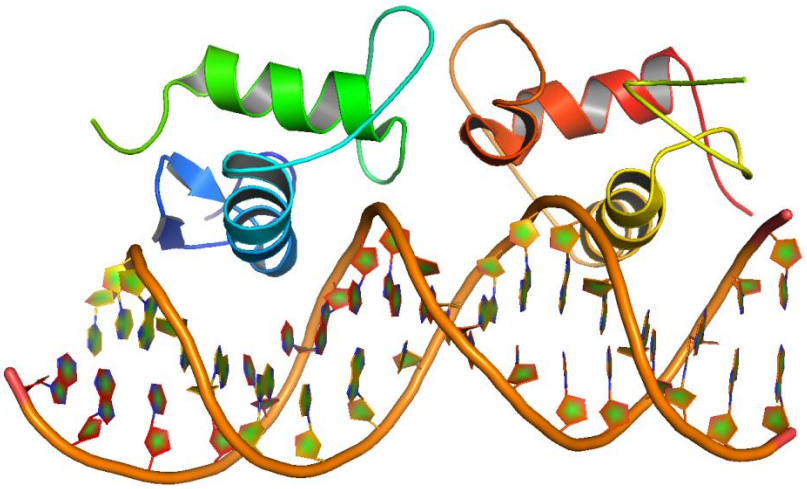


Figure 4.1. Crystal structure of AR DBD with DNA (PDB code: 1R4I).

4.1.3 Disruption of Protein-DNA Interaction

Diversified DNA binders could interrupt with the protein-DNA interaction and transcription through covalent or noncovalent binding, such as DNA intercalators and minor groove binders.[221, 222] However, it is difficult to achieve specificity for DNA binders and most of them have significant side effects. Thus, it is important to develop small-molecule inhibitors that directly and specifically bind to the protein and inhibit the transcription through disrupting the protein-DNA interaction.

We studied the AR DBD crystal structure using computational tools, which suggested plausible binding sites in the AR DBD, including one cavity underneath the P-box region. To interrupt the AR function and inhibit transcription using a novel mechanism of action, we proposed to target the AR DBD with small-molecule inhibitors by disrupting the DBD-DNA

interaction. Disruption of protein-DNA interactions by small-molecule inhibitors is generally considered as very challenging and undruggable interaction due to high protein-DNA binding affinity and big, flat binding interface.

However, there exist successful examples of small-molecule inhibitors binding to the protein and disrupting protein-DNA interactions,[223-225] which demonstrated that it is doable to disrupt protein-DNA interaction by small-molecule inhibitors. The Signal Transducer and Activator of Transcription 3 (STAT3) inhibitors such as flavopiridol and galiellalactone were reported to directly binding to STAT3 and prevent protein-DNA binding.[224, 225] In addition, a series of small-molecule inhibitors of xeroderma pigmentosum group A (XPA) were confirmed to inhibit the XPA-DNA binding.[223] These studies provided confidences for our hypothesis of targeting AR DBD to potentially disrupt the DBD-DNA binding.

4.2 Results

4.2.1 Site Identification on AR DBD

The rat AR DBD dimer bound to two hexameric half-site AREs (PDB code: 1R4I) is the only crystal structure of the DBD region of the receptor available to date.[41] As the sequences of rat and human AR DBDs are identical, the 1R4I structure was used as a template to build a homology model of the human AR DBD. The "hot spots" on the AR DBD dimer-ARE complex were predicted by a Site Finder module within MOE 2011. A cavity underneath the P-box region of the AR DBD was considered as a potential site for small-molecule binding which may disrupt the AR DBD-ARE complex (**Figure 4.2**). This cavity is mainly enveloped by residues Ser579, Val582, Phe583 and Arg586 of the recognition helix, as well as by polar residues Gln592 and Tyr594 belonging to the lever arm loop, and residues Pro613 and Arg616 from the other α-helix, together with the loop residues

Arg609 and Lys610. The site is solvent exposed with specific residues predicted to play a key role in anchoring possible binding of small molecules. Thus, polar residues Ser579, Gln592 and Try594 around the periphery of the site could be characterised as available for hydrogen bonding, whereas Phe583 in the core of the site may provide additional hydrophobic interactions with potential binders.

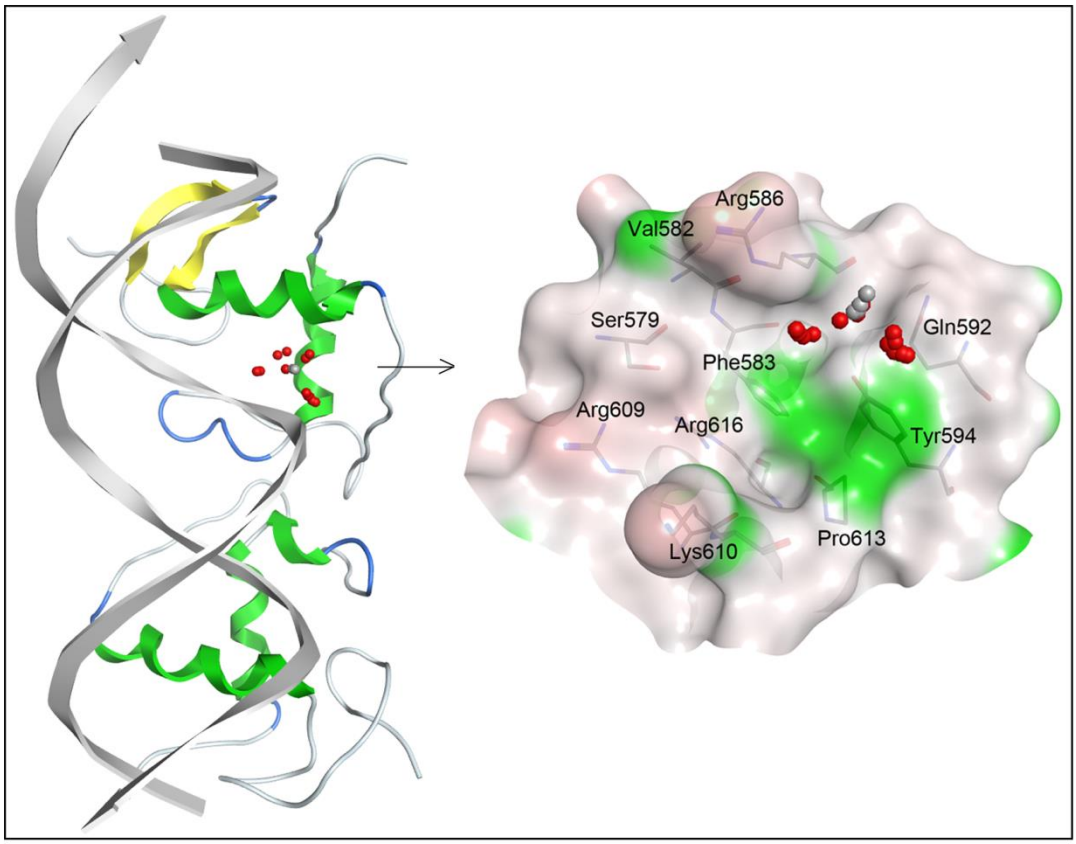


Figure 4.2. Predicted binding site on the human AR DBD.

The human AR DBD was homology modeled using the rat AR DBD (PDB code: 1R4I) as a template. This site was enveloped by residues Ser579, Val582, Phe583, Arg586, Gln592, Tyr594, Arg609, Lys610 and Pro613 in human AR DBD. The green and pink surfaces indicate hydrophobic and polar area, respectively.

4.2.2 MD Simulation of the AR DBD

As the AR DBD monomer was used for the site detection for VS against the intended site, the monomer conformation is important for further investigation. MD simulation was performed on a subunit of the AR DBD-dimer to investigate if the monomer retains the conformation. The force field parameters for the two zinc ions in the AR DBD were generated using the Zinc AMBER force field (ZAFF). After the simulation, it was found that the overall geometry is reserved (**Figure 4.3**), indicating the crystal structure may represent the AR monomer in solution.

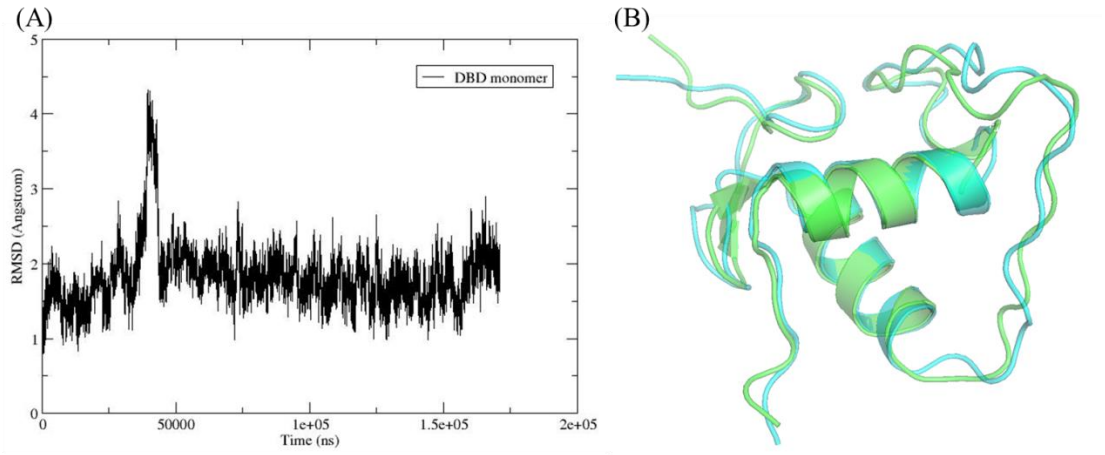


Figure 4.3. The MD simulation results of AR DBD monomer.

(A) The RMSD of the backbone of residues between the simulated structure and the crystal structure during the whole simulation process; (B) the superimposition of the average structure after simulation and the crystal structure.

4.2.3 Hit Identification and Optimization of AR DBD Inhibitors

A stepwise workflow for the discovery of AR DBD inhibitors is summarized as below (**Figure 4.4**). The discovery process started with *in silico* screening against the detected site

for the identification of hit compounds, followed with an iteration of computational prediction and biological evaluation in the hit to lead optimization.

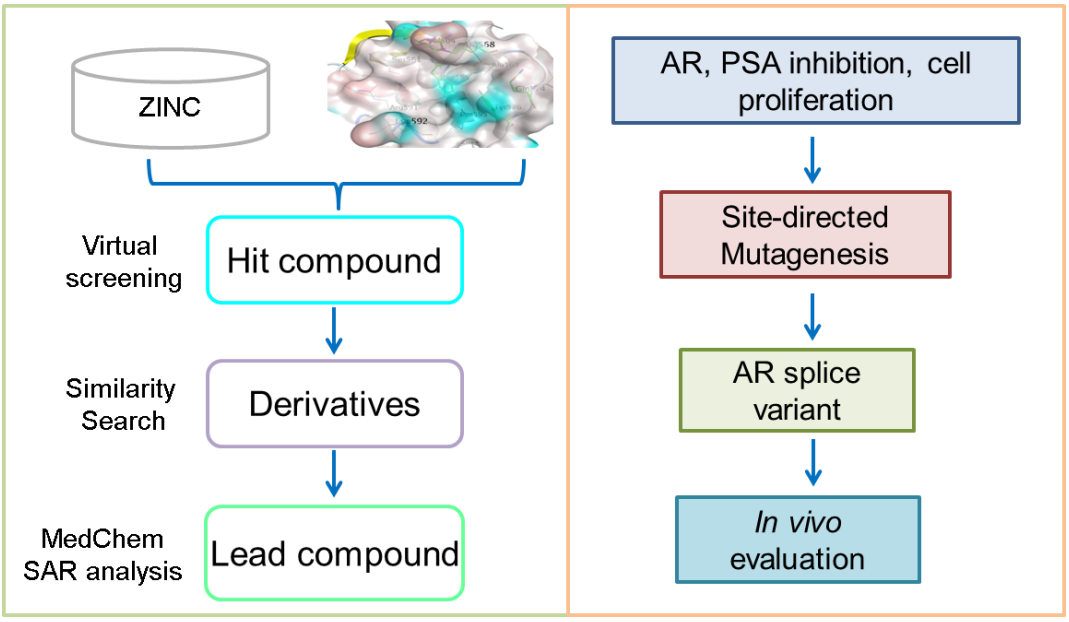


Figure 4.4. Pipeline for the discovery of AR DBD inhibitors.

The *in silico* workflow is presented in left panel, and the biological evaluation and characterization using in vitro and in vivo assays are in the right panel.

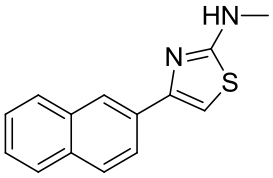
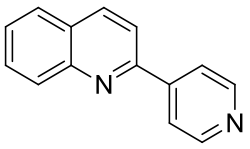
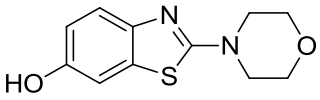
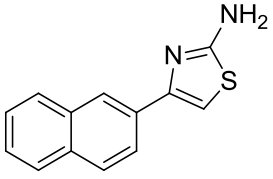
"*In Silico*" Identification of Hit Compounds Targeting the AR DBD Site

A subset of ZINC database,[172] containing approximately 3 million purchasable chemicals, was screened against the identified binding site in the AR DBD using docking program Glide.[173, 174] After filtration by the generated docking score, ligand efficiency, and physicochemical properties, top 5,000 ranked structures were clustered using the fingerprint method implemented by MOE, and a final selected set of 48 chemicals was purchased.

The purchased compounds were then assessed using the eGFP AR transcription assay,[180] which quantifies the AR-driven transcriptional activity in LNCaP cells. Compounds which inhibited AR activated transcription by more than 40% at 3 μM administration, were

further evaluated to obtain corresponding IC_{50} values. To avoid possible false positive detection by the eGFP assay, the complementary PSA screen was employed for most active molecules to confirm their AR inhibitory effect. As the result, five hits corresponding to three different chemical scaffolds were identified (**Table 4.1**) showing moderate AR and PSA inhibition with corresponding IC_{50} values around 10µM. Among these, molecule **VPC-14203** (compound 1) demonstrated the most potent inhibition of both AR transcription (eGFP IC_{50} = 3.17 ± 0.3 µM) and PSA expression (PSA IC_{50} = 3.91 µM) (**Figure 4.5**), and was selected as the starting structural template for similarity search.

Table 4.1. Initial hits of AR DBD from VS.

ID	Structure	eGFP IC_{50} (µM)	PSA IC_{50} (µM)
1 (VPC-14203)		3.17 ± 0.3	3.91
2		4.20 ± 0.6	2.26
3		7.41 ± 0.4	8.08
4		9.16 ± 0.5	10.6

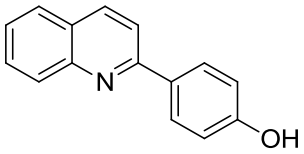
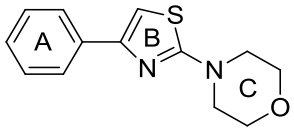
ID	Structure	eGFP IC_{50} (μM)	PSA IC_{50} (μM)
5		9.84 ± 3	N/A

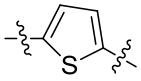
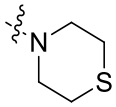
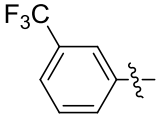
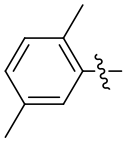
Figure 4.5. Dose-response curves of the hit VPC-14203 (1) in eGFP and PSA assays. (A) LNCaP eGFP cells were treated with VPC-14203 at various concentrations (0.2-100 μM) for three days in the presence of 0.1 nM R1881, and AR transcriptional inhibition of compounds was evaluated by measuring the fluorescence. (B) The PSA suppression by VPC-14203 was evaluated by measuring the PSA secreted into the media using the same LNCaP eGFP cells.

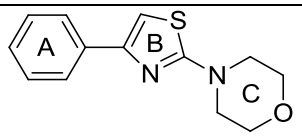
Subsequent similarity search was conducted on a full version of the ZINC database, containing about 12 million entries, and molecular docking was employed to position identified analogues into the AR DBD binding site. The conducted hit-based similarity

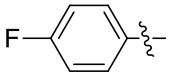
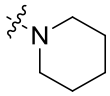
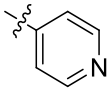
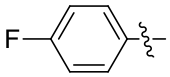
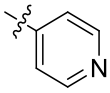
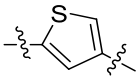
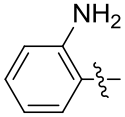
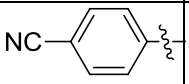
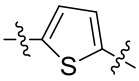
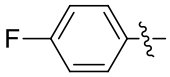
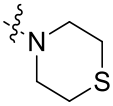
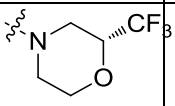
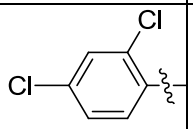
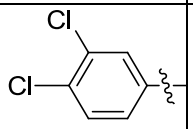
search resulted in the identification of a series of 4-(4-phenylthiazol-2-yl)morpholine analogues, with 18 active compounds of corresponding IC_{50} (eGFP) values below 10 µM, all listed in **Table 4.2** (23 additional chemicals with lower activities were also presented in **Table 4.3**). Among the identified similarity-based hits, compound VPC-14228 (**6**, 4-(4-phenylthiazol-2-yl)morpholine) demonstrated complete inhibition of AR-mediated transcription at a dose of 3 µM. When subjected to a concentration-dependent titration, compound VPC-14228 exhibited an IC_{50} (eGFP) value of 0.33 ± 0.12 µM, corresponding to a 10-fold improvement compared to the parental chemical VPC-14203. The PSA expression was reduced by compound VPC-14228 with the corresponding IC_{50} value of 0.28 µM, confirming its potent AR inhibitory effect (**Figure 4.6**).

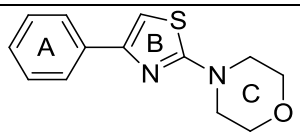
Table 4.2. Analogues of VPC-14228 from hit-based similarity search.



ID	A ring	B ring	C ring	eGFP IC_{50} (µM)	PSA IC_{50} (µM)
6 (VPC-14228)				0.33 ± 0.12	0.28
7				0.52 ± 0.03	0.51
8				0.62 ± 0.06	N/A
9				0.61 ± 0.02	0.58
10				0.62 ± 0.06	0.52



ID	A ring	B ring	C ring	eGFP IC_{50} (μM)	PSA IC_{50} (μM)
11				0.65 ± 0.06	0.41
12				1.03 ± 0.03	0.69
13				1.20 ± 0.23	0.63
14				1.27 ± 0.32	N/A
15				1.24 ± 0.10	0.84
16				1.38 ± 0.19	2.03
17				1.44 ± 0.04	0.89
18				1.59 ± 0.13	N/A
19				1.65 ± 0.11	0.16
20				1.66 ± 0.12	1.32
21				3.32 ± 0.16	2.96



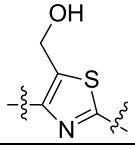
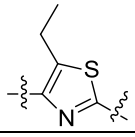
ID	A ring	B ring	C ring	eGFP IC_{50} (μM)	PSA IC_{50} (μM)
22				5.04 ± 0.19	N/A
24				5.80 ± 0.38	8.43

Figure 4.6. Dose-response curves of VPC-14228 in eGFP and PSA assays.

(A) LNCaP eGFP cells were treated with VPC-14228 and Enzalutamide at various concentrations (0.006-3.125 μM) for three days in the presence of 0.1 nM R1881 and the AR transcriptional inhibition of compounds was evaluated by measuring the fluorescence. (B) The PSA suppression by these compounds was evaluated by measuring the PSA secreted into the media using the same LNCaP eGFP cells.

Structure-Activity Relationship (SAR) for 4-(4- Phenylthiazol-2-yl)Morpholines.

The predicted binding mode of compound VPC-14228 inside the AR DBD binding site implied that the morpholine group could form hydrogen bond interaction with Tyr594 and the thiazole ring is engaged in hydrophobic contacts with Val582 above and Phe583 below. The pair of Arg609 and Lys610 forms a clamp-like shape, anchoring the phenyl ring through hydrophobic interactions by their aliphatic side chains (**Figure 4.7**). The docking configuration of 4-(4-phenylthiazol-2-yl)morpholines in the AR DBD target site and the corresponding established *in vitro* activity allowed developing the preliminary structure-activity relationship (SAR) on this series.

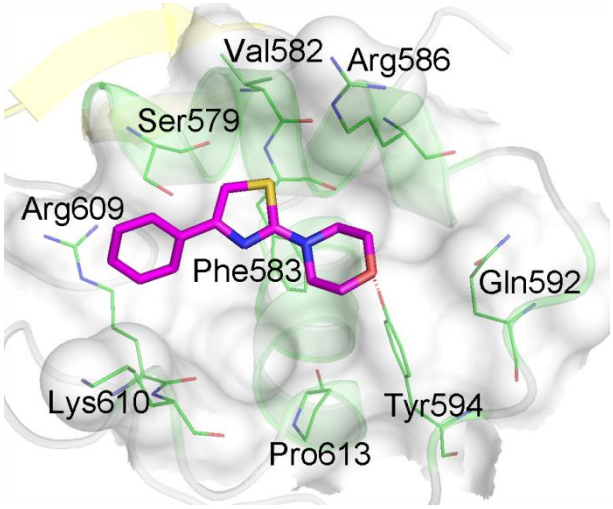


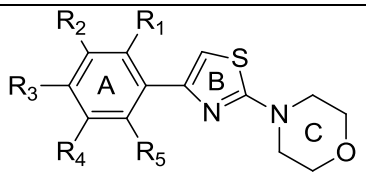
Figure 4.7. Binding mode of VPC-14228 in the AR DBD binding site.

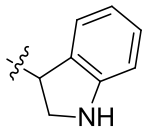
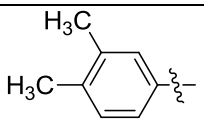
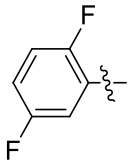
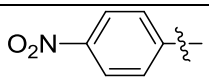
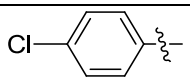
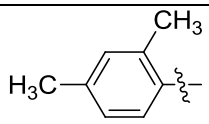
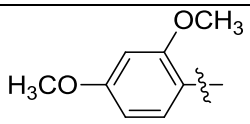
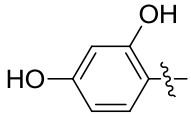
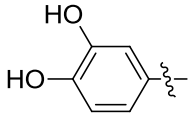
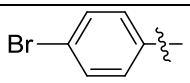
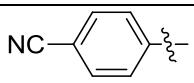
Thus, anti-AR activities of compound VPC-14228 analogues appeared to be rather sensitive to structural changes on the phenyl ring, especially at its para-position. The activity was almost completely abolished by bulky and hydrophobic chemical groups, such as $-C_2H_5$, $-OCH_3$ and $-SO_2CH_3$ (59, 60, 61 in **Table 4.3**) on that position. According to the docked pose, the para- position substituents of the ligands are solvent-exposed, and unfavourable solvation

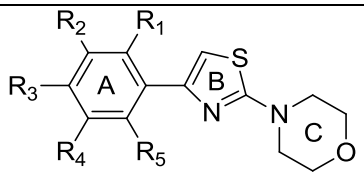
of bulky and hydrophobic groups is likely to account for the activity loss. This trend also maintains for halogen substitutions in the para position. When adding -F, -Cl and -Br to para-position (11, 50, 55 in **Table 4.2** and **Table 4.3)**, the percentage of inhibition at 3 μM decreases in the series, as the bulk and hydrophobicity of a substituent increases. Although -F group was tolerated (11 in **Table 4.2**), the activity dropped about 2-fold compared to the unsubstituted 6. Bulky groups at the ortho- and meta- positions may form hydrophobic clamp with the Arg609 and Lys610, which seem to be tolerated by the ligands, although their activity also decreases somewhat (9, 10, 20, 21 in **Table 4.2**).

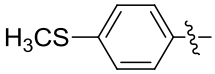
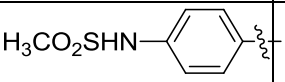
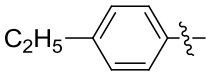
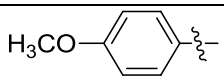
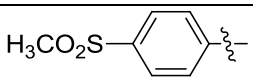
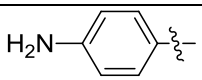
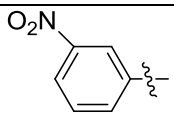
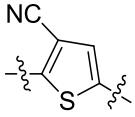
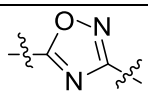
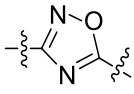
It was observed that the addition of substituents to the thiazole ring such as -CH_3 led to decreased activity (22, 24 in **Table 4.2)**. When the thiazole was replaced with other heterocyclic rings, such as thiophene, the resulting activity was reduced while still retaining in submicromolar level (15, 17 in **Table 4.2**), and the oxadiazole replacement abolished the activity completely (65, 66 in **Table 4.3**). According to the docked pose of these analogues, the morpholine ring forms a hydrogen bond with the hydroxyl group of Tyr594 in the active site (**Figure 4.7**). To capitalize on this observation, a hydrogen bond acceptor was incorporated in the form of thiomorpholine and pyridine groups as an alternative to maintain H-bonding to Tyr594. Such replacement allowed retaining the activity of derivatives in low- and sub-micromolar range (8, 13 in **Table 4.2**), confirming the crucial role of H-bonding anchoring to the Tyr594 residue in the AR DBD target site.

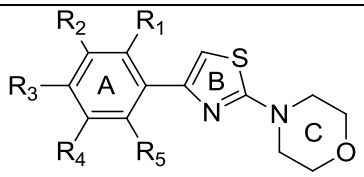
Table 4.3. Hit-based analogues with IC_{50} (eGFP) values above 10uM.

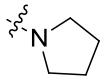
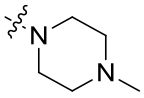
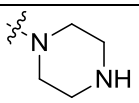
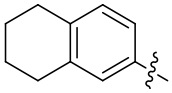
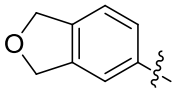


ID	A ring	B ring	C ring	%Inhibition at 3 μM	eGFP IC_{50} (μM)
46					10.14
47					10.31
48					10.45
49					15.80
50					39.86
51				<40	
52				<40	
53				<40	
54				<40	
55				<40	
56				<40	



ID	A ring	B ring	C ring	%Inhibition at 3 μM	eGFP IC_{50} (μM)
57				<40	
58				<40	
59				<40	
60				<40	
61				<40	
62				<40	
63				<40	
64				<40	
65				<40	
66				<40	



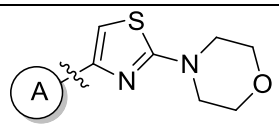
ID	A ring	B ring	C ring	%Inhibition at 3 μM	eGFP IC_{50} (μM)
67				<40	
68				<40	
69				<40	
70				<40	
71				<40	

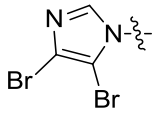
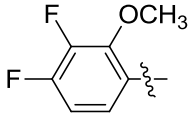
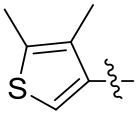
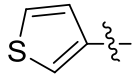
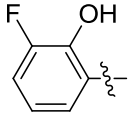
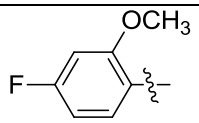
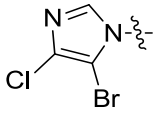
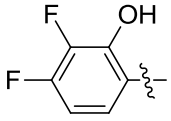
Rational Design, Synthesis, and Evaluation of 4-(4-Phenylthiazol-2-Yl)Morpholine Derivatives.

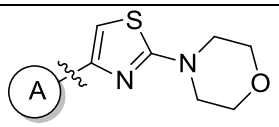
Based on the above SAR considerations, medchem investigations were conducted using compound VPC-14228 as a parental structure. As modifications on the phenyl ring could remarkably change the activity, derivatives of compound VPC-14228 were designed by modifying the phenyl ring or replacing the phenyl ring with heteroaryl groups. A total of 34 derivatives of VPC-14228 (listed in **Table 4.4** and **Table 4.5**) were ordered from Life Chemicals (http://www.lifechemicals.com/) and Enamine (http://www.enamine.net) companies. These efforts resulted in the creation of 21 active AR inhibitors (eGFP IC_{50} < 10

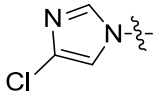
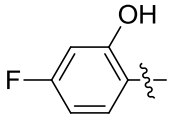
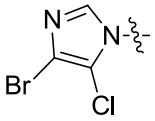
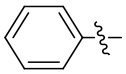
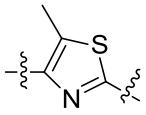
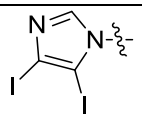
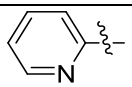
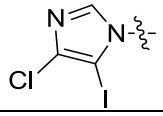
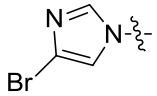
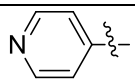
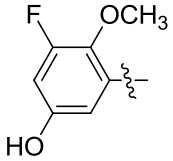
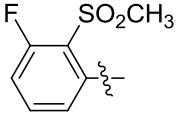
μM), with 6 of them demonstrating superior anti-AR potency compared to the template compound VPC-14228.

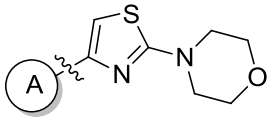
Table 4.4. Synthesized derivatives against AR DBD.



ID	Ring A	Ring B	eGFP IC_{50} (μM)	PSA IC_{50}(μM)
25 (VPC-14449)			0.10 ± 0.05	0.17
26			0.18 ± 0.01	0.25
27			0.25 ± 0.05	0.43
28			0.26 ± 0.02	0.22
29			0.27 ± 0.04	0.15
30			0.30 ± 0.02	0.23
31			0.33 ± 0.01	0.44
32			0.68 ± 0.01	0.57
33			0.80 ± 0.06	0.70



ID	Ring A	Ring B	eGFP IC_{50} (μM)	PSA IC_{50}(μM)
34			0.96 ± 0.09	0.72
35			1.19 ± 0.68	0.81
36			1.45 ± 0.42	1.28
37			1.46 ± 0.31	N/A
38			1.73 ± 0.66	1.81
39			2.58 ± 0.31	N/A
40			2.61 ± 0.49	4.02
41			2.80 ± 1.07	1.74
42			2.94 ± 0.86	N/A
43			3.02 ± 0.32	N/A
44			3.56 ± 0.42	N/A



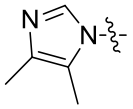
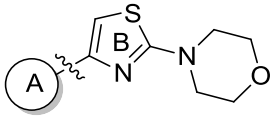
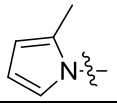
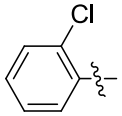
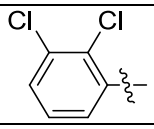
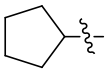
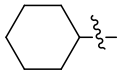
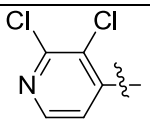
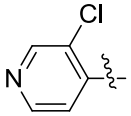
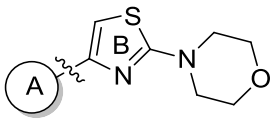
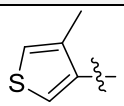
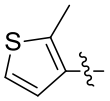
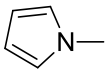
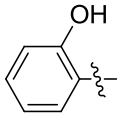
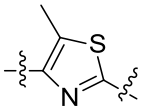
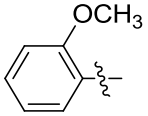
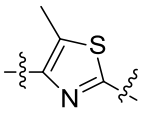
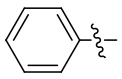
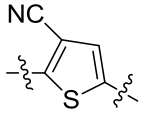
ID	Ring A	Ring B	eGFP IC_{50} (μM)	PSA IC_{50}(μM)
45			3.56 ± 0.42	2.42

Table 4.5. Synthetic derivatives with IC_{50} (eGFP) values above 10uM (structural variations at ring A and B).



ID	Ring A	Ring B	%Inhibition at 3 μM
72			45.00
73			45.04
74			<40
75			<40
76			<40
77			<40
78			<40



ID	Ring A	Ring B	%Inhibition at 3 μM
79			<40
80			<40
81			<40
82			<40
83			<40
84			<40

In particular, a synthetic analogue VPC-14449 (compound 25), bearing 4,5-dibromo-imidazole, displayed 2-fold improved activities (eGFP IC_{50} = 0.12 ± 0.01 μM; PSA IC_{50} = 0.17 μM, **Figure 4.8**) compared to compound VPC-14228, reaching the level of activity of the latest FDA-approved PCa drug, Enzalutamide (eGFP IC_{50} = 0.11 ± 0.01 μM; PSA IC_{50} = 0.12 μM). With such promising inhibitory activity towards AR transcription and PSA expression, we further evaluated the ability of compound VPC-14449 to inhibit the growth of AR-dependent PCa cells in the AR-positive LNCaP system. Following hormone activation of the AR (0.1 nM R1881), compound VPC-14449 elicited a concentration-dependent inhibition of the cell growth. A similar potency for cell-growth inhibition was achieved when VPC-14449 were evaluated against the newly developed Enzalutamide-resistant cell line, MR49F.[28] Importantly, compound VPC-14449 were ineffective in inhibiting the proliferation

of AR-negative PC3 cells, supporting the mechanism of its action through specific interaction with the AR rather than by means of generic toxicity.

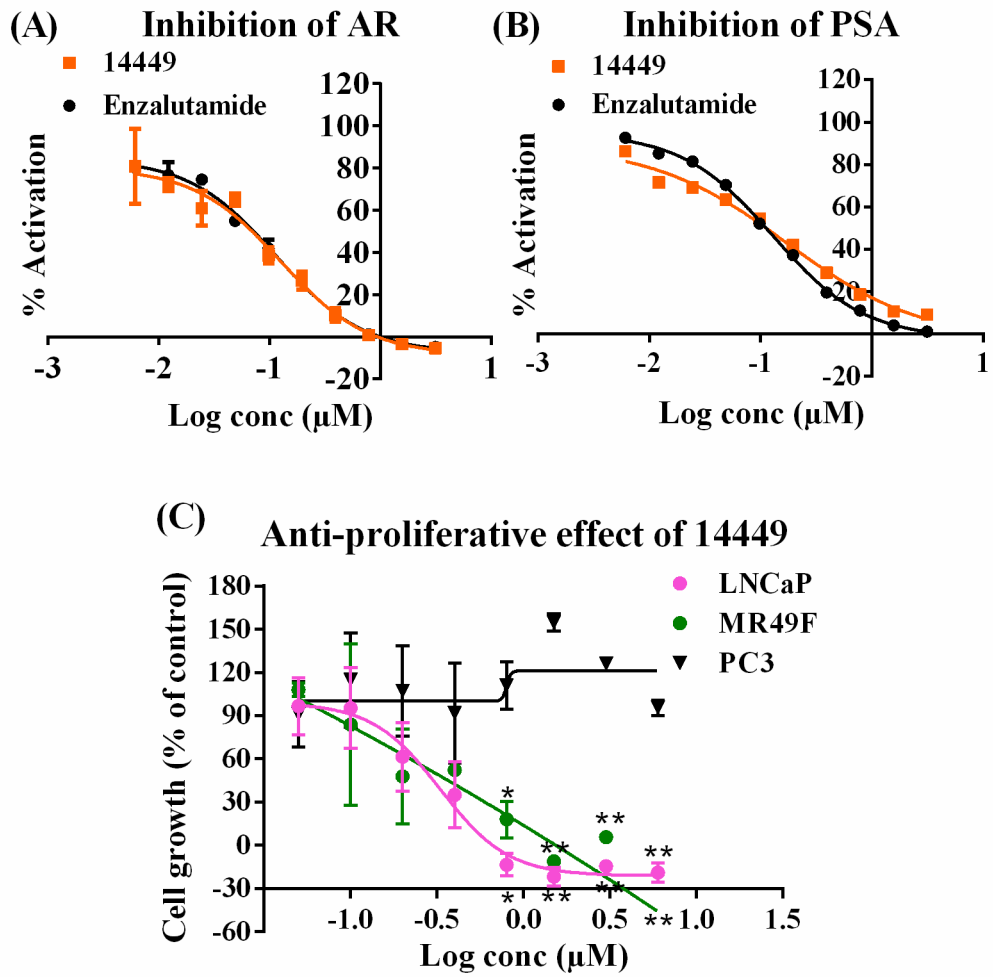


Figure 4.8. Dose-response curves of compound 6 (VPC-14449).

(A) The AR inhibitory activity of Compound VPC-14449 in comparison with Enzalutamide using LNCaP eGFP cells in the presence of 0.1 nM R1881. The AR transcriptional inhibition of compounds was evaluated by measuring the fluorescence. (B) The PSA suppression by these compounds was evaluated by measuring the PSA secreted into the media using the same LNCaP eGFP cells. (C) The anti-proliferative effect of VPC-14449 on LNCaP, MR49F

(enzalutamide-resistant) and PC3 cells using MTS assay. The LNCaP, MR49F, PC3 cells were treated with the inhibitor at various concentrations for three days in the presence of 0.1 nM R1881.

Consistent with the initial docking hypothesis for compound VPC-14228, compound VPC-14449 adopted the same docking pose in the AR DBD model where it forms similar receptor-ligand interactions (**Figure 4.9**). The morpholine group retained the hydrogen bond interaction with Tyr594, and the thiazole ring maintains the van der Waals interactions with Val582 and Phe583. The 4,5-dibromo-imidazole group intercalates into the clamp-shaped groove formed by Arg609 and Lys610, making hydrophobic interactions. This structural basis was further used to guide the site-directed mutagenesis experiments on key binding residues on the AR DBD.

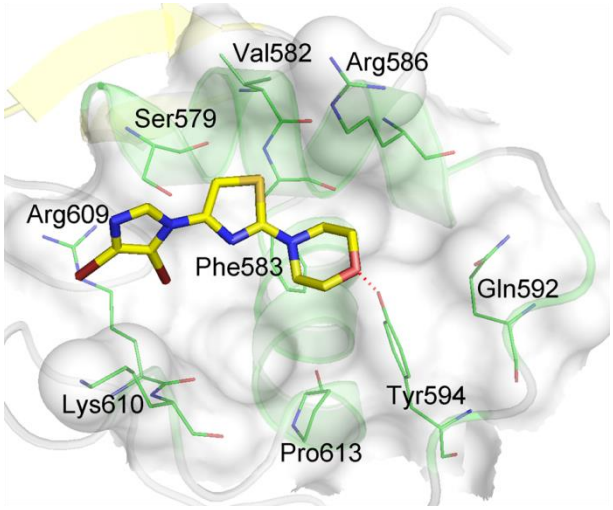


Figure 4.9. Binding mode of VPC-14449 in the AR DBD model.

4.2.4 Confirmation of Developed Compounds Binding to the AR DBD

To prove that the developed DBD inhibitors directly bind to the intended target site on the AR, we designed and constructed amino-acid mutations that may alter the predicted binding site of the compounds while not affecting the AR function. Based on the molecular modeling, residues Ser579, Val582, Phe583, Arg586, Gln592, Tyr594, and Lys610 in human AR DBD are embedded in the binding site and provide important coordinating interactions with the developed ligands. We created aspartic acid point mutations at these amino-acid positions in the full-length human AR, and co-transfected them with an ARR_3tk-luciferase reporter into AR-negative PC3 cells.[226] Among those, two mutants Gln592Asp and Tyr594Asp allowed maintaining the AR transcriptional activity, while the remaining point mutations destroyed the AR function and were not considered further (**Figure 4.10**). Notably, western blots confirmed no change in AR expression levels for all the mutants regardless of their transcriptional activity or presence of our compounds.[227] Compounds VPC-14228 and VPC-14449 were tested on the two active mutants and compared to the luciferase-detected activities obtained with the wild-type AR. As expected, the inhibitory effect of VPC-14228 and VPC-14449 on the AR was diminished by Gln592Asp and Tyr594Asp mutations confirming that that the compounds interact with these residues in the proposed AR DBD target site.

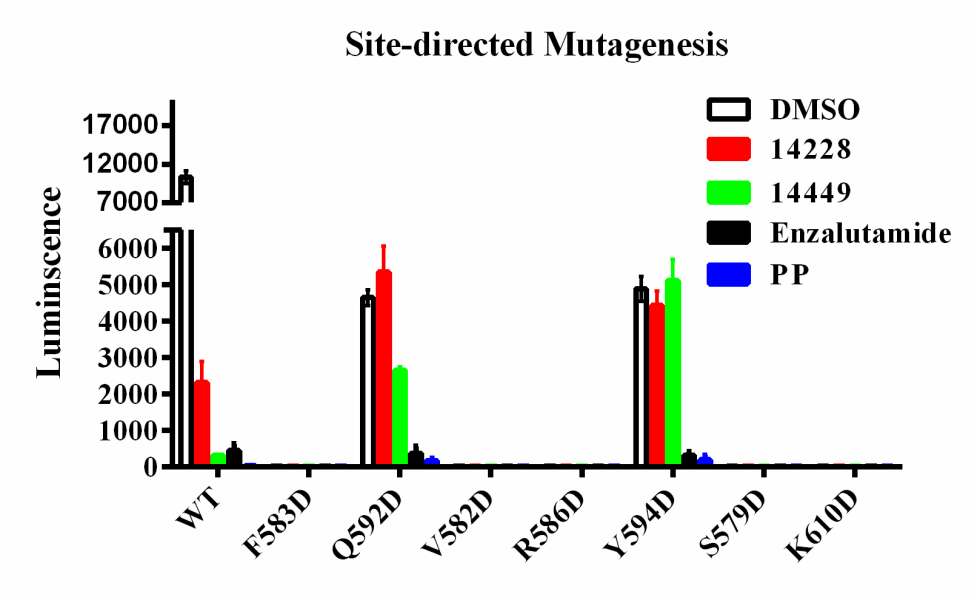


Figure 4.10. Site-directed mutagenesis study on the predicted binding site residues. Wild-type hAR and hAR mutant (S579D, V582D, F583D, R586D, Q592D, Y594D, and K610D) plasmids were transfected with a ARR_3tk-luciferase reporter construct into PC3 cells and the transcriptional activities of tested compounds VPC-14228, VPC-14449 and Enzalutamide at 10 μM were measured based on the luminescence on the AR mutants and wild-type AR.

We next sought to rule out possible interaction of VPC-14228 and VPC-14449 with known functional sites in the AR LBD including the ABS, Activation Function 2 (AF2) and Binding Function 3 (BF3) sites, which are known to interact with various small molecules.[65, 66, 89, 90, 181, 228, 229] To perform this task, we conducted androgen displacement experiments with VPC-144228 and VPC-14449 (**Figure 4.11**), and demonstrated that neither of the chemicals could displace bound androgen from the AR. To exclude binding to the other sites, an *in vitro* biolayer interferometry (BLI) assay using recombinant AR LBD was carried out and also demonstrated the lack of binding of VPC-14228 and VPC-14449 to the LBD,

effectively excluding the possibility of the compounds binding to all known sites on this domain. Together, these findings suggest that the identified compounds act beyond known binding sites in the C-terminal LBD of the receptor.

In addition, we also used BLI to exclude the possibility of these compounds binding to the ARE. The corresponding experiments detected no direct interaction between compounds VPC-14228 and VPC-14449 and the DNA (data not shown)**.**

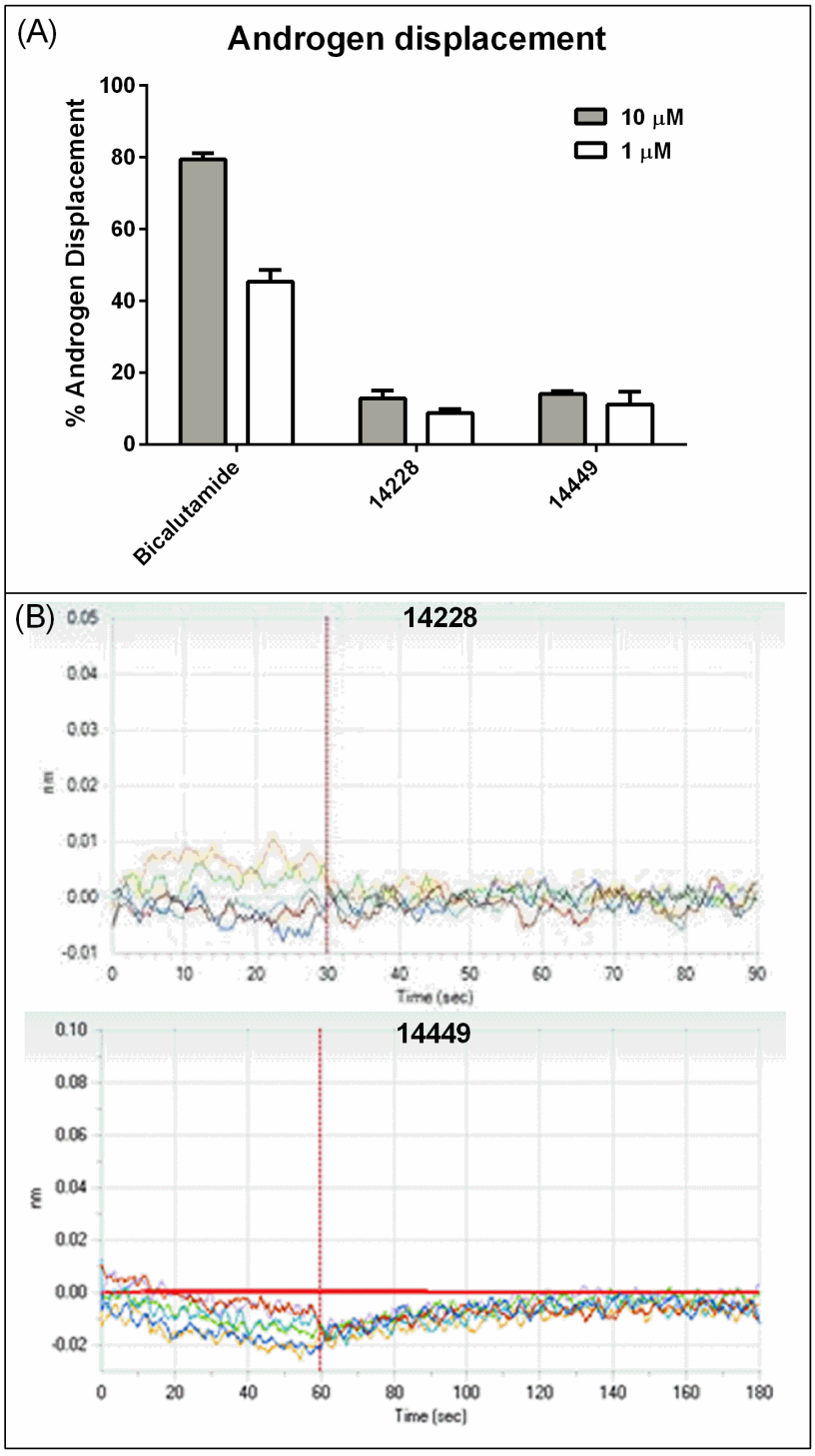


Figure 4.11. AR DBD inhibitors not binding to AR LBD.

(A) Androgen displacement assay was used to measure the direct binding of VPC-14228 and VPC-14449 (50 μM) with androgen binding site with buffer and Bicalutamide (50 μM) as

controls. (E) BLI demonstrates that there is no binding of VPC-14228 and VPC-14449 with AR LBD at different concentrations (3.125-50 μM).

4.2.5 The Developed AR DBD Inhibitors Inhibit ARVs

As the developed AR DBD inhibitors have been confirmed to bind to their intended target area, they were also expected to inhibit the activity of the truncated ARV splice variants, which lack the entire AR LBD and thus, are completely resistant to all currently used antiandrogens including Enzalutamide as illustrated in **Figure 4.12**. To measure whether the developed chemicals can inhibit such ARVs, we co-transfected plasmids encoding for AR-V7 and the ARR3tk-luciferase reporter into AR-negative PC3 cells and evaluated the effects of VPC-14228 and VPC-14449 on the corresponding luciferase expression. As anticipated, both VPC-14228 and VPC-14449 demonstrated effective inhibition of the AR-V7 activity in a concentration-dependent manner, which further validated the developed compounds as effective AR DBD inhibitors. A more detailed study of the action of the identified inhibitors on AR splice variants and in-depth discussion around the developed ARV assays is presented in a specialized publication.[230]

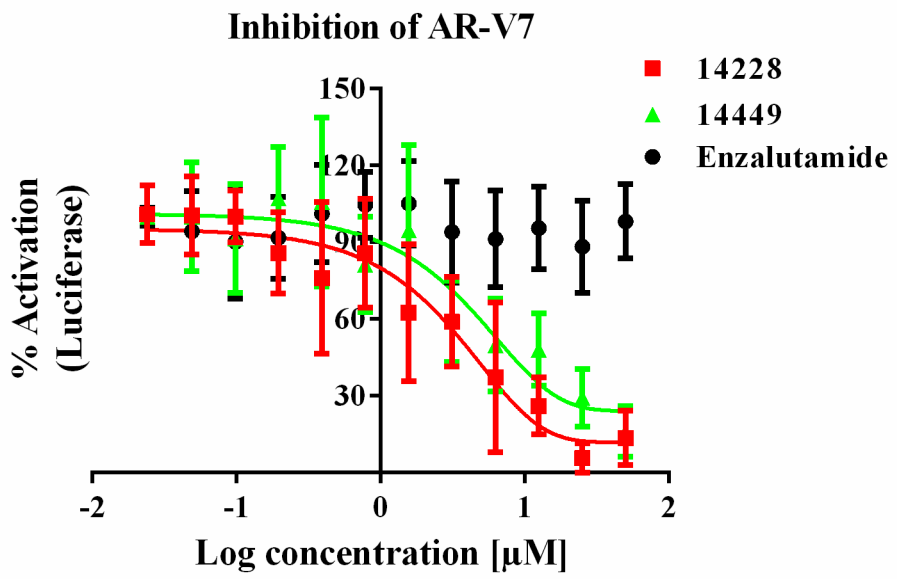


Figure 4.12. AR DBD compounds inhibits the transcription of AR-V7.

Wild-type hAR/ARV7 plasmids were co-transfected into PC3 cells with ARR_3tk-luciferase, and treated with compound VPC-14228, VPC-14449 and Enzalutamide.

4.2.6 Selectivity of the Developed AR DBD Inhibitors.

As the DBDs of steroid nuclear receptors, such as progesterone receptor (PR), glucocorticoid receptor (GR) and estrogen receptor (ER) are highly conserved, compounds targeting this domain may suffer from poor selectivity. However, the sequence alignment of the steroid nuclear receptors reveals distinct amino acid differences highlighted in Figure S3. The Gln592 residue in human AR located in the proposed DBD binding site is different from residues in any other steroid nuclear receptors, which may determine the specificity of small-molecule inhibitors, such as 6 and 25, rationally designed to engage Gln592 into binding (as it has been confirmed by the mutation experiments). Accordingly, when tested against PR,

GR and ER, the developed inhibitors 6 and 25 displayed negligible inhibition of PR or GR, and exhibited only weak cross-activity toward ER (**Figure 4.13**).

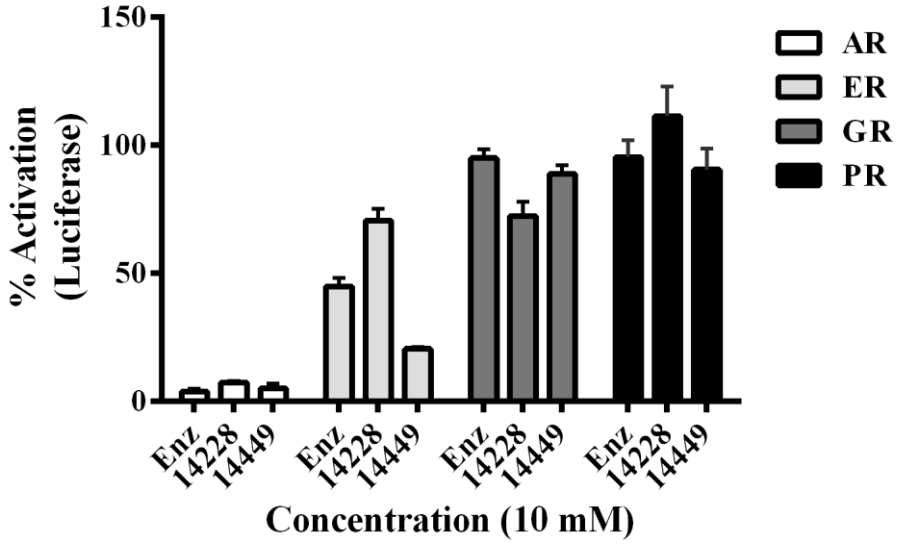


Figure 4.13. The transcriptional inhibition of developed compounds on other SHRs. VPC-14228, VPC-14449 and Enz (Enzalutamide) were measured for their inhibition on AR, ER-α, GR and PR at a concentration of 10 μM.

4.2.7 *In Vivo* Effects of AR DBD Inhibitors

The effect of compound VPC-14449 was evaluated in mice by monitoring the growth of androgen-sensitive LNCaP xenografts. Initial experiments demonstrated no systemic toxicity and doses of up to 100 mg/kg administered i.p. twice daily could be tolerated by the mice with no decrease in body weight over a total duration 4 weeks. The in vivo screening for tumor growth inhibition was initially performed using our previously established castration-resistant tumor xenograft model in castrated hosts. Post-castration, animals were monitored for regrowth of LNCaP tumors and precastration levels of serum PSA, at which point the mice were treated with compound VPC-14449 (100 mg/kg) or enzalutamide (10

mg/kg) twice daily. Tumor volume and serum PSA were effectively suppressed by VPC-14449 in this model comparable with enzalutamide treatment (**Figure 4.14**). The results demonstrate that compound VPC-14449 is as effective as enzalutamide in blocking androgen signaling in vivo. Thus, DBD inhibitor prototypes may yield useful AR targeting drugs that could provide benefit in treating castration-resistant prostate cancer patients.

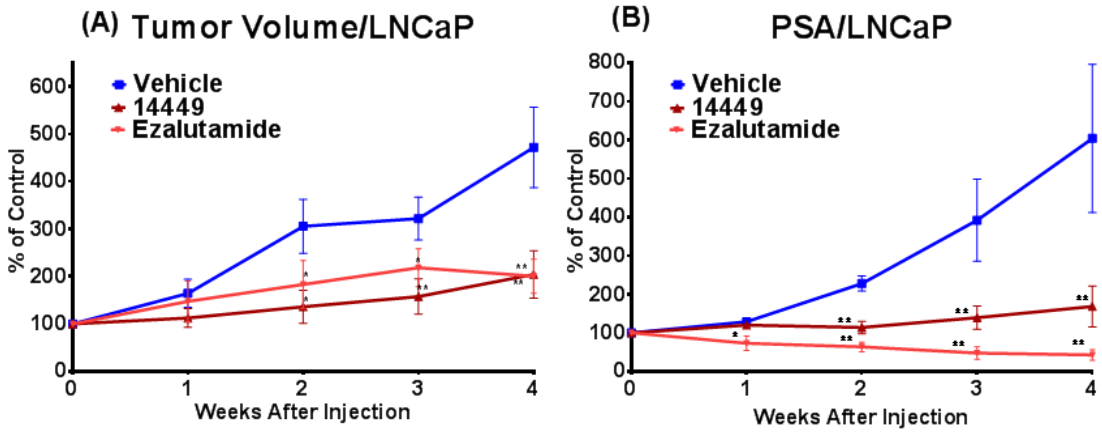


Figure 4.14. The *in vivo* effect of VPC-14449.

VPC-14449 reduces tumor volume and abolishes PSA production in LNCaP xenograft model. Castrated mice were dosed twice daily with VPC-14449 (100 mg/kg) or enzalutamide (Enz) (10 mg/kg) for 4 weeks and assessed for LNCaP xenograft tumor volume (A) and serum PSA (B). Data are presented as mean ± S.E., n = 4. p value < 0.05 was considered significant (*) compared with vehicle control; p value < 0.001 was considered extremely significant (**) compared with vehicle control.

4.3 Discussion

Although the etiology of PCa is largely unknown, aberrant regulation of the transcriptional machinery through AR plays an important role in the progression and

development of PCa. To circumvent existing resistance problem from current antiandrogens, we proposed to target an unexplored domain - the AR DBD by small-molecule inhibitors with a novel mechanism of action. In this study, we identified a binding site in the AR DBD and utilized virtual screening to discover a set of micromolar hits for the target. Through further exploration of the most potent hit VPC-14203, a structural analogue VPC-14228 was identified demonstrating 10-fold improved anti-AR potency. Further optimization resulted in a more potent synthetic analogue VPC-14449 with anti-AR potency comparable to a newly FDA approved drug enzalutamide. Site-directed mutagenesis demonstrated that the developed AR DBD inhibitors do interact with the intended target site. Importantly, the AR DBD inhibitors could effectively inhibit the growth of enzalutamide-resistant cells as well as block the transcriptional activity of constitutively active ARVs, such as ARV7.

Further characterization of developed AR DBD inhibitors demonstrated that different from the conventional antiandrogen enzalutamide, these inhibitors don't impede the nuclear translocation as seen in a confocal microscopy experiment. This suggests the developed inhibitors inhibit AR and its splice variants in the nucleus through directly interacting with AR DBD. Consistent with our hypothesis, it was observed that these inhibitors blocked the DBD-DNA interaction in nucleus demonstrated by the ChIP assay, and in addition, the binding of DNA to DBD was weakened by AR DBD inhibitors.[230] Further lead optimization is ongoing to improve the physicochemical properties, and acquire a better microsomal stability and PK for preclinical study.

There are no preceding studies on targeting the DBD of AR or other nuclear receptors by small-molecule inhibitors. This study provides the first proof-of-concept of targeting the AR DBD by small-molecule inhibitors for the treatment of advanced PCa, which promises a

new field of developing therapeutics using AR DBD as a target. More importantly, this work also demonstrated the DBD is potentially a drugable target, and provided a new avenue of targeting nuclear receptors through a different mechanism of action, which is less likely to be associated with drug resistance.

Chapter 5: Conclusions

5.1 Summary of the Study

This thesis describes the discovery of novel AR inhibitors as prospective therapeutics for advanced PCa using a combination of computer-aided drug discovery and experimental evaluation. We initially targeted the conventional druggable site of the AR ABS, to develop competitive small-molecule inhibitors. Two computational strategies were applied to maximally take advantage of available resources of protein structures and known ligands – a) structure-based alone and b) a combination of structure- and ligand-based approaches. Both strategies generated hit molecules, while hits emerged from campaign b) appeared to be more diverse and potent. Notably, one chemical class of identified ABS inhibitors demonstrated appealing AR degradation profile in multiple PCa cell lines and effective *in vivo* potency to inhibit the tumor growth.

The developed AR ABS inhibitors described in Chapter 3 are structurally distinct from the current antiandrogens, which are expected to help partly address the resistance. However, since mutation-induced antagonist-to-agonist conversion represents a major limitation, new AR ABS inhibitors may encounter the same undesired transformation. The underlying mechanism of mutation-induced transformation is largely unknown, and in addition, the antagonism of AR is unclear yet, which makes it difficult to build a sound predictive model and get rid of agonistic effect. The MD simulations were conducted while it didn't give explicit explanation of the agonism versus antagonism.[81, 219, 231] Thus, continuing efforts are expected to understand the mutation-induced transformation as well as antagonism of antiandrogens. In addition, the synthesis of ARVs lacking the entire LBD was reported to drive resistance to antiandrogens although the biological and clinical significance of ARVs in

PCa is not well-established yet. Thus, it is important to develop therapeutics effective to ARVs. AR inhibitors targeting the LBD are ineffective to ARVs, which drove us to abandon ABS inhibitors and to explore alternative sites for inhibiting both full-length AR and ARVs.

The most novel work described in the thesis is the discovery and development of inhibitors for the DBD portion of the AR outlined in Chapter 4. This study proposed to target the AR function by disrupting DBD-DNA interaction and investigated the AR DBD as a drug target for the first time. Our results demonstrated that the identified compounds directly interact with AR DBD, suggesting this domain of AR is druggable. After structural modifications on the hit compounds, the developed lead chemicals demonstrated comparable potency towards AR inhibition and PSA suppression compared to conventional antiandrogen Enzalutamide. Importantly, the lead compound potently inhibits the growth of Enzalutamide-resistant cells[183] *in vitro* as well as Enzalutamide-resistant xenograft model *in vivo* (unpublished data). Since the developed inhibitors directly bind to AR DBD, they inhibit ARVs as expected, which is a desired profile differentiating the developed AR DBD compounds from current drugs. Importantly, the lead chemical demonstrated effective suppression on the tumor growth in a xenograft model driven by ARVs, which further confirmed the inhibitory effect on ARVs (unpublished data).

The proof-of-concept work of targeting AR DBD showed a great promise in circumventing the resistance. So far, our study indicated that the AR DBD is a viable drug target, and the developed inhibitors indeed bind to the intended site and inhibit constitutively active ARVs. However, its druggability still requires more direct and solid evidence such as x-ray co-crystallization or NMR. As we are targeting the protein-DNA interface, the binding site in AR DBD is rather shallow, presumably belonging to the "difficult" target category.[232,

[233] Thus, the character of the site limited us to get a clear SAR of developed chemical series. For this type of site, covalent inhibitors may lead to stronger binding. Another concern of developing DBD inhibitors lies on the specificity due to the high conservation of DBD among SHRs. Although our results showed that the cross-activity with ER, GR and PR is not significant or almost negligible, which may be attributed to few distinct residues around the binding site, the specificity still represents an important issue for DBD inhibitors.

Of note, dimerization via the AR DBD is an essential process for AR transcription, and disrupting AR dimerization may be an effective intervention for PCa. Recently, it has been reported that ARVs can homodimerize or heterodimerize with themselves and full-length AR, and the ARV dimerization is required to transactivate target genes and confer castration-resistant cell growth.[234] This provides rationale of targeting the DBD dimerization for drug discovery. The AR DBD dimerizes by forming hydrogen bond interactions in the dimerization interface, and this interface may represent another viable site on the AR DBD for the development of AR inhibitors with novel mechanisms of action. A virtual screening was performed against the dimerization interface (data not shown), and hit compounds were identified which could be used for future investigation of dimerization disruptors.

5.2 Future Directions

Although a vast majority of CRPC patients remain dependent on AR signaling for viability, a multi-institutional integrative clinical sequencing analysis on a cohort of 150 metastatic CRPC patients revealed other genomically aberrant pathways in PCa, including PI3K, Wnt, cell-cycle and DNA repair pathways.[235] Thus, in addition to the AR-directed therapy, targeting other pathways and transcription factors could be considered as alternative strategies to treat CRPC.

5.2.1 Targeting Kinase-Dependent Pathways

The PI3K/AKT/mTOR signaling pathway plays an important role in the proliferation and survival of a varitety of cancers.[236] In PCa, in addition to the AR pathway, the PI3K/AKT/mTOR pathway is another frequently activated one, especially in PCa with the phosphatase and tensin homolog gene (PTEN) loss.[136, 235] As shown in Figure 5.1, the receptor tyrosine kinase can recruit and activate the PI3K, which converts the phosphatidylinositol-4, 5-diphosphate (PIP2) into phosphatidylinositol-3, 4, 5-triphosphate (PIP3). The PTEN regulates the PI3K/AKT/mTOR pathway by converting the PIP3 back to PIP2, and thus the deletion of PTEN leads to over-activation of AKT, which will cause cell proliferation, reduced apoptosis and enhanced angiogenesis.

The AR and PI3K/AKT/mTOR pathways cross-regulate each other via a negative feedback.[136, 237, 238] AKT interacts with AR in an androgen-independent manner, and regulates the AR transcriptional activity and expression. Therefore, the PI3K/AKT/mTOR pathway is partly involved in the reactivation of AR in CRPC. And the AR inhibition activates AKT signaling by reducing the AKT phosphatase. Due to the existence of the reciprocal regulation of two pathways, tumor cells compensate the inhibition of one pathway with the other for the survival. Thus, the inhibition of either single pathway is not very effective as it leads to up-regulation of the other. However, a combination therapy of targeting both PI3K/AKT/mTOR (for example, AKT or mTOR inhibitor) and AR (such as antiandrogens) pathways demonstrated synergistic effects on cancer regression in hormone-sensitive PCa and CRPC.[136, 137]

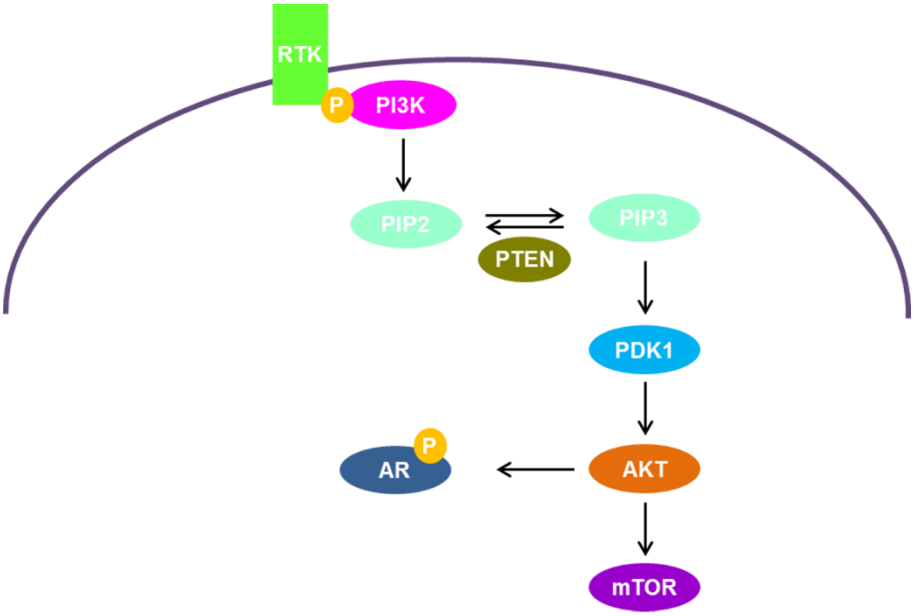


Figure 5.1. The PI3K/AKT/mTOR signaling pathway.

Growth factors binds and activates RTK, which in turn activates the PI3K/AKT/mTOR pathway. In this pathway, PTEN converts PIP3 back into PIP2. Abbreviation: RTK, receptor tyrosine kinase; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4, 5-bisphosphate; PIP3, phosphatidylinositol 3, 4, 5-triphosphate; PDK1, 3-phosphoinositide dependent protein kinase-1; PTEN, phosphatase and tensin homolog; AKT, protein kinase B; mTOR, mammalian target of rapamycin.

5.2.2 Targeting ERG

The E-26 transformation-specific (ETS)-related gene (ERG) is a transcription factor belonging to the ETS family,[239] which has been emerged as an important therapeutic target for PCa in the past decade. In 2005, Tomlins et al. reported a gene fusion of the promoter region of TMPRSS2 gene and the coding region of ERG, which results in the ERG overexpression in a high proportion of PCa.[240] ERG is not expressed in normal prostate epithelia,[241] while it is overexpressed in PCa and associated with advanced cancer stage,

metastasis and shorter survival time.[242] In PCa, there exists a crosstalk between ERG and AR that impacts androgen-regulated transcription and cell differentiation.[243] ERG is able to bind to the promoter of AR, and the knockdown of ERG results in AR induction. In addition, ERG upregulates an oncogene MYC which is a transcription factor and plays a role in cell survival, invasion as well as the androgen-independent cell growth.[244] The loss of ERG recruits AR to the promoter of c-MYC and blocks the transcription.[245]

Since ERG is implicated in multiple processes of PCa such as the invasion, metastasis and differentiation, it becomes an appealing drug target for novel therapies. The ERG protein has a highly conserved ETS DNA-binding domain (EBD) which recognizes a GGA(A/T) motif, a protein-protein interaction pointed domain (PNT), a transcriptional activation domain (TAD), the C-terminal inhibitory domain (CID) and N-terminal inhibitory domain (NID) which flank the EBD.[246] Small-molecule inhibitors were reported to directly target ERG, such as YK-4-279, which was claimed to bind to ERG and inhibit its transcriptional activity by interrupting ERG-protein interaction.[247] A DNA binding inhibitor was reported to target the GGA(A/T) and disrupt the EDB binding to the DNA.[248] In addition, targeting the ERG-related cofactors such as poly ADP ribose polymerase (PARP) and histone deacetylase (HDAC) with small-molecule inhibitors also demonstrated therapeutic potential for PCa.[249, 250]

5.2.3 Targeting Bromodomain

The epigenetic proteins are important targets in the drug discovery such as the bromodomain and extra-terminal (BET) family. The BET family consists of BRD2, BRD3, BRD4 and BRDT, which are characterized by two conserved bromodomains BD1 and BD2. Among them, BRD4 is the most studied BET member as an anticancer target, which recruits

the positive transcription elongation factor P-TEFb and plays a role in transcription by RNA polymerase II. The BET bromodomain is a protein-protein interaction site, and a variety of BET bromodomain inhibitors have been developed, such as the small molecule (±)JQ1 selectively targeting the BRD4.[251]

A recent study on JQ1 by Dr. Chinnaiyan's group established the BET bromodomain as a compelling drug target for the treatment of CRPC.[252] JQ1 was demonstrated to induce apoptosis and cell cycle arrest in AR-positive PCa cells, and it also down-regulates AR-mediated gene transcription. BET proteins may be involved in AR-mediated transcription as suggested by the repression of AR target genes after JQ1 treatment. Furthermore, it was found that AR, BRD4 and RNA polymerase II may form a large multi-protein complex, and the BD1 and BD2 are sufficient for AR-BRD4 binding. JQ1 could reduce the recruitment of AR to AR-responsive genes as potently as antiandrogen Ezalutamide. When evaluated in mice xenograft models, JQ1 is more effective in reducing the tumor volume than Enzalutamide.[253] This study provides a novel epigenetic approach to develop therapeutics for CRPC, and highlights the potential of BET proteins as therapeutic targets for CRPC.

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